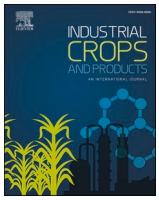
Industrial Crops & Products 180 (2022) 114702

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Integrated lipidomic and transcriptomic analysis reveals triacylglycerol accumulation in castor bean seedlings under heat stress

Yanyu Zhang [a], Yelan Li [b], Bing Han [c], Aizhong Liu [d], Wei Xu [c,*]

[a] College of Life Sciences, Northwest University, Xi'an 710069, Shaanxi, China
[b] College of Life Sciences, Shaanxi Normal University, Xi'an 710119, Shaanxi, China
[c] Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China
[d] Key Laboratory for Forest Resources Conservation and Utilization in the Southwest Mountains of China, Ministry of Education, Southwest Forestry University, Kunming 650224, China

ARTICLE INFO

Keywords:
Heat stress
Triacylglycerols
Lipid remodeling
Polyunsaturated fatty acids
Monogalactosyldiacylglycerol
Castor bean

ABSTRACT

Heat stress is one of the most challenging environmental factors for plants, which have evolved various mechanisms to cope with it. However, the role of lipid remodeling caused by heat stress is not yet fully understood. Here, we integrated UPLC-QTOF/MS-based lipidomic and transcriptomic analyzes to uncover the molecular basis of lipid remodeling under heat stress in castor bean (*Ricinus communis*), an important non-edible oil crop. We detected 297 lipid compounds in castor bean seedlings, of which 54 displayed altered abundances under heat stress. In particular, polyunsaturated triacylglycerols (TAGs) (e.g., TAG54:6, TAG54:7, TAG54:8, and TAG54:9) and diacylglycerols (DAGs) (DAG36:6) significantly increased, while polyunsaturated monogalactosyldiacylglycerols (MGDGs) (MGDG34:3) markedly decreased. When heat stress was subsequently relieved, these lipid molecules recovered to their normal levels. We found that heat-induced TAGs primarily accumulate in the cytosol. Transcriptomic analysis indicated that polyunsaturated fatty acids (FAs) used for TAGs are not derived from *de novo* synthesis, but likely from the lipids remodeling via lipase activity, and are subsequently esterified into TAGs via diacylglycerol acyltransferase (DGAT). Our results suggest that TAGs may act as intermediates in lipid turnover and provide one mechanism by which plants respond to heat stress, broadening our understanding of how lipid remodeling functions in plant adaptation to heat stress.

1. Introduction

Heat, a particularly damaging abiotic stress, poses an enormous threat to global crop yields and food security that has been aggravated by the recent drastic and rapid changes in the global climate (Lobell et al., 2011; Wang et al., 2012; Deryng et al., 2014). Plants have evolved a series of sophisticated mechanisms for coping with heat stress. Researchers have made great progress in advancing our understanding of the physiological, genetic and epigenetic mechanisms underlying these plant responses (reviewed in Liu et al., 2015; Ohama et al., 2017; Zhao et al., 2020). For example, several highly conserved evolutionary mechanisms for sensation and response to heat stress have been elucidated, including reactive oxygen species (ROS), heat shock factors and proteins (HSFs and HSPs), and calcium and hormone signaling pathways (Song et al., 2021; Liu et al., 2015; Zhao et al., 2020). The epigenetic mechanisms including stress memory in response to heat have also been analyzed extensively, revealing the involvement of DNA methylation, histone modification and chromatin remodeling (Liu et al., 2015, 2019; Lämke et al., 2016; Lämke and Bäurle, 2017; Brzezinka et al., 2019; He et al., 2021; Balazadeh, 2021).

