

# Integrative Analysis of Lipidomics and Transcriptomics Revealed Dynamic Details of Lipids Metabolism and Accumulation in Developing Tiger Nut (*Cyperus Esculentus*) Tubers

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## Research Article

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# Abstract

**Background:** Oil crop varieties are currently subjected to an increasing worldwide demand and the tiger nut (*Cyperus esculentus* L.) attracts significant attention because of its huge capacity of lipids production. In eukaryotic cells, the balance between the accumulation of lipids and the distribution of certain pivotal molecules is fundamental for the regulation of many complex transcriptional regulatory networks. However, many studies have struggled to understand the dynamic of lipids and the transcriptomic mechanisms governing their biosynthesis and accumulation process during plant development.

**Results:** Our results displayed dynamic patterns for key lipids like glycolipid, phospholipid, and glyceride during the development of tiger nut tubers. Lipidomic analysis showed molecular species distribution of lipid class during developing stages. Here, we also characterized transcription profiles of key transcripts that determined biosynthesis and distribution of natural lipids in tiger nuts tuber. The expression of FAD2 exhibited a significant influence on the molecular composition of phosphatidylcholines (PC) and phosphatidyl ethanolamine (PE) in tiger nuts. Moreover, during lipids accumulation, the expression pattern of three candidate transcripts of oleosin genes (OLE9, OLE10 and OLE11) also displayed significant leverage on the size of lipid drops.

**Conclusion:** We described the significant alterations in the composition of lipids class during developing stages of tiger nut tuber, we also revealed transcriptional profiles of genes involved in lipid biosynthesis and accumulation. These results provided new landscapes for research on lipid composition, synthesis and accumulation during different developmental stages of plant tubers.

## Background

Oil plants constitute a key source of nutrients and energy for humans and are also very important for animals. Due to the limited cultivated land, and the increasing demand for oil consumption, novel oil plants or genetic breeding varieties with high oil content, short growing season, and wide adaptability have been receiving increasing attention as an alternative resource to traditional oil crops.

*Cyperus esculentus* is a monocotyledonous perennial plant and a member of the family of *cyperaceae*. The *Cyperus esculentus* morphology looks similar to that of the grasses or rushes appearance, but exhibits a hypogeal rhizome similar to that of potato, and is commonly called tiger nut. Tiger nut is a prominent source of lipids, oleic acid, carbohydrates, vitamin E,  $\beta$ -carotene, and mineral substance. This small plant also has high crude oil content, which is about 24.9–28.9% according to varieties[1]. Tiger nut is also an easy and fast-growing plant, with approximately 110 days cultivated period, presenting a high average of yield ranged from 4.5 to 12t ha<sup>-1</sup> depending on the ambient temperature. Due to its amazing potentialities such as high oil content, high nutritional value, and perfect animal feed, the tiger nut is emerging as a novel economic oil plant with expanded worldwide cultivation including Africa, Australia, China and Spain. As a valuable resource of lipids, the tiger nut remains underestimated and its potential in the aspect of oil production and crop breeding demands to be more investigated.

In 1994, the first electrospray ionization mass spectrometry (ESI-MS) on lipidomics reported various phospholipid molecules through the quantitative analysis. The study revealed the physiological importance of lipids[2], identified their metabolic networks, and demonstrated their involvement in multiple signaling pathways of eucaryotic cells. Over the last decades, ESI-MS usage has experienced considerable improvements in plant biotechnology, accelerating the research rhythm on lipidomics as well. Similar techniques have been applied to describe regulation of lipid modeling to various plant tissues[3, 4, 5] and such studies have been conducted on several plants. Lipidomics was also used to restore lipid modeling species under varied growth conditions, providing insights into the biochemical features of lipid metabolism during plant tissue's development.

The modulation of the both production and accumulation of lipid plays a very important role in plant tissues development. Lipidomic analysis identifies existing correlations among varied polar lipids including phosphatidylcholines (PC), phosphatidyl ethanolamine (PE), and some neutral lipids such as diglycerides (DG) and triglycerides (TG). In addition, lipidomic researches were also implemented in the successful characterization of certain groups of unsaturated lipids. Nevertheless, application of gene expression patterns associated with lipidomic studies in plant tissues development is a rare process. Meanwhile, the mechanism governing the production, regulation, and accumulation of lipids in plants including the involved transcriptional response pathway remains unclear. Thus, it is fundamental to understand the molecular mechanisms that regulate various aspects of lipid metabolism could facilitate genetic improvement of the oil plants.

Presently, metabolic pathways regulating fatty acids synthesis and lipids metabolism in oilseed plants such as *Arabidopsis thaliana* and *Brassica napus* were achieved through the application of enzymology coordinated with the flux of carbon[6, 7]. The general mechanism of lipids production contains high levels of lipid substrates such as phosphatidylcholine (PC), diacylglycerol (DAG), or triacylglycerols (TAG) which are the most prominent precursors of lipids in plants. However, the signaling pathway involved in TAG synthesis through the Kennedy pathway appears to be distinct in terms of flux of fatty acids and enzymology among plant species[8].

Transcriptional analyses in many plant models have shown that the regulation of the seed oil production is related to the developmental stages[9, 10]. Many enzymes and transcriptional factors involved in TAG biosynthetic pathways were identified and confirmed as positive regulators in various plant species. Among those, fatty acids synthesis (FAS), FAD2, FAD3[11], KCSs[12] or MYB1[13] represent the key for fatty acids composition alteration. FAX1 and FAX2 facilitate fatty acids transport from plastids for improving seed oil accumulation[14, 15]. In the Kennedy pathway associated with TAG and membrane lipid synthesis (PULL), acyltransferases such as Lysophosphatidic acid acyltransferase (LPAAT)[16], diglyceride:acyltransferase (DGAT)[17], and Phospholipid diacylglycerol acyltransferase1 (PDAT1)[18] are important for TAG synthesis. Recent lipid accumulation studies revealed that DGAT1 and DGAT2 in *Cyperus esculentus* strongly affected TAG production and lipid accumulation[19]. The SDP1 also regulates TAG accumulation by maintaining FA turnover in *Arabidopsis lipins*[20]. Several transcription factors like those of the APETALA2 transcription factor family, WR1[21], MYB96[22], B3-type containe

DNA binding domain FUSCA3[23] and Lec1[24, 25] which are all reported as the key regulators initiating lipid assembly in higher plants.

Oleosin is necessary for natural lipids storage and stability in seeds[26]. In this study, candidate genes responsible for lipid metabolism and regulatory processes were selected based on their transcriptomic analysis to provide new gene targets for genetic engineering operation. Therefore, lipidomics and RNA-Seq analysis were applied to explore the lipids metabolism processes and molecular mechanisms that are involved at different developmental stages of tuber in *Cyperus esculentus*. Thus, the understanding of the alternations between lipid categories and biological mechanisms of oil biosynthesis may provide a new landscape for tiger nut germplasm utilization and other plant breeding programs.

## Materials And Methods

### Plant materials and treatments

The sampled plants of *Cyperus esculentus* were cultivated in Jilin province, China, under natural conditions. The collection of samples was done at the optimal developmental stage, which is a crucial step for an optimal comprehensive transcriptional profiling analysis of plant tubers. Based on observations on the *Cyperus esculentus* tubers development, five developmental stages were identified. In the present study, we namely arranged these five developmental stages as the 35 days after sowing (initial stage, 35 DAS); 50 days after sowing (early stage, 50 DAS); 70 days after sowing (middle stage, 70 DAS); 90 days after sowing (late stage, 90 DAS); finally 120 days after sowing (mature stage, 120 DAS) corresponding to complete mature of tubers. Comparative transcriptome analysis was also monitored to explore gene expression patterns during lipid biosynthesis in the tubers of *Cyperus esculentus*. For this step, samples (gathered tubers) were collected in three replicates for each developmental stage (five developmental stages), then we immediately froze the collected materials in liquid nitrogen and stored at -80°C until further uses.

### Extraction of Lipids Drops and Measure of particle size

Lipid droplets (LDs) were isolated using 0.1 M PBS solution buffer (PH 7.4), the *Cyperus esculentus* tubers were peeled and crushed carefully. The tuber powders (30 g) were transferred to a beaker in deionized water at a tubers-to-water ratio of 1:5 (w/v). This mixture was then stirred with a pulverizer Fluko homogenizer (18,000 rpm, FM200) for 2min, and Viscozyme L was added to the mixture to adjust the final concentration of 1.35% (v/w, based on raw peanut weight) The latter was therefore incubated for 90 min at 52 °C in the bath shaker (Huafeng, THZ-82, Jintan, China). After a sufficient enzymatic hydrolysis, we transferred the mixture into a boiling water bath for 5 min to inhibited the enzymes, this step help in avoiding the impact of enzymes on subsequent experiments. The mixture was further filtered through four layers of cheesecloth and transferred to a 250mL tube, then centrifuged at 4,000 rpm for 30 min (Anting, DZ267-32C6 centrifuge, Shanghai, China). The appearing LDs as a cream at the top of each centrifuge tube, were carefully collected in a tube and stored at 4°C. Further analyses were performed within 24 hours and sodium azide (0.02%, w/v) was added to all media to avoid microbial spoilage.

Particle size measurement LDs were diluted to an approximately 0.1% oil concentration, by adding PBS solution buffer (PH 7.4), and then leached using ultrasonic baths to ensure homogeneity. The particle diameter distributions were analyzed using a Nanometer particle size and ZETA potentiometer (NANO ZS90). Particle size measurements were completed after the LDs suspensions had been stored for 24 hours at room temperature.

### **Extraction of lipid drops**

The LDs obtained during the oil body extraction were dissolved in 0.1 M phosphate buffer, shaken at 0°C for 30 min, and then centrifuged at 10,000× *g* for 20 min. The upper layer of the LP suspension was collected and mixed with excess acetone at 0°C to isolate the triacylglycerols. After repeating twice, the remaining extract was membrane protein.

### **Nile Red staining**

Five development tubers were gained, peeled, cut into small pieces of 0.5×0.5cm, and transferred into fix solution of 4% tissues for 24h at 4°C. Then, the tissues were embedded into paraffin by use embedding cassettes, blocked, put at 4°C until the complet solidification, and cut at 5–10 µm, dewaxing with a 60°C Electro Thermostatic Blast Oven. The sections were deparaffinized, rehydrated, and stained with Nile Red for 2 min. Sections were then rinsed in running tap water, dehydrated, mounted, and captured, the sheet was sealed with neutral gum at last.

### **Measurement of oil content**

Five milligrams (5 mg) of dry seeds were used for oil content and fatty acid analysis. After placing all seeds into a glass tube, we added 1 ml of 2.5% sulfuric acid-methanol solution, 0.4 ml of toluene, and 0.2 ml of a 2 mg/ml C17:0 solutions in toluene. The final solution was therefore mixed by vortex and heated at 90 °C water bath for 1 h; then, 1.8 ml of ddH<sub>2</sub>O and 1 ml of hexane were added after the tube cooled. After 15 min, the supernatant was filtered using a 0.45-µm microporous membrane. The filtrate was used to determine the fatty acid GC content using an Agilent 7890A instrument. At least three replicate samples were examined for all of the experiments.

### **Lipid extraction**

For the lipide extraction, 100 mg of each sample were transferred into the 5 mL centrifuge tubes, in which we added 1500 µL of mixed solution of chloroform and methanol (2:1) pre-cooled at -20°C and divided in five steel balls (all insufficient samples size were reduced to an equal scale). Furthermore, we grind all samples by a high flux organization grinding apparatus for 1.5 min at 60 Hz, put on the ice for 30 min, add 0.38 mL ddH<sub>2</sub>O, vortex for 30 s, and put back on the ice for 10 min, centrifuged at 12, 000 rpm for 5 min at room temperature and transfer 0.6 mL lower layer fluid into a new centrifuge tube, add 1000 µL of previous mixed solution , vortex for 30 s, centrifuged at 12, 000 rpm for 5 min at room temperature and transfer 0.8 mL lower layer fluid into the same centrifuge tube above. Samples were concentrated to dry

in a vacuum, dissolve samples with 200  $\mu$ L isopropanol, and the supernatant was filtered through a 0.22  $\mu$ m membrane to obtain the prepared samples for LC-MS, take 20  $\mu$ L from each sample to the quality control (QC) samples (these QC samples were used to monitor deviations of the analytical results from these pool mixtures and compare them to the errors caused by the analytical instrument itself). Use the rest of the samples for LC-MS detection[51].

### Lipidomic analysis by LC/MS

Chromatographic separation was carried using a Thermo ultimate 3000 system equipped with an ACQUITY UPLC® BEH C18 (100 x 2.1 mm, 1.7  $\mu$ m, Waters) column maintained at 50°C. The temperature of the autosampler was 8°C; gradient elution of analytes was carried out with acetonitrile: water = 60:40 (0.1% formic acid +10 mM ammonium formate) (A) and isopropanol : acetonitrile = 90:10 (0.1% formic acid +10 mM ammonium formate) (B) at a flow rate of 0.25 mL/min. Injection of 2  $\mu$ L of each sample was done after equilibration. An increasing linear gradient of solvent A (v/v) was used as follows: 0~5 min, 70~57% A; 5~5.1 min, 57%~50% A; 5.1~14 min, 50%~30% A; 14~14.1 min, 30% A; 14.1~21 min, 30%~1% A; 21~24 min, 1% A; 24~24.1 min, 1%~70% A; 24.1~28 min, 70% A[52].

The ESI-MS<sup>n</sup> experiments were executed on the Thermo Q Exactive Focus mass spectrometer with the spray voltage of 3.5 kV and -2.5 kV in positive and negative modes, respectively; sheath gas and auxiliary gas were set at 30 and 10 units respectively; finally, the capillary temperature was set to 325°C. The Orbitrap analyzer scanned over a mass ranged of m/z 150-2000 for a full scan at a mass resolution of 35 000. Data-dependent acquisition (DDA) MS/MS experiments were performed with an HCD scan. The normalized collision energy was 30 eV. Dynamic exclusion was implemented to remove some unnecessary information in MS spectra [52].

## Results

### Raw oil contents in developing tiger nut tuber

To investigate the lipid accumulation during different plant tuber developing stages, we analyzed the oil content of different development stages of *C. esculentus*. Tiger nut tubers started to sprout after approximately 3-6 days upon seed sowing under regular growth conditions. The growth period of tubers took around 120 days, while their appearances turned from white to brown; the white appearance was observed during the initial 35 day after sowing (35 DAS) and early (50 DAS) stages, light brown on the middle stage (70 DAS), moderate brown on the late stage (90 DAS), and finally turned to dark brown in mature stage (120 DAS). The tuber inside is sustainable oyster white when the coat is removed (Figure 1A). During tuber development, the fresh weight and diameters of each tuber increased significantly following averages of 0.2g and 0.2mm per day, respectively (Figure 1B, 1C). We also determined the oil content during tuber development; it showed that raw oil content in tubers increased continuously from 4.6% to 22.2% throughout all developmental stages (Figure 1D). There was a rapid oil accumulation rate for the early and middle stages, which increased approximately on average 0.26% d<sup>-1</sup> from 35 to 50 DAS

and 0.33% d<sup>-1</sup> from 50 to 70 DAS, respectively. That fascinating increase was followed by the late and mature stage, which increased approximately on average 0.25% d<sup>-1</sup> from 70-90 DAS and 0.11% d<sup>-1</sup> from 90-120 DAS, respectively.

The reserves of lipid droplets represent the main storage organelles for natural lipids in plants[27]. To evaluate the variation of lipid accumulation during the developmental process inside the tuber, we stained tubers of different stages with Nile Red to observe the structure and amount change of lipid droplets (LDs) (Figure 1E). The LDs showed irregular spherical structure and a constantly increasing amount with tuber development. The accumulation of neutral lipids in tubers was visually evident; this may indicate that the increase in the expression level of the TAG leads to an increase in the crude oil content; this classify TAG as a major contributor to the enhancement of oil accumulation in oil plants.

### **Lipid species analysis and variation of lipid content**

To determine the changes in the overall lipid composition and distribution in *C. esculentus* tubers, we isolated metabolite from tubers and explored them by LC-MS analysis. Over 430 different lipid species in tubers, consisting of 133 triacylglycerols (TG) species (31.1%), 23 diacylglycerols (DG) species (5.4%), 21 phosphatidylglycerols (PGs) species (4.9%), and other lipid molecular species (Figure 2A). Neutral lipids (glycolipids and AcHex) composition was continuously increasing during all five different stages (Figure 2B). As the main neutral lipids component found in tubers, TG content continuously increased until the fully mature stage (Figure 2C). It implied that the increased proportion of neutral lipids is mainly justified by the continuous accumulation of TAG. Subsequently, the relative composition of polar lipids in the development of tiger nut tuber showed that expect neutral lipids, glycolipids, phospholipids, and sphingolipids main components of polar lipids (Figure 3A). During the phospholipid profiling analysis, the relative composition of PC was continuously decreased along the five different stages of tubers, the profiling of PE displayed a minor decrease at the five stages and only periodical increase at the late stage (Figure 3B). The decrease of PC, periodic decreased of PE and relative content increased of TAG suggests that during the development of tubers, PC is probably convert into TAG and PE is probably convert into TAG obviously on the early and middle stage. Other polar lipids profile changes were lack in regularity, only the Hexcer increased at all developing stages (Figure 3C, 3D), that the metabolism correlation with glycolipid is weak.

### **Molecular species dynamic changes during lipid accumulation**

Rapid lipid accumulation was observed in both the early and middle stages of tuber development. Then, we analyzed the changes of molecular species during lipid accumulation stages in tubers. The most prominent variety of TAG at all time points was 16:0/16:1/18:1, followed by TAG species containing a single saturated fatty acid (16:0/16:1/18:1, 20:0/18:1/18:2, 26:0/18:1/18:1) and higher unsaturated species (16:1/18:2/18:2, 18:1/18:1/24:1, 18:1/18:2/24:1) (Figure 4A). The detected TAG molecular species are all shown in figure S1. The proportions of molecular species almost all showed an increasing trend compared with the initial stage (35DAS) for TAG. This trend of lipids increase explained the overall



lipid composition and oil content of *C. esculentus* tubers. During the oil accumulation period a general shift towards more unsaturated TAG molecular species, leading to an increase of unsaturated fatty acid content in the lipids.

Major molecular species of DAG are displayed in figure 4B. The most abundant species in DAG at all stages of oil accumulation were 16:0/18:2 and 18:1/18:1, both of these two species increase in abundance during the developmental stage; the proportion ranged from 10% to 60% of the total DAG accumulation. Other significant molecular species of DAG were those carrying palmitoyl, oleoyl, or linolenyl (16:0/18:2, 18:3/18:2); the proportion of molecular species of 16:0/18:2 strongly decreased during lipid accumulation stages; finally, molecular species of 18:3/18:2 only increased in the last development stage. The complete analysis of all detected species in figure S2.

As immediate precursors of DAG in the Kennedy pathway, the main molecular species of PA are well displayed in Figure 5A. The major species of PA were 16:0/18:1, 18:0/18:2, and 18:3/18:2. Between 35 and 120 DAS, the abundance of 18:0/18:2 species significantly increased, while 18:3/18:2 undergo a constant decrease. The 16:0/18:1 species kept the increase in the previous four-time points and slightly decreased in the last time points. Other significant PA species were 16:0/16:0, 16:0/16:1, 16:0/18:3 and 24:0/18:2. In general, proportions of accumulation of the major PA species constantly increased during the whole process.

In oil crops, almost all fatty acyl in TAG flux through PC [28], so PC played a very important role during oil accumulation. The main molecular species of PC during oil accumulation in *C. esculentus* tubers are shown in figure 5B, while a complete analysis of all detected species is depicted in the figure S3. The main molecular species at 35 DAS were 16:0/18:3 and 18:2/18:2 but both showed a significantly decreased trend in all development stages. Molecular species 16:0/16:0 and 18:1/18:2 showed an increasing trend during developmental process. Some other molecules also presented a discontinuous trend of its proportion, molecular species 16:0/18:1 and 18:1/18:1, which increased between 35 and 70 DAS and slightly decreased in proportion between 90 and 120 DAS.

The DAG from the Kennedy pathway serves as a substrate for the synthesis of PC as well as PE, the two phospholipid have a strong correlation in the metabolism[29]. Therefore, we examined PE molecular species to compare with those of PC. The main molecular species of PE are shown in figure 5C with a complete breakdown of all species detected in figure S4. The pattern of molecular species is similar to that of PC, which is 16:0/18:3 and 18:2/18:2, showed significantly decreased tendency in proportion accordingly to the plant development. In figure 5C, the major species of PE showed the most significant were 16:1/18:1 and 26:0/18:2, which increased in proportion. In general, other molecular species maintained relatively steady proportions of PE during oil accumulation. This results demonstrate that the PE is synthesized through the same pathway as the PC, but have differential distribution in molecular species proportions.

## Transcriptome analysis of *C.esculentus* during tuber development

Studies on the molecular mechanism of lipid biosynthesis during the development of tiger nuts are limited and the genetic resource of the tiger nut plant is insufficient. In this study, the expression analysis of relevant metabolic genes correlated with lipid metabolic (PAP2→PLD→FATA→SAD→DGAT etc.) was carried out by Illumina RNA-seq approach. More than 744 million clean reads and 150,153 transcripts were generated from five libraries. A total number of 8, 2,008, 3,967, and 7,345 different expressed genes corresponding to 50 VS 35, 70 VS 35, 90 Vs 35 and 120 VS 35 DAS were computed out, respectively. When comparing gene expressions across the five stages of tiger tubes development, it appeared that the proportion of the expressed genes during the developing stage simultaneously increase along with the plant growth.

Additionally, physiological analyses revealed different genes expression pattern of each developmental stage, which were mapped to the pathways in the biological pathways database of Kyoto Encyclopedia of Genes and Genomes (KEGG). These differentially expressed genes participated in various lipid metabolic related processes including linoleic acid metabolism, glycerophospholipid metabolism, etc. (Figure 6A). All the expressed genes involved in carbohydrate metabolic pathways such as the starch and sucrose metabolic and glycolysis were abundant during the late-developing stages. This suggesting that G6P and Acyl-CoA served as a precursor for the synthesis of lipid is of great abundant for supply in late developing stage.

The GO enrichment analysis of differentially expressed genes in tiger nut identified several biological processes, which probably represented specific or common conserved functions of those expressed genes throughout the plant development (Figure 6B). Various functions, such as RNA processing, DNA binding, and protein binding were significantly enriched in the genes with higher expression pattern, particularly at 90 and 120 DAS developing stage. Likewise, GO terms, response to oxidative stress, oxidoreductase activity and peroxidase activity were also significantly enriched for down-regulated genes at 90 and 120 DAS developing stage when compared with 35 DAS. In contrast, these terms were also significantly enriched for two gene sets exhibiting up-regulated expression pattern at 50 VS 35 DAS stages. Gene sets enriched on the metabolism process of macromolecular compounds like lipid metabolic process, lipid metabolic process, glycosphingolipid metabolic process, sphingolipid metabolic process, exhibited down-regulated expression pattern at 70, 90, and 120 when compared with 35 DAS stage. on the contrary, polysaccharide biosynthetic process indicated higher activity of lipids biosynthetic at initial developing stage. Interestingly, for 120 VS 35 DAS stage, we observed more significant enrichment of GO term in fatty acid metabolism, including the fatty acid derivatived from the metabolic process and unsaturated fatty acid metabolism, here these genes exhibited down-regulated patterns of tuber development. In addition, genes enriched on transferase activity and phospholipase C activity seemed to be less active at 120 DAS when compared with 35 DAS.

### **Majors genes are responsible for the molecular species composition of TAG**

Plant oil quality usually is determined by oil content and fatty acid composition. The unsaturated fatty acyl group composition of TAG is an important factor in evaluating the oil quality. 12 candidate genes

involved in fatty acid metabolism, elongation in the plastid, lipid desaturation, and synthesis mechanisms in the endoplasmic reticulum (ER) were characterized through the lipid metabolic pathway of maize[30] (Figure 7). Among these candidate genes, most transcripts of GPAT1, DGAT, PLC, LPAAT, FATA, and SAD showed an abundant transcription profile in the early stage and showed dynamic decreased expression patterns during late and mature stages. Most transcripts of ACSL, PAP2, PLD and KAS have low abundance expression patterns during the initial early and middle stage and up-regulated expression patterns during the late and mature stage. Two of the PDAT transcripts were mainly expressed at a high level in 70 DAS; the other two had higher expression in the 90 DAS.

The comparative lipidomic analysis also revealed that the unsaturated linolenic acyl group (C18:3) carried by PC, and PE had a more significant decreased level in the mature stage compared with the initial stage, from 6.76% to 1.45% for PC and from 3.85% to 2.93% for PE, respectively. The composition rate of the oleic acyl group (C18:1) decreased by approximately 10.87% in PC and 11.47% in PE during the mature stage when compared with the initial stage (Figure S5). Higher transcriptional level of PDAT, PLC, and DGAT activity could therefore result from the reduced levels of monounsaturated and polyunsaturated phosphatidylcholine or phosphatidylethanolamines, which are substrates for the Flavin adenine dinucleotide FAD2 desaturase that responsible for the production of polyunsaturated fatty acyl groups in phospholipid. Furthermore, FAD2 had low transcriptional abundance during developing stages, except for the middle stage. The different expression patterns of FAD2 in ER during developmental stages could explain a portion of the downward trend of oleoyl composition for PC and PE, respectively. In addition, the flow of polyunsaturated acyl in PC or PE would partially influence the dynamic variation composition of molecular species in TAG.