Notably, accumulating evidence suggests that lipid remodeling, and triacylglycerol (TAG) accumulation in particular, plays a vital role in plant stress responses, especially at high temperatures (Yang and Benning, 2018; Lu et al., 2020; deVries and Ischebeck, 2020). Heat stress usually leads to higher membrane fluidity and permeability (Lee, 2000; Los and Murata, 2004), and as a response to this, plants generally decrease the ratio of unsaturated to saturated fatty acids (FAs) in their membrane glycerolipids (Falcone et al., 2004; Higashi et al., 2015, 2018). Studies in Arabidopsis revealed that unsaturated membrane glycerolipid is reduced mainly through the removal of polyunsaturated FAs from monogalactosyldiacylglycerol (MGDG) in the chloroplast membrane under heat stress (Burgos et al., 2011; Szymanski et al., 2014;

* Corresponding author.
E-mail address: xuwei@mail.kib.ac.cn (W. Xu).

https://doi.org/10.1016/j.indcrop.2022.114702
Received 14 December 2021; Received in revised form 17 February 2022; Accepted 19 February 2022
Available online 25 February 2022

Higashi et al., 2015, 2018). Subsequently, these unsaturated free fatty acids (FFAs) are immediately assembled into the neutral glycerolipid TAGs to reduce the toxicity of FFAs to cells (Mueller et al., 2015; Lu et al., 2020). Generally, TAGs are not soluble in the aqueous phase of cells; thus, their accumulation would not contribute to the osmotic potential of the cell, providing one mechanism by which plants respond and adapt to heat stress. However, the precise pathways of TAG biosynthesis under heat stress remain to be fully elucidated in plants.

In higher plants, TAG (three FAs esterified to a glycerol backbone) is synthesized by diverse routes, as summarized in Fig. 1 (Li-Beisson et al., 2013; Vanhercke et al., 2017). The major route of TAG biosynthesis involves two biological processes: *de novo* FA synthesis in plastids and assembly in the endoplasmic reticulum (ER). In the plastids, FA synthesis is initiated by the irreversible carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACCase) to form malonyl-CoA. Subsequently, the utilization of acetyl-CoA as a building block FA progresses via sequential condensation catalyzed by fatty acid synthases (FASs). FAs are released from FAS machinery, ultimately activated to CoA esters by a long-chain acyl-CoA synthetase (LACS) and exported to the ER. Within the ER, FAs are in turn esterified to glycerol-3-phosphate (G3P) via the Kennedy pathway. FAs are first esterified at the *sn*-1 position of G3P by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA), which is then acylated at the *sn*-2 position by lysophosphatidic acid acyltransferase (LPAAT) to produce phosphatidic acid (PA). Subsequently, PA is converted into *sn*-1,2-diacylglycerol (DAG) by phosphatidic acid phosphatases (PAPs) or other phospholipids such as phosphatidylglycerol (PG) and phosphatidylinositol (PI). In the final step, DAG is acylated on the *sn*-3 position using a fatty acyl-CoA