### **Particle size analysis of lipid drop and expression profile of candidate oleosin genes**

As intracellular storage organelles for neutral lipids, lipid droplet (LP) packed with natural lipids originated from the endoplasmic reticulum and mainly exist in a wide range of density distinct cytoplasm, together with starch and storage proteins [31,32]. We extracted lipid droplets and measured the particle size of the lipid droplet at different developing stages (figure 8A). Diameter sizes were range from 2,500 nm to 3,500 nm; diameters were undergoing of significant increase in 70 DAS (Figure 8B). Lipid droplet associated proteins (LDAPs), which are uniformly expressed in all cells, facilitated lipid droplet biogenesis and prevented lipid droplets from aggregation; this activity is associated with the dynamic regulation of lipid droplet size, number, and distribution in response to cellular metabolism and lipid composition. As plant small molecular LDAPs, oleosin derived from green algae and universally in advanced plants[33]. Oleosins were helping to stabilize and protect neutral lipids by protecting against degradation. 15 putative oleosin-like (OLE) and two putative caleosin (CaLE) transcripts were characterized according to our transcriptome result of developing tubers. To determine the expression pattern of LDAP in developing tubers of tiger nut, the transcriptional levels of LDAPs were analyzed. Candidate oleosin transcripts were expressed comprehensively during tuber development. In particular, OLE9, OLE10, and OLE11, which are expressed predominantly in developing tubers, the three transcripts were exhibiting similar expression patterns and showed comparable decreased levels in 70 DAS (Figure 8C). However, the expression

pattern of these three candidate oleosin transcripts was opposite correlated from LD size. This observation may indicate that these three candidate oleosin transcripts were probably involved in LD formation related processes, relevantly responsible for the raised particle size of LD 70 DAS stage.

## Discussion

As an oil crop with high nutritional value, the oil-rich tuber of *C. esculentus* could serve as an ideal plant model to characterize the specific functions and regulation of lipid synthesis and storage in plants. Different from sweet potato and potato, oil represent the major natural products of tiger nuts.

Lipid metabolism was found to be significant in tiger nut according to be gene function annotation of KEGG and Gene ontology, so *C. esculentus* is a special model for lipid metabolism research. However, not much is known about the dynamic structure of lipid composition during tiger nut tuber development and maturation. In this study, we have carried out comprehensive lipidomic and transcriptome analyses related to lipid metabolism in tiger nut tubers. Through the analysis of dynamic changes for lipid composition and major gene expression profile toward TAG synthesis as to disclose the underlying mechanism of synthesis and storage of lipid for *C. esculentus* during developing tubers. Our results revealed that the accumulation rate of oil in the tuber at the middle stage (50-70 DAS) was higher than that of tuber oil of another developing stage. This observation is consistent to a previous study on *C. esculentus* which showed a rapid lipid accumulation rate in the middle stage (50–85 DAS)[34]. However, in our study the second higher rate for lipid oil accumulation was in the early stage (35-50 DAS) not the late developing stage. It may be associated with the time point selection of morphological development of tiger nut tubers. Any how, increasing the oil content in plants is still a target for the breeding program.

Neutral lipid represent the highest proportions of lipid in tiger nut; as a major and important lipid stocker[35], the proportion of TAG in tiger nut lipid is nearly 87% for the initial stage, and the proportion of TAG is still increasing when the tiger nut developing and accumulate approximately 90% for the mature stage. Also, the proportion of PC and PE significantly decreased during plant development and early and middle stage development. It had been revealed that PC is the main source for TAG synthesis in plant developing seeds[36], and in the accumulation of TAG, PE does not seem to be as effective as PC[37]. Our results implies that, during development of plant tubers, PC is actively involved in the synthesis of TAG and PE may participate in TAG synthesis actively on early and middle stage.

Lipids biosynthesis is involved in a series of pathways including PUSH, PULL, and PACKAGE, and fatty acids synthesis (PUSH) is located in plastids. The formation of 18:0-ACP from Palmitoyl-ACP that is catalyzed by KAS while the desaturation of 18:0-ACP to form 18:1-ACP is catalyzed by stromal stearyl-ACP desaturase (SAD) to form 18:1-ACP. During synthesis, acyl-ACP is transferred into free FA through the action of acyl-ACP thioesterase (FATA). Free fatty acids are reactivated by acyl-CoA synthetase (ACSL) and exported from plastids to enter the eukaryotic glycerolipid metabolic pathways[38]. Based on our transcriptome results, abundant FATA, SAD and ACSL transcripts guaranteed 16:0, 18:0, and 18:1 free fatty acids synthesized continuously in the plastid especially in early stage.

TAG biosynthesis (PULL) in ER is involved in the acylation of glycerol-3-phosphate (G3P) with acyl-CoA and its late dephosphorylation. This phenomenon mainly occurs in the endoplasmic reticulum. During the catalysis of glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid (LPA) was generated from glycerol-3-phosphate (G3P) and transformed into PA through the enzymatic action of LPAAT. Then, DAG was by catalytic action of PAP2 and transformed into TAG by the catalytic action of DGAT. DGAT is the rate-limiting enzyme in the Kennedy pathway and plays an important role in the process of TAG synthesis and accumulation. Four DGAT have been discovered: DGAT1, DGAT2, WS/DGAT, and soluble DGAT. Ectopic expression DGAT gene to improve seed oil content has been verified in Arabidopsis[39], soybean[40], corn[41], and other plants. DGAT1 and DGAT2 may play different roles in different plants at differential development stages, and produce TAG with different fatty acid components[42]. For example: in the seeds of castor and tung tree, the expression level of DGAT2 is higher than that of DGAT1, and plays an important role in the accumulation of oil[43,44]. In tung tree, DGAT2 has a strong effect on tung oil acid substrate selectivity[43]. In *Cyperus esculentus* L. tubers, we obtained two transcripts of DGAT which were down-regulated from 35DAS to 120DAS. This may be an effective factor for the accumulation rate of lipids.

TAG can also be synthesized by PDAT using PL and DAG as substrates[45]. During the developmental stages, the proportion of C18:1 in PC and PE were decreased drastically in the mature stage, the C18:2 and C18:3 fatty acyl species are also declining in the proportion during the PE accumulation course. For the fatty acyl composition of TAG, the 18:1 fatty acid of PE and PC was transported from the plastid to the endoplasmic reticulum, it was desaturated into C18:2 and C18:3 fatty acid under the catalysis of FAD2 or FAD3[46]. Our RNA-Seq results suggested that a low abundance of FAD2 and high expression patterns of PDAT, PLC, and DGAT likely to be factors for the decent proportion of polyunsaturated fatty acyl in phospholipid, and affect the molecular species composition of TAG. The increased level of PE in the polyunsaturated fatty acyl composition observed in the middle stage is probably related to high expression of FAD2 in the middle stage. Similar changes were not observed during the PC content analysis, this may be due to the specificity of the enzyme or the phospholipid variety[47].

LDs are widely present in all types of plant cells and mostly active in the endoplasmic reticulum. In vegetative tissues, LDs are required for neutral lipid homeostasis and prevent lipids degeneration in the cytoplasm. The mechanisms underlying the formation, stabilization, and turnover of plant LDs are associated with LDAPs. Among all LDAPs, oleosin is the tightest one which associated with LDs. The study of oleosin is originated from seed, and it was believed that the gene is abundantly expressed in seeds. In the process of lipids drops formation, expression of seed oleosins was associated with the sub-micrometer size of LDs[48]. Oleosin evolved from green algae and has evolved into different lineages in advanced plants. Lineage SL and SH oleosins was kind of seed-specific oleosins[49]. The study of oleosins originated from plant seeds, which contribute to the accumulation of a high level of oil during seed development[50]. The structural and functional role of oleosin homologs in LD formation in other types of cells is unclear. Our transcriptome results characterized three candidate oleosin transcripts with abundant expression patterns and compared their diameters to that of LDs during tuber development.

The results indicated that putative oleosin transcripts might principally interact with the generation and the stablition of plant LDs in tuber development.

In combination with the lipidomic and transcriptome analysis of tubers during different developmental stages, this study elucidate the diversity of lipid composition and dynamic changes by lipidomic analysis. The transcriptome analysis also provided a landscape of fatty acids flow direction and effective database about the lipids compositions among different developmental stages of tubers.

## Conclusion

In conclusion, Our study reports a dynamic lipid composition during different developemental stages of tiger nuts. We used an integrated approach to construct the acyl flow of fatty acids involved in the biosynthesis of TAG. Gene expression patterns have been particularly important to carry out the catalytic rate of the reaction related to the glyceride accumulation. Our analyses also revealed eight candidate genes including encoding candidate oleosin 9, 10, and 11, which encode the key functional structure related to oil lipid diameter responsible for lipid storage.

## Abbreviations

TAG:triacylglycerol; ESI-MS:electrospray ionization mass spectrometry;

PC:phosphatidylcholines; PE:phosphatidyl ethanolamine; DG:diglycerides;

TG:triglycerides; LPAAT:Lysophosphatidic acid acyltransferase; DGAT:diglyceride acyltransferase; PDAT1:Phospholipid:diacylglycerol acyltransferase 1; DAS:days after sowing; OBs:Oil bodys; LDs:lipid drops; QC:quality control; LDAPs:Lipid droplet associated proteins; OLE:oleosin-like; SAD:stromal stearoyl-ACP desaturase; FA:Free fatty; LACS:long-chain acyl-CoA synthetase; G3P:glycerol-3-phosphate;

GPAT:glycerol-3-phosphate acyltransferase; LPA:lysophosphatidic acid.

## Declarations

### Acknowledgment

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### Authors' Contributions

L.W., M.J., and Y.D. performed some of the data analysis. M.J., Y.W. and J.L. managed the samples and tissues. J.L., Y.W. and Y.W. performed some of the data analysis and prepared graphics. W.L., N.W., X.L. and Y.Z. performed the experiments. Y.D. F.W. and H.L. assisted in data analysis and in the overall design

of the project. Y.D., and H.L. developed the overall design of the study and assisted with manuscript preparation. L.W., M.J., Y.D., X.L. and N.W. wrote the manuscript. All of the authors reviewed and approved the final manuscript.

## Funding

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## Availability of data and materials

The datasets has been uploaded to the NCBI SRA database. SRA accession:PRJNA681247. SRA records will be accessible with the following link: <https://www.ncbi.nlm.nih.gov/sra/PRJNA681247>.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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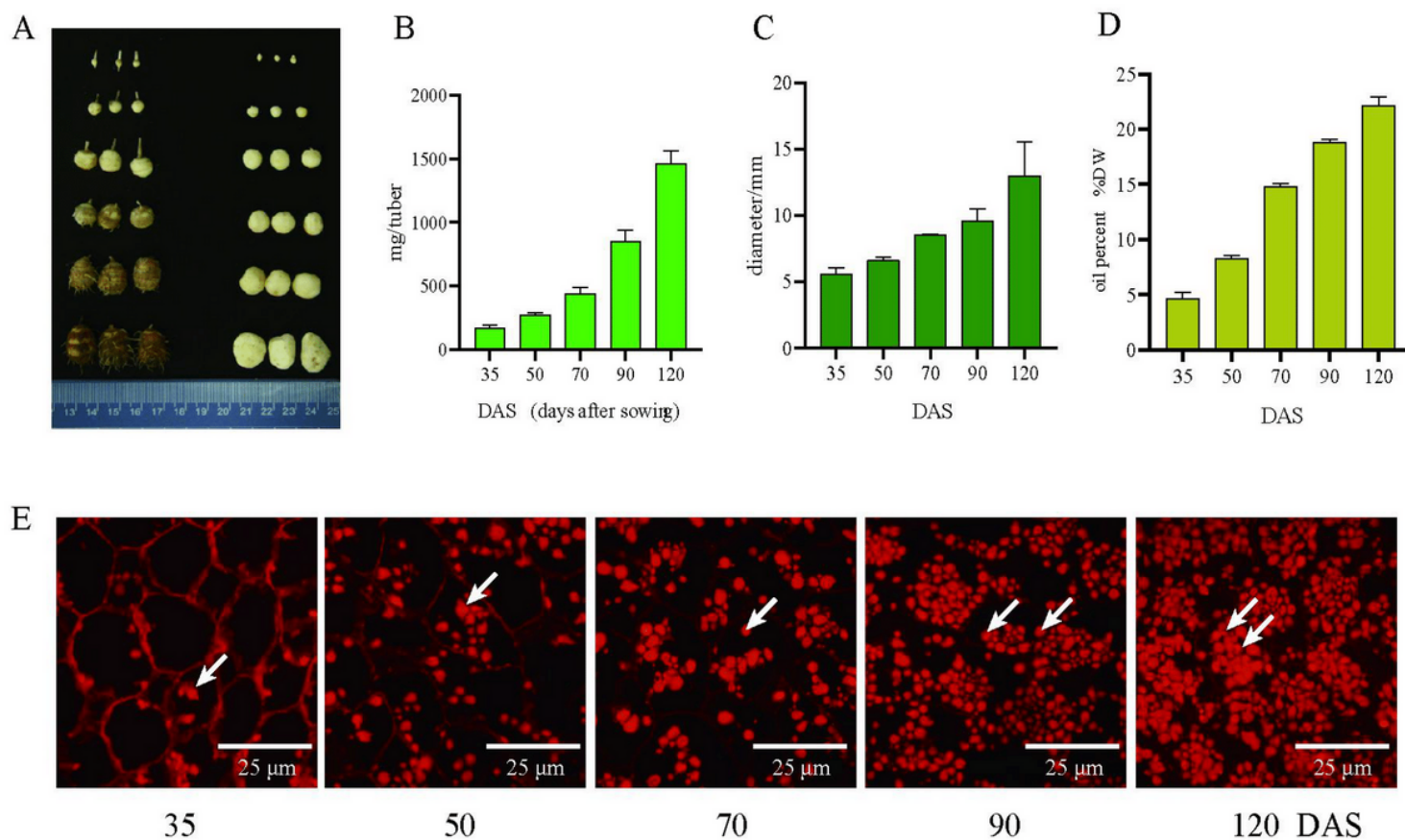


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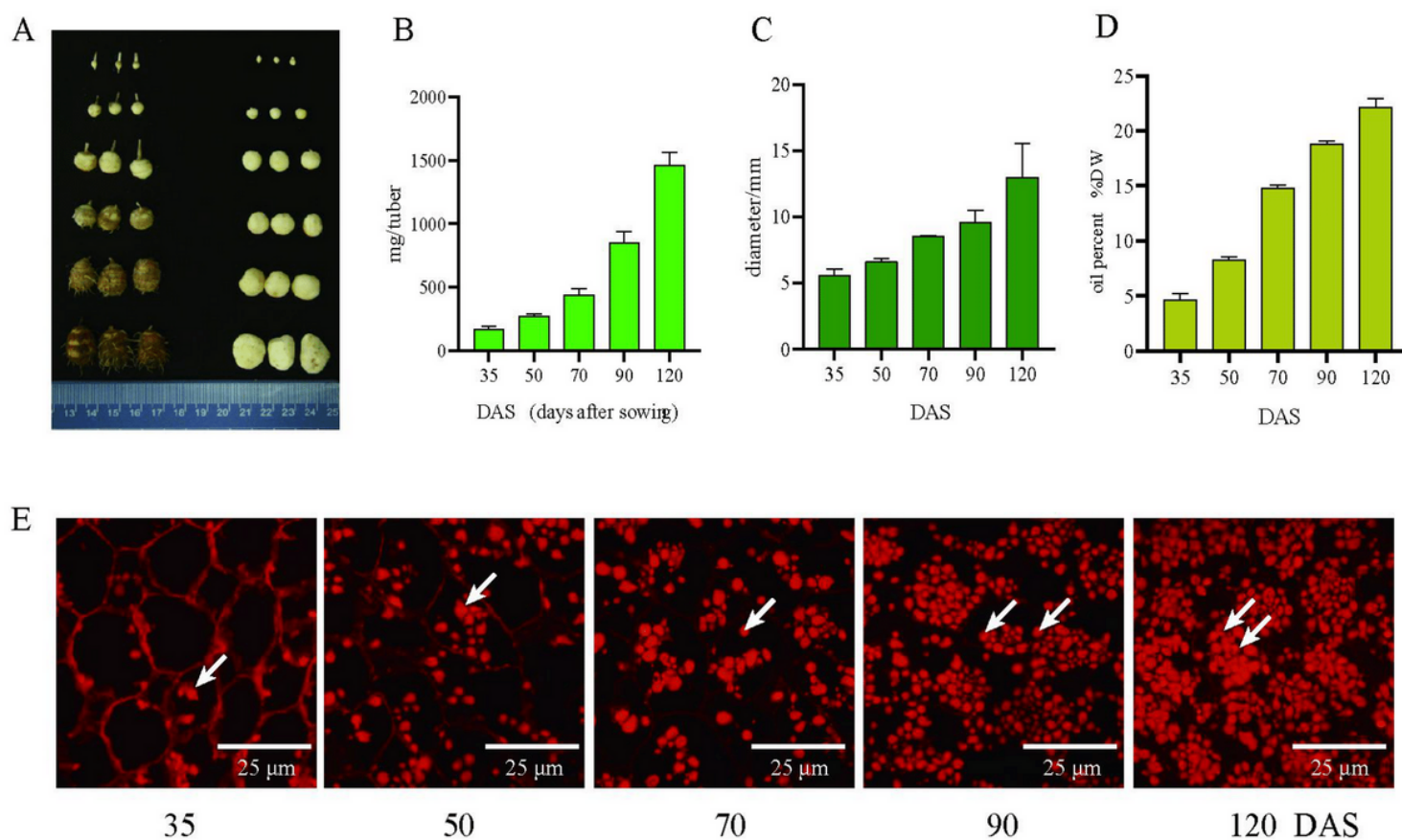
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## Figures



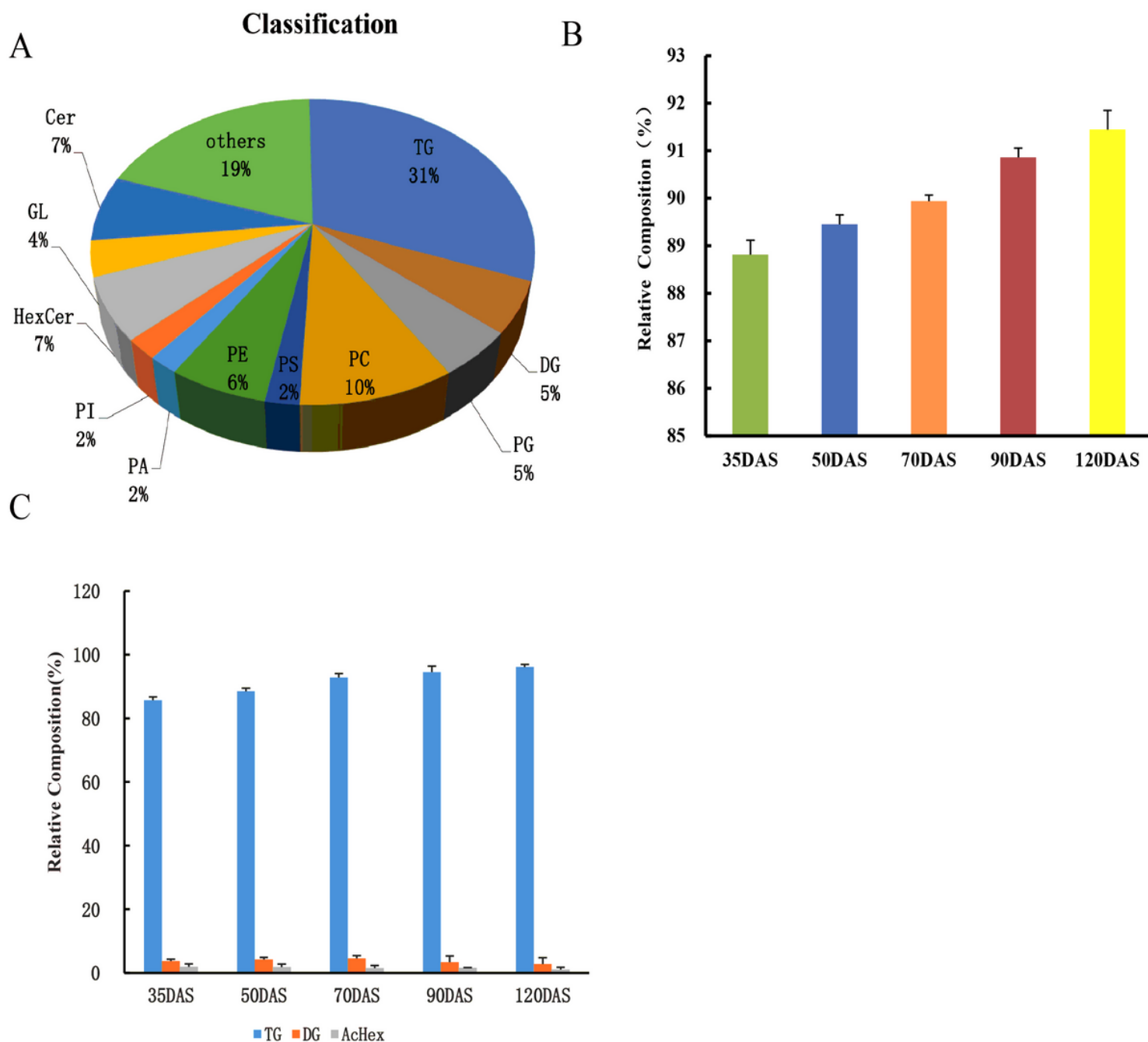
**Figure 1**

Changes in Lipid content and lipid droplets accumulation during tuber development in *C.esculentus*. A. Changes in appearance and growth status of *C.esculentus* tubers. B. Average weight changers per tuber under different development stages. C. Average diameter changes per tuber. D. Draw oil content in dry tubers. E. Lipid droplets accumulation inside the *C.esculentus* tubers.