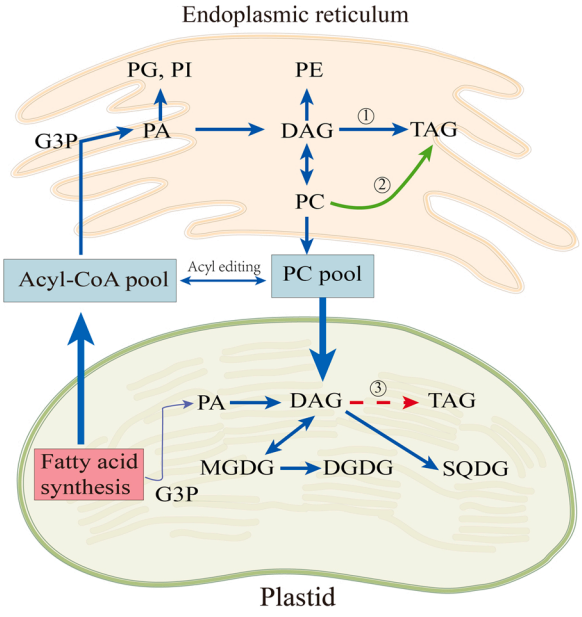


Fig. 1. Biosynthetic pathways of triacylglycerol (TAG) and chloroplast lipids (MGDG, DGDG and SQDG) in higher plants. In brief, fatty acids are de novo synthesized in plastid and exported to acyl-CoA pool in endoplamic reticulum (ER). Subsequently, these FAs were in turn esterified to glycerol-3-phosphate (G3P) to product PA, DAG and TAG. TAG in ER is synthesized and involves two different pathways: Kennedy pathway (Route 1, from DAG to TAG) and acyl-CoA-independent pathway (Route 2 indicated by green arrow, from PC to TAG). Meanwhile, phospholipids such as PG, PI, PE and PC are produced in ER. Besides, in plastid, TAG (Route 3 indicated by red arrow) and other chloroplast lipids including MGDG, DGDG and SQDG can be synthesized using DAG as substrate. Abbreviations: DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; G3P, glycerol-3-phosphate; MGDG, Monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidyl glycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol.

molecule by diacylglycerol acyltransferase (DGAT) to yield TAG. TAG can also be synthesized via an acyl-CoA-independent pathway, in which DAG is acylated using phosphatidylcholine (PC) as the acyl donor to produce TAG, a process catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT). Once synthesized, TAGs are packaged to form oil bodies or lipid droplets and stored in the cytosol. However, it should be noted that TAG can be synthesized and stored in the chloroplast (within plastoglobules), where it is referred to as chloroplastic TAG (Lippold et al., 2012). In chloroplasts, most DAG molecules are used to synthesize glycerolipids, including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and the sulfolipid sulfoquinovosyl diacylglycerol (SQDG) (reviewed in Hölzl and Dörmann, 2019). Meanwhile, TAG and other lipids can be hydrolyzed by cellular lipases to release FFAs, participating in lipid remodeling or turnover.

Castor bean (*Ricinus communis* L. Euphorbiaceae) is an important non-edible oil crop. The castor bean plant originates from tropical East Africa and is extensively cultivated in tropical and subtropical regions (Moshkin, 1986; Chan et al., 2010; Xu et al., 2021). Owing to the high level of ricinoleic acid (over 90%) in its seeds, the castor bean oil has been widely used in industry for making lubricants, cosmetics, coatings, inks, plastics, and biodiesel (Ogunniyi, 2006). Therefore, there has been considerable interest in breeding to improve varieties with high seed yield or oil content. Significant progress has furthered our understanding of how ricinoleic acid and TAGs are synthesized in castor bean seeds (Chan et al., 2010; Brown et al., 2012). Several key genes involved in this pathway were identified and functionally characterized, including those encoding oleate 12-hydroxylase (Van de Loo et al., 1995; Broun and Somerville, 1997; Venegas-Calerón et al., 2016), DGAT (Burgal et al., 2008), and PDAT (Kim et al., 2011). Meanwhile, castor bean displays a striking ability to thrive and produce high yields in adverse environments where most other crops would not grow (Kumar et al., 1997; César and Batalha, 2010; Sausen and Rosa, 2010). Specifically, castor bean is able to grow despite drastic daily and seasonal temperature cycles between 15 ℃ and 45 ℃ (Kumar et al., 1997) and therefore provides an ideal candidate in which to study the molecular basis of plant adaptation to high temperatures. However, whether TAG is involved in castor bean high-temperature tolerance remains unclear. Therefore, in our study, we explored the accumulation of TAGs in castor bean seedlings in response to heat stress via integrative lipidomics and transcriptomics analyzes.

2. Materials and methods

2.1. Plant material and heat treatment

Seeds from castor bean var. ZB306 (kindly provided by the Zibo Academy of Agricultural Sciences, Shandong, China) were surface sterilized and placed on wet filter paper for germination at 25 ℃. After 2 days, the germinated seeds were transplanted into potting soil and grown in a glasshouse under standard light conditions (16-h-light/8-h-dark cycle, 25 ℃/22 ℃) at Kunming Institute of Botany (Kunming, Yunnan, China). Four-week-old seedlings were then divided into two groups, each with 20 seedlings. Seedlings grown at 25 ℃ for 12 h-light condition were defined as the control group (hereafter named CK), and those grown at 45 ℃ for 12 h-light condition were considered the heat stress group (named HS). After heat treatment, half of seedlings were returned to normal conditions (25 ℃ for 12 h-night condition) and were defined as the recovery group (RHS). Seedlings of each group were collected separately, frozen immediately in liquid nitrogen, and stored in an ultra-low-temperature freezer at − 80 ℃ for subsequent analyzes.