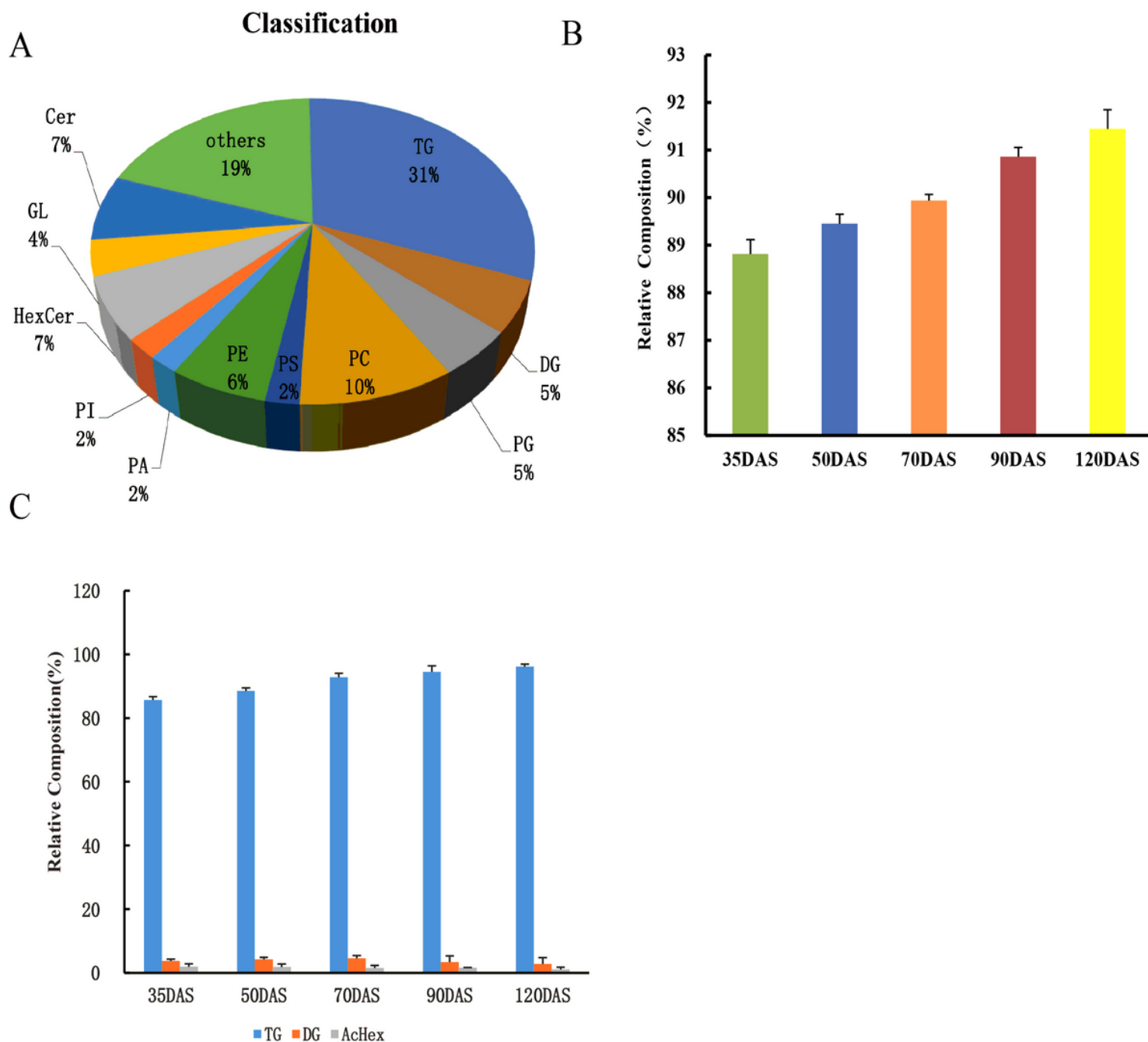
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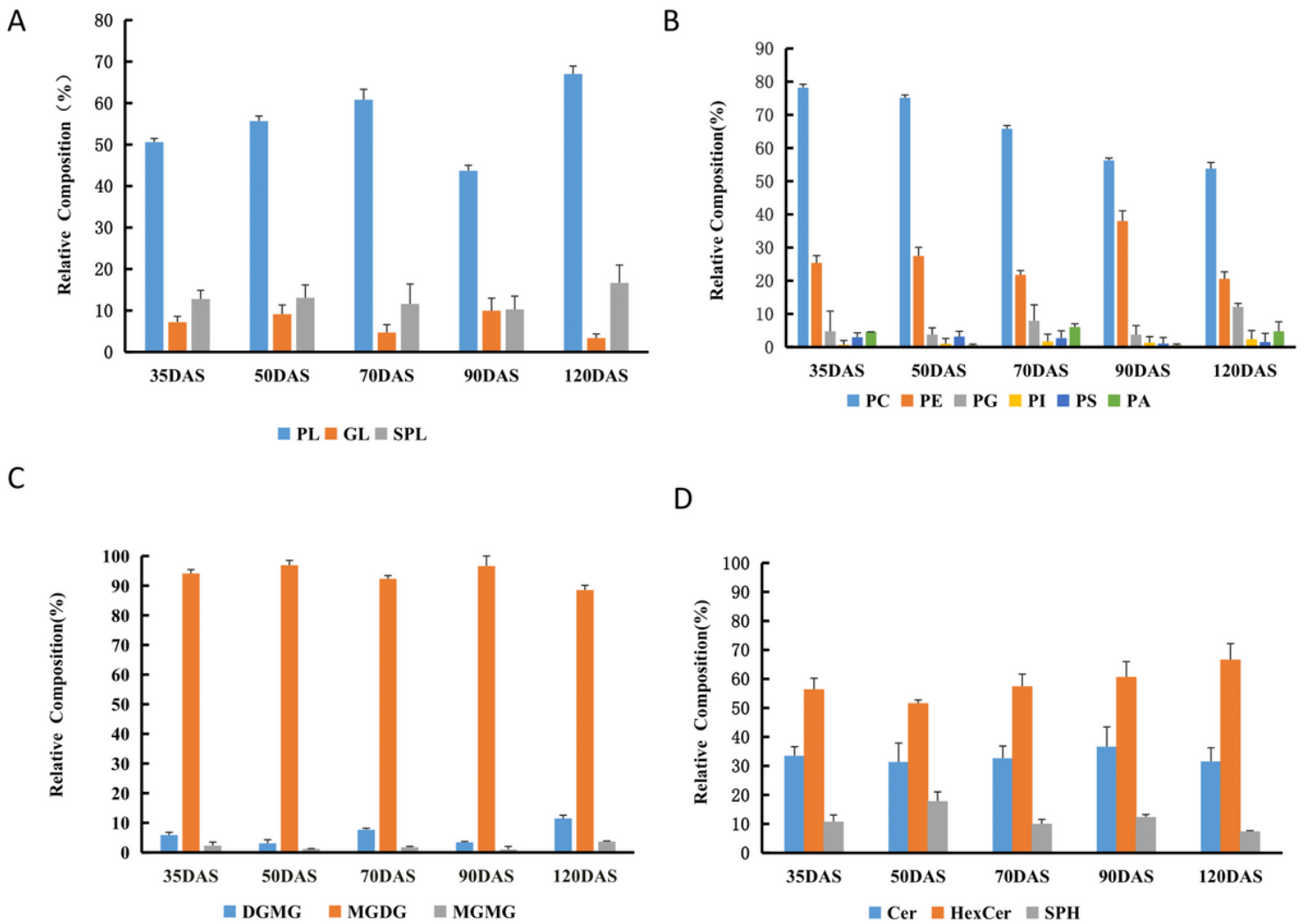
**Figure 2**

Lipids classification and composition. A. Lipid species and proportion; B. The ratio of neutral lipid to total lipids; C. The relative composition of Triacylglycerol account for neutral lipids. Five individual biological samples are depicted, each of which was analyzed with technical six duplicates (means  $\pm$  SD). Five developmental stages were analyzed, 35, 50, 70, 90, and 120 days after sowing (DAS).



**Figure 2**

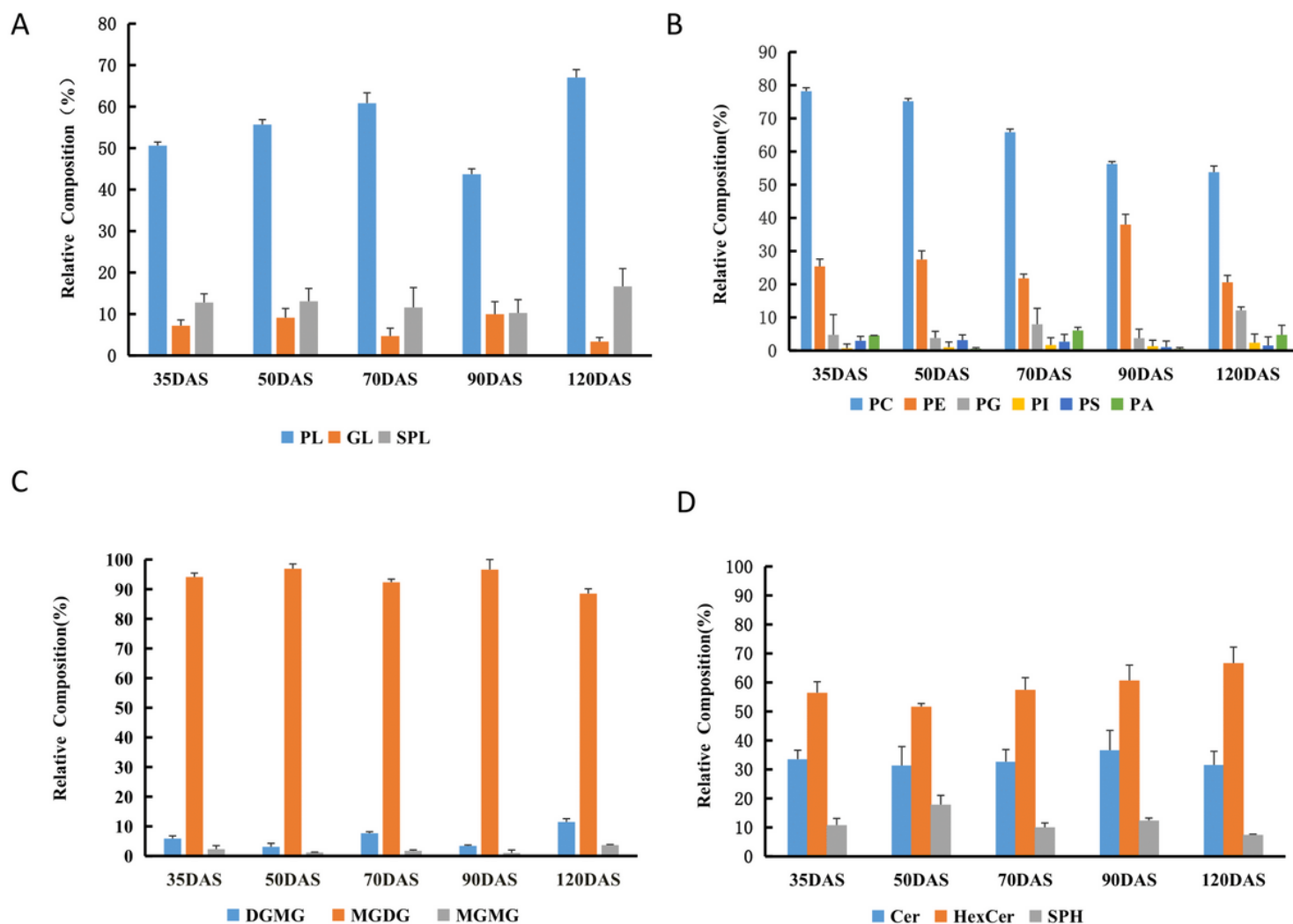
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**Figure 3**

Profiling of polar lipid classes under developing tuber. A. The relative composition of glycolipids, phospholipids, and sphingolipids in the polar lipid. B. Proportion of phospholipid composition during developing; C. Proportion of glycolipid composition during developing; D. Proportion of sphingolipids composition; Five individual biological samples are depicted, each of which was analyzed with technical six duplicates (means  $\pm$  SD shown). Five developmental stages were analyzed.

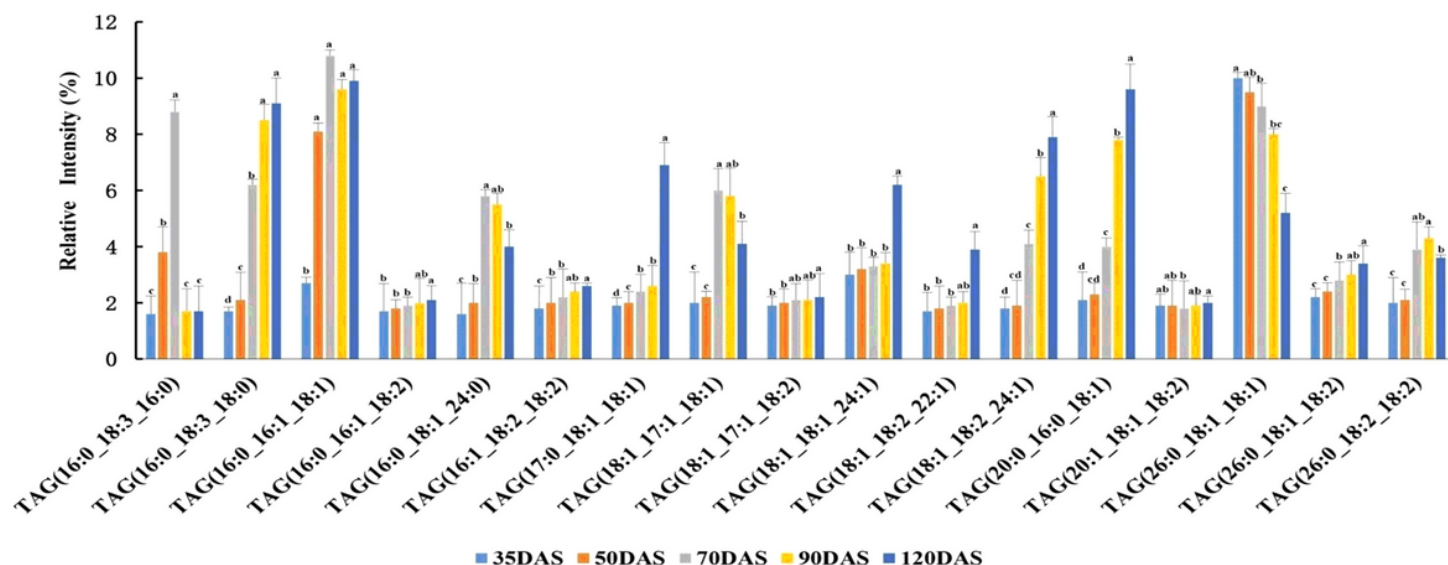




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A



B

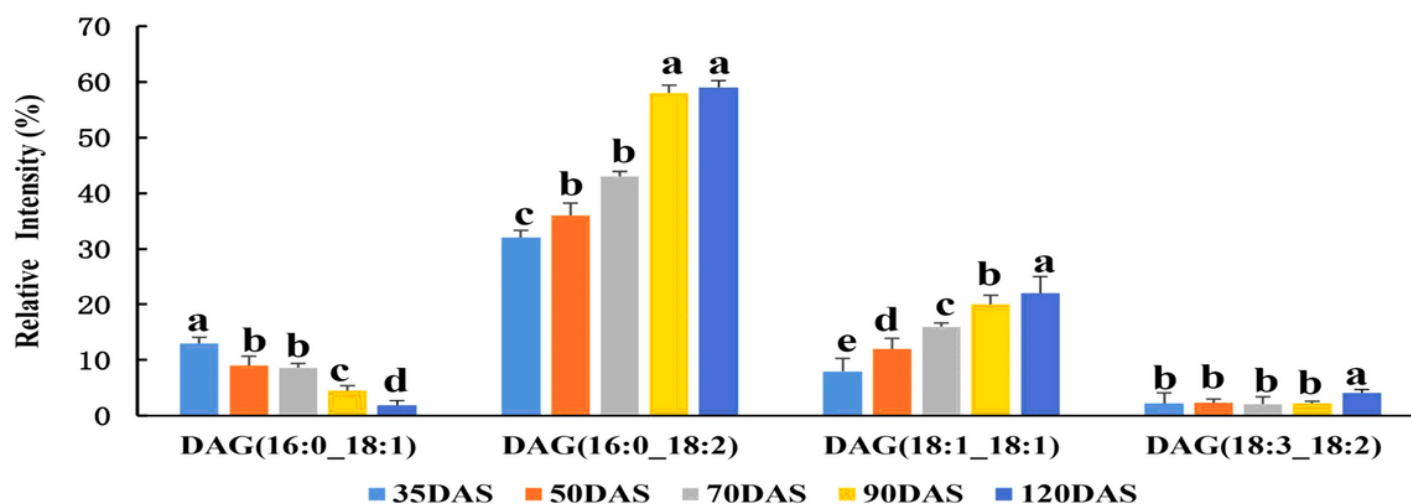
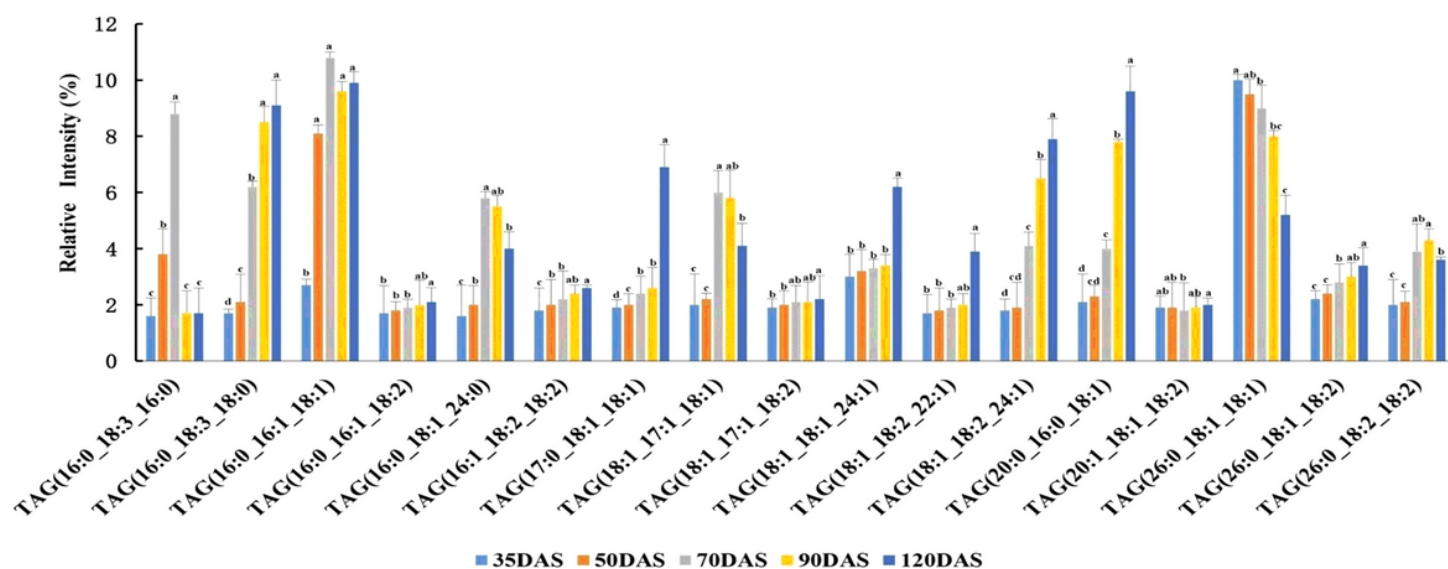


Figure 4

Profiling of molecular species of glycerolipid classes during tuber development. Triacylglycerol (A) and Diacylglycerol (B) molecular species were analyzed by LC/MS as described in Materials and methods. Five developmental time points were analyzed, 35, 50, 70, 90, and 120 days after sowing (DAS) for oil accumulation. (a, b, c, d, e represented significant difference during developing stages with  $p < 0.05$ ).

A



B

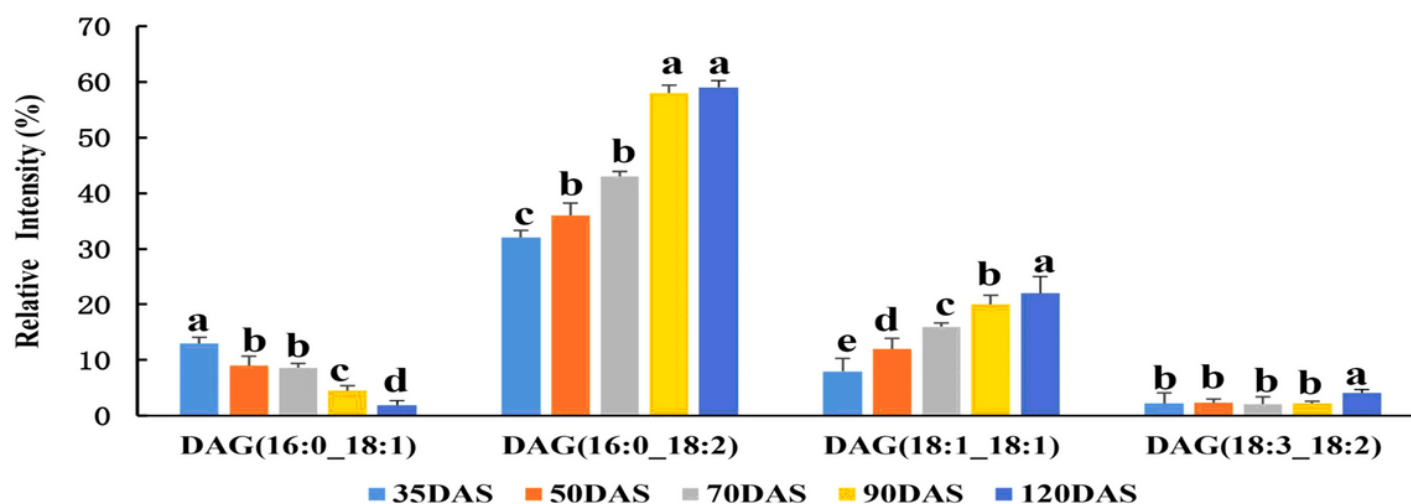
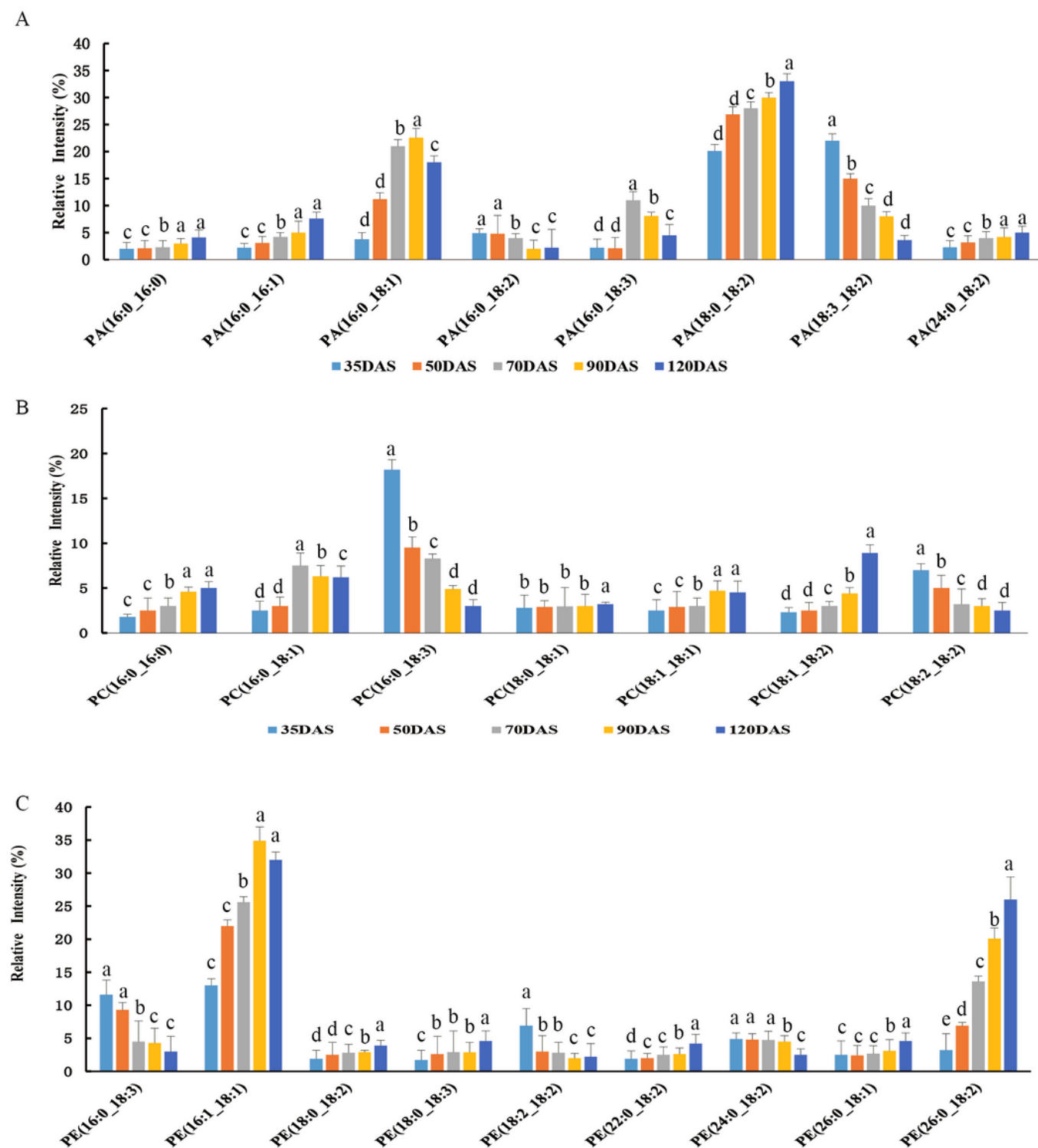