2.2. Lipid analysis by thin-layer chromatography

To investigate lipid remodeling under heat stress, we first measured abundance changes of different lipid molecules by thin-layer chromatography (TLC), including neutral glycerolipids (TAG and DAG), free

fatty acids (FFAs), phospholipids (PG, PA, and PC), and membrane glycerolipids (MGDG, DGDG, and SQDG). An abbreviated list is provided in Table S1. These different lipids were extracted from seedlings of CK, HS and RHS with three independent biological replicates, and identified using standards purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Briefly, approximately 0.2 g of frozen fresh tissue was homogenized using a mortar and pestle with liquid nitrogen, and then total lipids were extracted with hexane/isopropanol (3:2, v/v). Total lipids were then redissolved in chloroform/methanol (1:1, v/v). The neutral glycerolipids and FFAs were separated on commercial silica gel 60 F254 TLC plates (Beijing Stronger Science Co., Ltd., Beijing China) that were dipped in a solution of hexane/diethylether/acetic acid (80:20:1, v/v/v). Subsequently, the neutral glycerolipids were visualized by placing TLC plates into iodine vapor and identified using standards run in parallel with samples. For phospholipid analysis, we employed high-performance thin-layer chromatography (HPTLC) to separate PG, PA, and PC using a solution of chloroform/methanol/water/triethylamine (35:35:7:35, v/v/v/v). The TLC plates were sprayed with anisaldehyde (Aladdin, Shanghai China) and oven-dried (120 ℃ for 1 h) for visualization. As for chloroplast membrane glycerolipid analysis, an HPTLC method was used to separate MGDG, DGDG, and SQDG with acetone/methylbenzene/water (91:30:7.5, v/v/v) as a solvent. The separated bands were sprayed with anisaldehyde and visualized by heating in the oven (120 ℃ for 1 h). TLC results were quantified using ImageJ.

2.3. UPLC-QTOF/MS-based lipidomics and statistical analysis

To precisely measure lipidome-wide alterations under heat stress, we performed ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS)-based lipidomic analysis (Higashi et al., 2015; Okazaki and Saito, 2018). Seedlings from the CK, HS, and RHS groups were collected with three independent biological replicates each. Approximately 35 mg of sample was ground in liquid nitrogen and then inner standards (80 μL, 10 μg/mL, Table S2), dichloromethane (2 mL), and methanol (2 mL) were added for each sample. The mixture was vortexed for 1 h at room temperature. Subsequently, dichloromethane (2 mL) and deionized water (1.6 mL) were added, and the mixture was vortexed for another 5 min and then spun for 3 min at 4500 rpm at 4 °C. After that, the lower, organic phase was collected and the remaining solution was extracted twice using the dichloromethane (4 mL). The entire lower, organic phase was combined and dried with gaseous nitrogen. Finally, the residue was dissolved with 1 mL of isopropanol and filtered through organic membranes.

One microliter of solution was first subjected to analysis with a Shimadzu UHPLC LC-30A with a Phenomenex Kinete C18 column (2.1 × 100 mm^2, 2.6 μm) at 60 °C. Chromatographic separation was performed by a gradient elution using H_2O/MeOH/ACN (1:1:1) containing 5 mM NH4Ac (A) and IPA/CAN (5/1) containing 5 mM NH4Ac (B) with a flow rate of 0.4 mL/min. The procedures of elution were based on the following criteria: 20% B from 0 to 0.5 min; 40% B from 0.5 to 1.5 min; 60% B from 1.5 to 3.0 min; 98% B from 3.0 to 13.0 min; 20% B from 13.0 to 13.1 min; and 20% B from 13.1 to 17.0 min. Next, the separated substances were introduced into the mass spectrometer (AB Sciex TripleTOF® 6600) and were detected in the positive mode from 100 to 1200 m/z according to the following parameters: curtain gas: 35,000 psi; ion source gas1: 50.000; ion source gas2: 50.00; ion spray voltage: 5500.00 V; temperature: 600 °C.