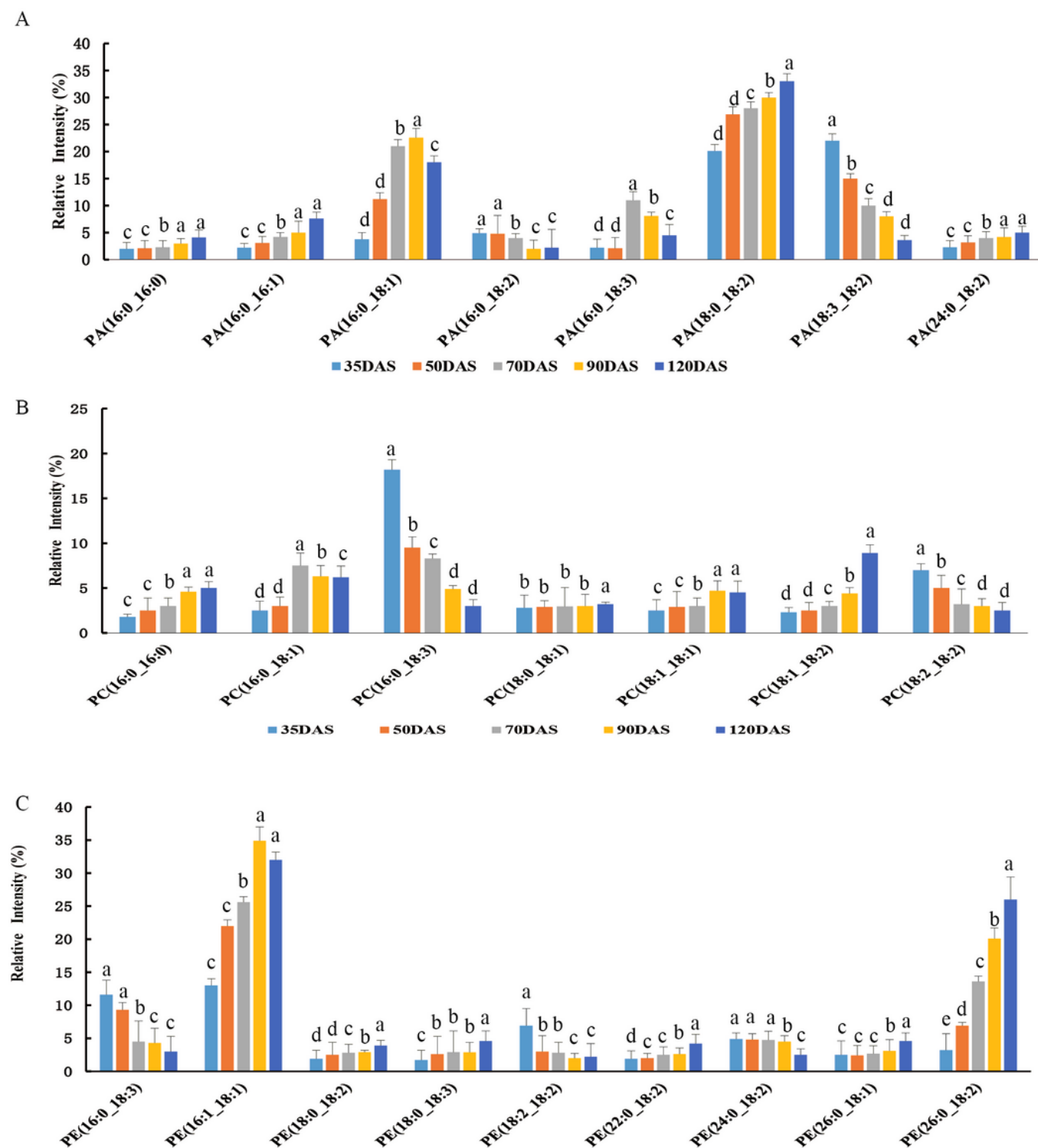
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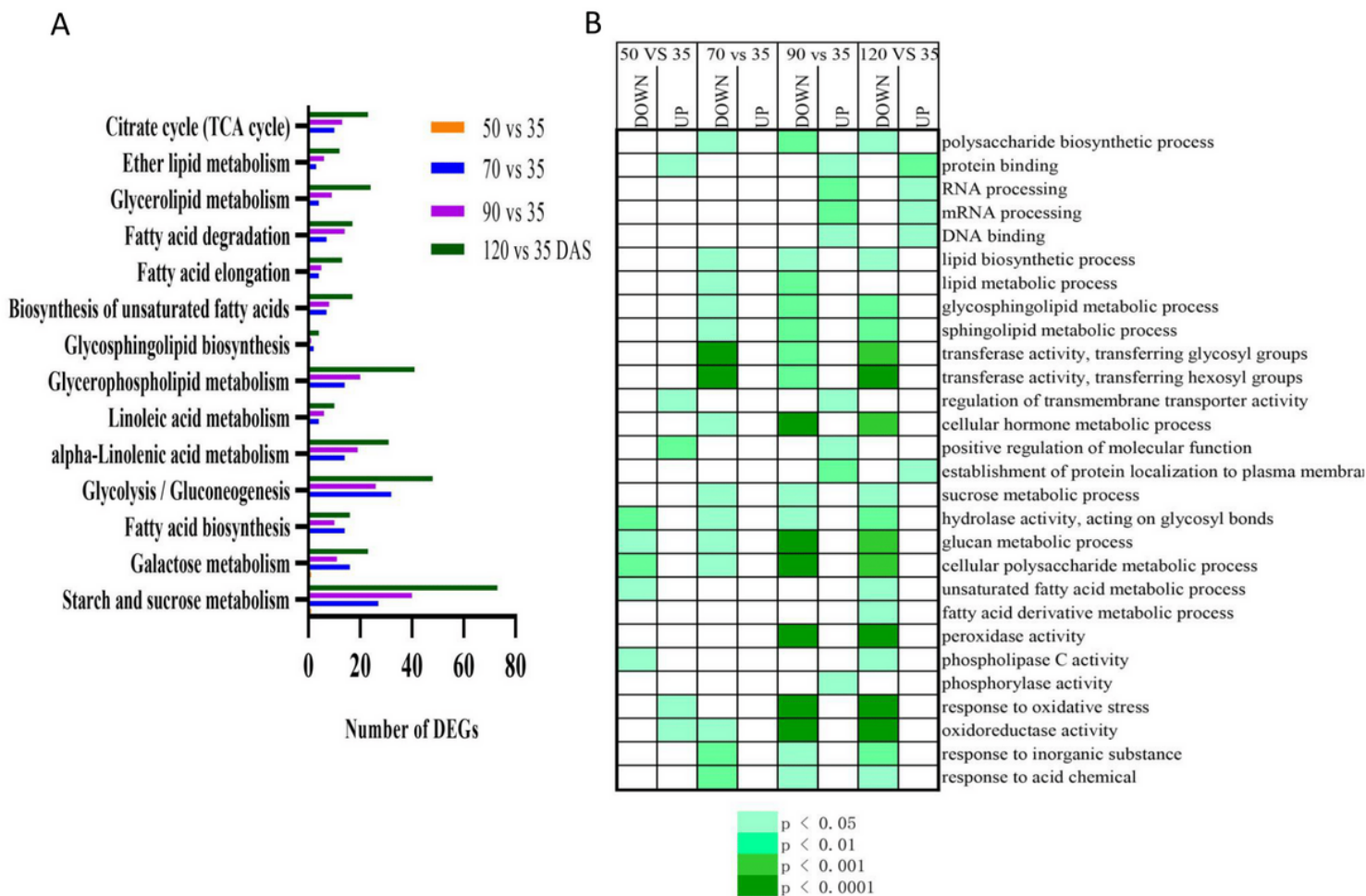
**Figure 5**

Profiling of molecular species of phospholipid classes during tuber development. Phosphatidic (A), phosphatidylcholine (B), and phosphatidylethanolamine (C) molecular species were separated by LC/MS as detailed in Materials and methods.



**Figure 5**

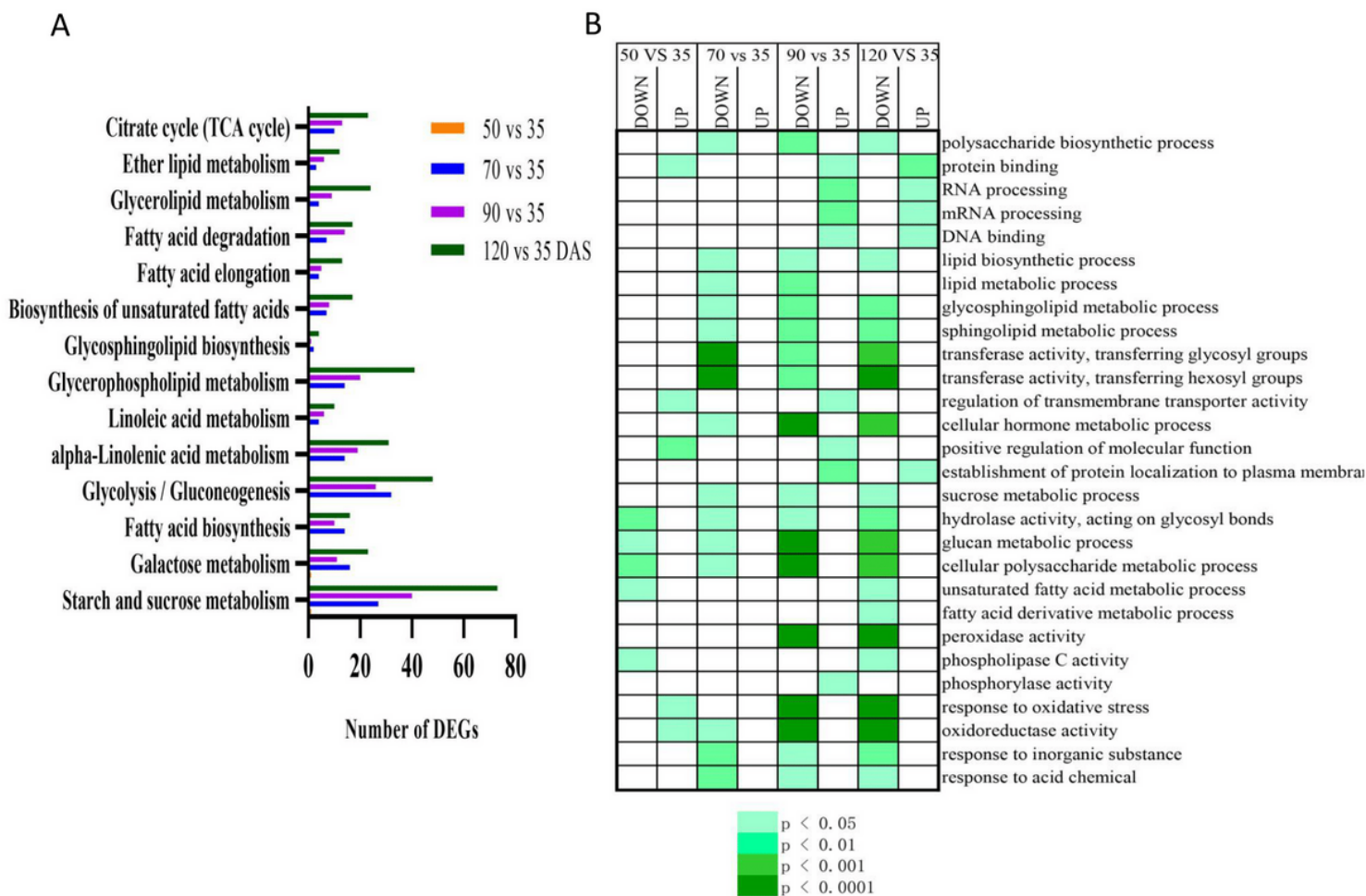
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**Figure 6**

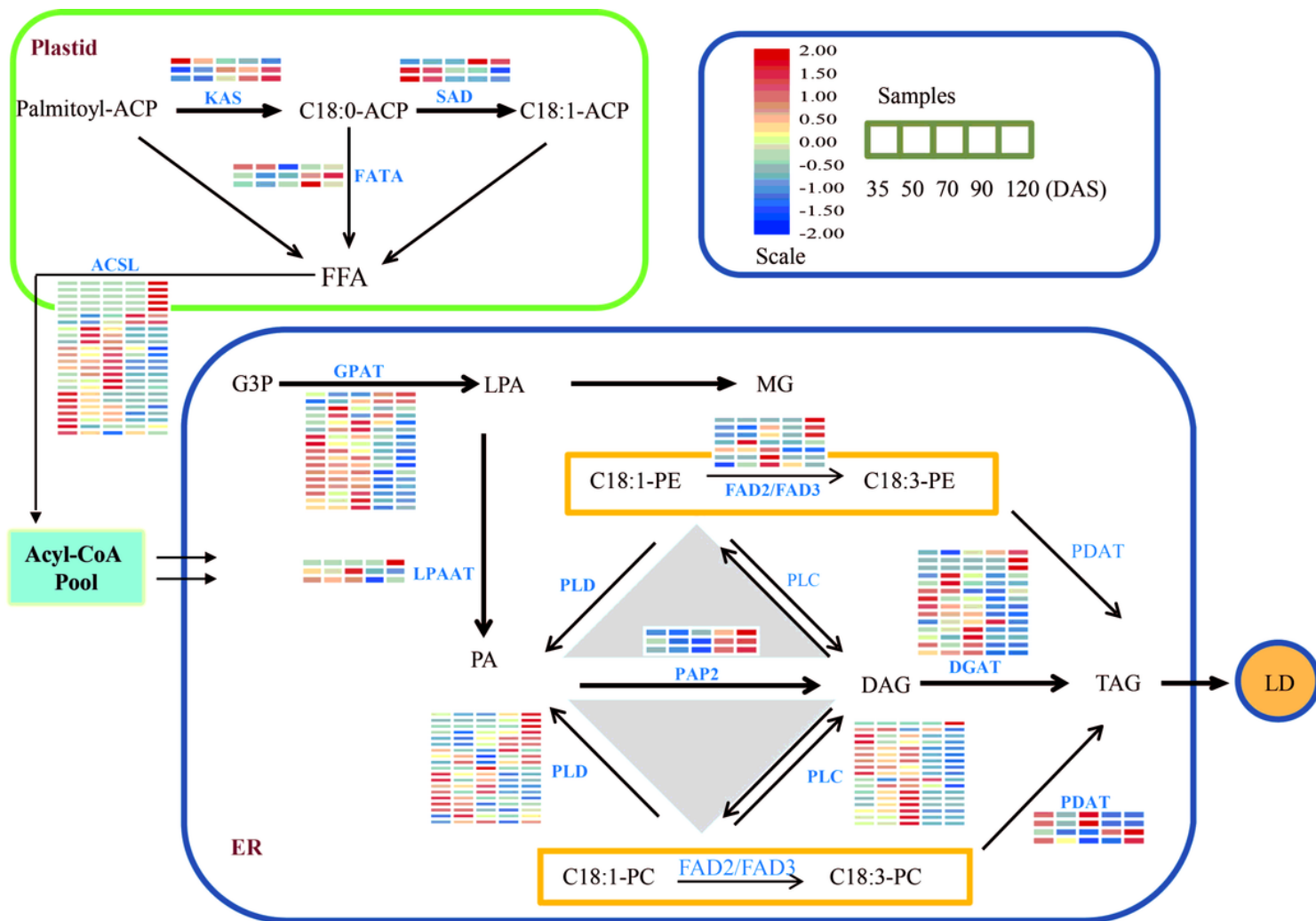
Function annotation of transcripts during tuber development. A. Functional enrichment analyses using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. B. Functional enrichment analyses using GO.