The acquired data were qualitatively and quantitatively analyzed by MSDIAL (ver. 4. 00), PeakView (ver. 2.1), and MultiQuant (ver. 3. 0. 2) software. To identify differential lipid metabolites between the two groups, we adopted partial least squares-discriminant analysis (PLS-DA), a supervised method for multivariate data discrimination. The variable importance projection (VIP), fold change (FC), and false discovery rate (FDR) were calculated in the PLS-DA model. Metabolites with significant differences were determined by VIP value ≥ 1 and FDR ≤ 0.05. Meanwhile, we identified the strongly altered lipid species as markers with FC ≥ 2.

2.4. Subcellular localization of TAGs

As mentioned above, TAGs can be synthesized and stored in the cytosol or chloroplasts. To determine where TAGs were stored, we performed Nile red staining experiments in castor bean protoplasts isolated from leaves of four-week-old seedlings of CK, HS and RHS according to previously described protocols (Yoo et al., 2007; Zhai et al., 2009). In brief, young leaves were sectioned into 1-mm slices using a fresh razor blade in sterile disposable Petri dishes. These slices were then rapidly treated with filter-sterilized enzyme solution (10 mL, pH 5.7) that contained 0.4 M D-mannitol, 10 mM MES hydrate, 20 mM $CaCl_2$, 20 mM KCl, 0.1% BSA, 1.5% cellulase, and 0.4% macerozyme (Onozuka R-10). Before the enzyme solution was used, it needed to be incubated in a 55 °C water bath for 10 min and then cooled to room temperature and filter-sterilized using a syringe filter. Next, a vacuum was applied for 30 min and then released. The reaction was incubated on an orbital shaker at 50 rpm for 3 h under dark conditions at room temperature. After enzyme digestion, the product was washed by adding an equal volume of W5 wash buffer (2 mM MES, pH 5.7, containing 154 mM NaCl, 5 mM KCl, and 125 mM $CaCl_2$) and filtered through a 100-mesh nylon mesh to remove undigested tissues. Protoplasts were collected by centrifugation at 100 × g for 5 min at room temperature and incubated using W5 wash buffer for 1 h on ice until precipitation of the protoplasts was complete. Finally, the supernatant was discarded and protoplasts were resuspended in sterilized WI solution containing 20 mM KCl, 0.5 M mannitol, and 4 mM MES. Approximately 500 μL of protoplast cell suspension was stained with 3 μL Nile red dye (1 mg/mL in dimethyl sulfoxide [DMSO]). The mixtures were then incubated in the dark for 10 min at room temperature. The stained protoplasts were imaged using a confocal laser-scanning microscope (Olympus FV-1000, Tokyo, Japan).

2.5. Transcriptome sequencing and analyzes

Total RNA was isolated from seedlings using Trizol reagent (GENEray, SHH, CHN) following the manufacturer's directions. Three biological replicates were used for each group (CK, HS, and RHS). The concentration and integrity of the RNA were measured on a Qubit 3.0 device (Thermo, Waltham, MA, USA) and by gel electrophoresis (Bio-Rad, Hercules, CA, USA), respectively. High-quality RNAs were reverse transcribed into cDNA and then sonicated to the fragment size of 300–500 bp. The RNA-seq libraries were constructed using the TruSeq® Stranded mRNA Sample Preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions and sequenced on an Illumina HiSeq X Ten system (Illumina, San Diego, CA, USA) at Shanghai OE Biotech Co., Ltd. (Shanghai, China).

Raw reads obtained from RNA-seq were first preprocessed to remove adapter sequences, low-quality reads, and contaminating sequences. Subsequently, the clean reads were aligned to the castor bean reference genome (downloaded from our website, http://oilplants.iflora.cn/) using HISAT2 version 2.0.4, and gene expression was calculated and normalized as FPKMs (fragments per kilobase million) using StringTie version 1.3.1 (Pertea et al., 2016). Differentially expressed genes (DEGs) between the two groups were determined by fold change ≥ 2 and adjusted $P \leq 0.05$ using DEseq software (Anders and Huber, 2010). The functional enrichment analysis including GO and KEGG terms was performed using the OmicShare tools (www.omicshare.com/tools).

2.6. Quantitative real-time PCR validation

We collected three independent seedlings from the CK, HS, and RHS groups to validate the expression levels of the candidate genes we

selected. Total RNA was isolated and purified as mentioned above. High-quality RNAs were reverse transcribed using the PrimeScript RT Reagent Kit (TransGen Biotech, Transgen Biomedical Technology Co., Ltd., Beijing, China). Quantitative reverse-transcription PCR (qRT-PCR) reactions were performed using SYBR Green Master Mix (TransGen Biotech) on the CFX96 machine (Bio-Rad, USA). The *ACTIN2* gene (30206.m000761) was used as an internal reference to normalize the relative expression level of all genes. All primers used in this study are listed in Table S3.

3. Results and Discussion

3.1. Heat induced the accumulation of TAGs in castor bean seedlings

The four-week-old seedlings of castor bean var. ZB306 were used for heat stress (HS) and recovery (RHS) as described in the Methods. Compared with seedlings from the CK group, the HS seedlings exhibited no obvious phenotypic differences, suggesting a strong tolerance of castor bean seedlings to heat stress (Fig. 2A). To investigate the changes in lipid molecules, we initially isolated the neutral glycerolipids (DAGs and TAGs), polar chloroplast membrane glycerolipids (MGDG, DGDG, and SQDG), free fatty acids (FFAs), and phospholipids (PG, PA, and PC) from the seedlings in the CK, HS, and RHS groups and performed TLC analysis. As a results, we found that the content of TAG was increased to 2.4-fold and MGDG was decreased to 47% in HS group relative to CK group (Fig. 2B). After recovery from HS, the content of TAG was reduced, but still relatively higher (1.5-fold) than in the CK group. The contents of other glycerolipids, such as DGDG and SQDG, were not obviously altered in HS and RHS relative to CK. As for phospholipids, we did not observe any difference in the contents of PG, PA, or PC in the seedlings from the CK, HS, and RHS groups (Fig. 2B). The content of FFA seems to be slightly higher (1.2-fold) in HS than in CK. These results show that heat stress can induce the accumulation of TAG and the reduction of MGDG in castor bean seedlings.

3.2. Lipidomic analysis reveals the dynamic changes of lipid compounds under heat stress and during recovery

To quantitatively measure the dynamic changes of lipid compounds under heat stress, we performed lipidomics for castor bean seedlings from CK, HS, and RHS with three biological replicates using the UPLC-QTOF/MS technique (see Methods). A total of 297 lipid molecules were identified (Table S4). Pearson correlation analysis showed a significant positive correlation between biological replicates ($r_p \geq 0.99$, Fig. S1A), suggesting that our data are reliable for subsequent analyzes. Principal component analysis (PCA) and hierarchical clustering revealed three major groups, corresponding to CK, HS, and RSH (Fig. S1B, C). These lipids were further classified into four categories according to LIPID MAPS comprehensive classification system (Fahy et al., 2009): (1) glycerolipids (including 91 TAGs, 17 DAGs, 4 Monoacylglycerols [MGs], 15 MGDGs, 16 DGDGs, 10 SQDGs, and 5 Diacylglycerylglucuronides [DGGAs]); (2) phospholipids (including 5 PAs, 16 PCs, 36 phosphatidylethanolamines [PEs], 13 PGs, and 5 PIs); (3) sphingolipids (including 5 hexosylceramides [HexCers] and 28 ceramides [Cers]); and (4) free fatty acids (31 FFAs) (Fig. S1D). Among them, MGDG and PI were abundant in castor bean seedlings (Fig. 3A), but only trace amounts of Cer, HexCer, and DGGA were detected (Table S4). Among FFAs, polyunsaturated FAs such as C18:2 and C18:3 were major components in