**Figure 6**

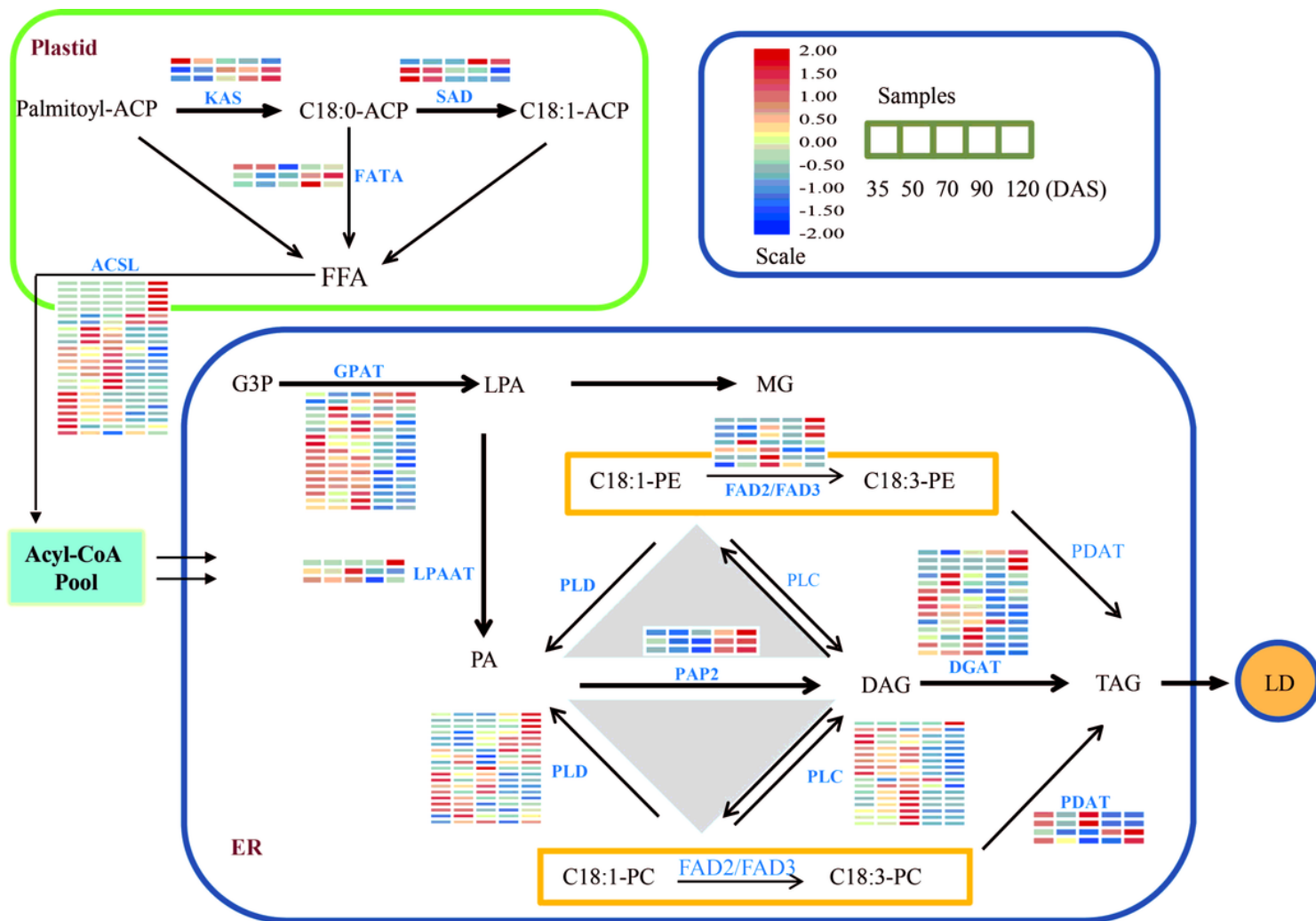
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**Figure 7**

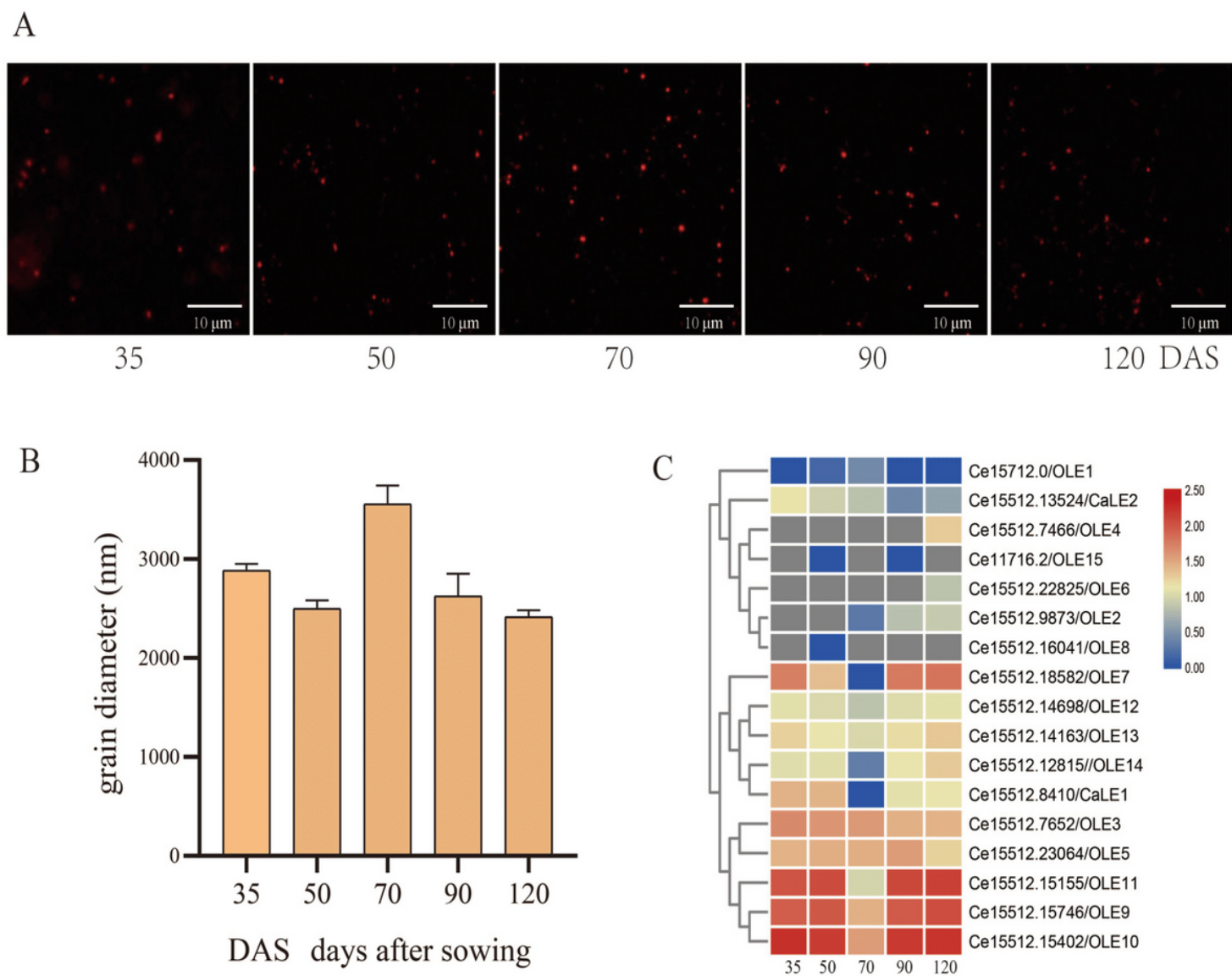
Summary of gene profiling in TAG biosynthesis pathway during tiger nut developing. The heat map squares represent the expression level of genes associated with TAG synthesis.





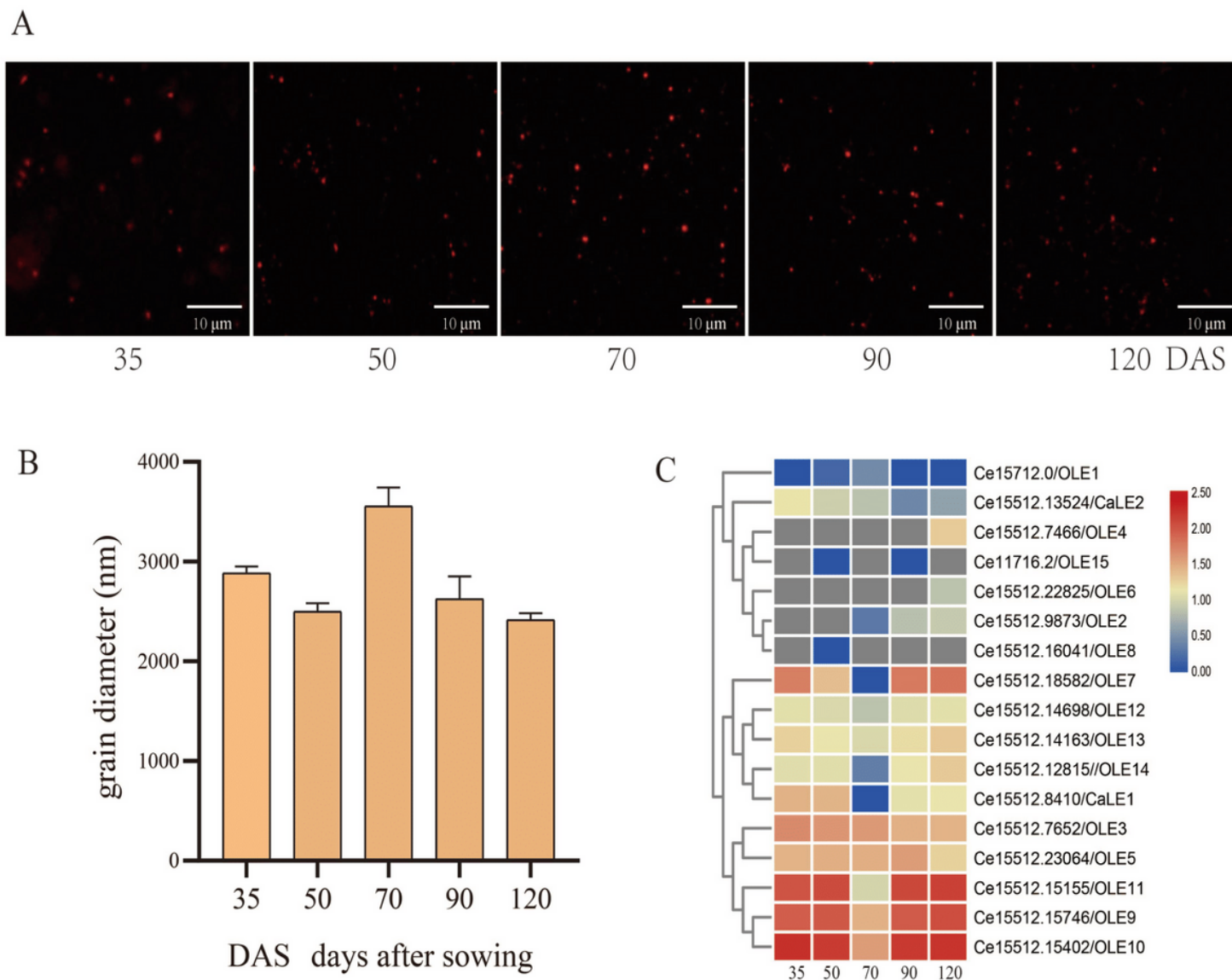
**Figure 7**

Summary of gene profiling in TAG biosynthesis pathway during tiger nut developing. The heat map squares represent the expression level of genes associated with TAG synthesis.



**Figure 8**

Particle sizes of lipid droplets. A. Microscopic image of lipid droplet during different developing stages. B. The particle size of lipid droplet. C. Heatmap of candidate genes associated with lipid droplet protein. The color scale demonstrates Log2FPKM values.



**Figure 8**

Particle sizes of lipid droplets. A. Microscopic image of lipid droplet during different developing stages. B. The particle size of lipid droplet. C. Heatmap of candidate genes associated with lipid droplet protein. The color scale demonstrates Log2FPKM values.

## Supplementary Files

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- [FigureS4.jpg](#)
- [FigureS4.jpg](#)
- [figureS5.png](#)
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