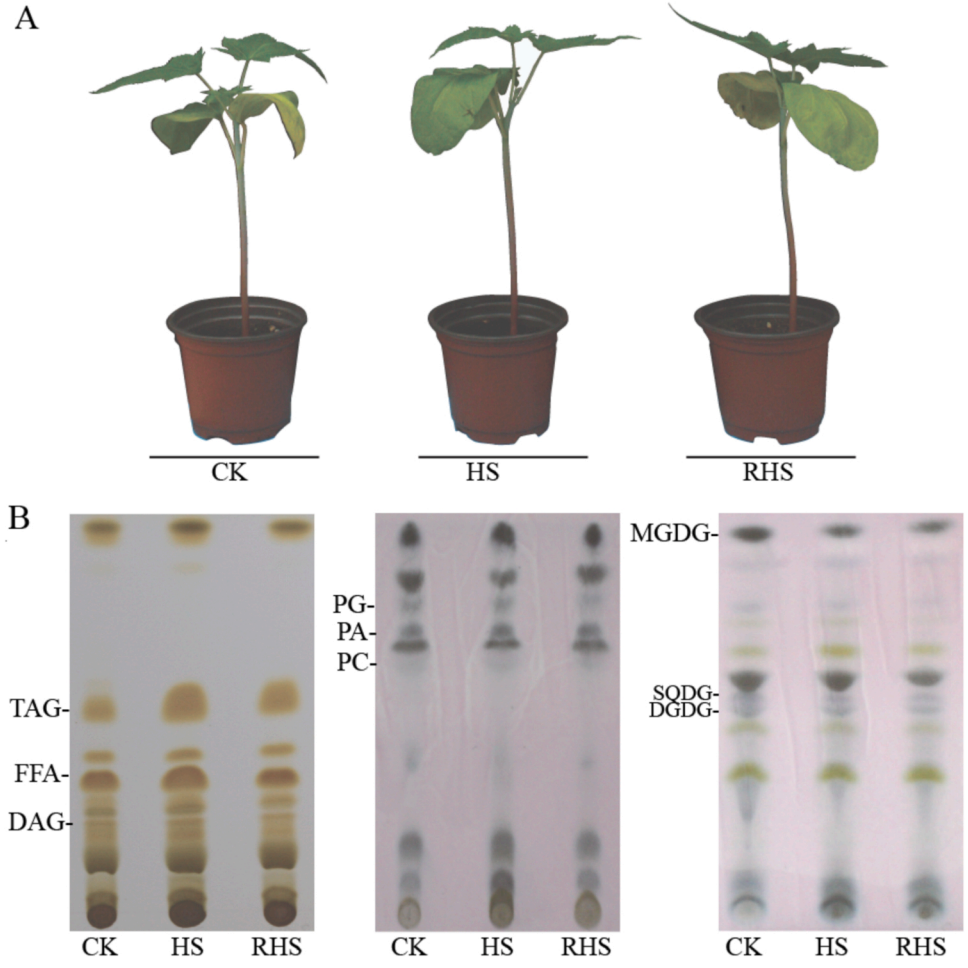


Fig. 2. Heat-induced phenotypic change and lipids remodeling in castor bean seedlings. (A) Appearance of normal seedling grown in 25 ℃ (CK), heat-treated seedling grown in 45 ℃ for 12 h (HS) and recovered seedling grown in 25 ℃ for 12 h (RHS). (B0Thin-layer chromatography (TLC) analysis for neutral glycerolipids lipids (DAGs and TAGs), free fatty acids (FFAs), phospholipids (PG, PA and PC) and membrane glycerolipids (MGDG, DGDG and SQDG). Abbreviations: DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; FFA, free fatty acids; MGDG, Monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidyl glycerol; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol.

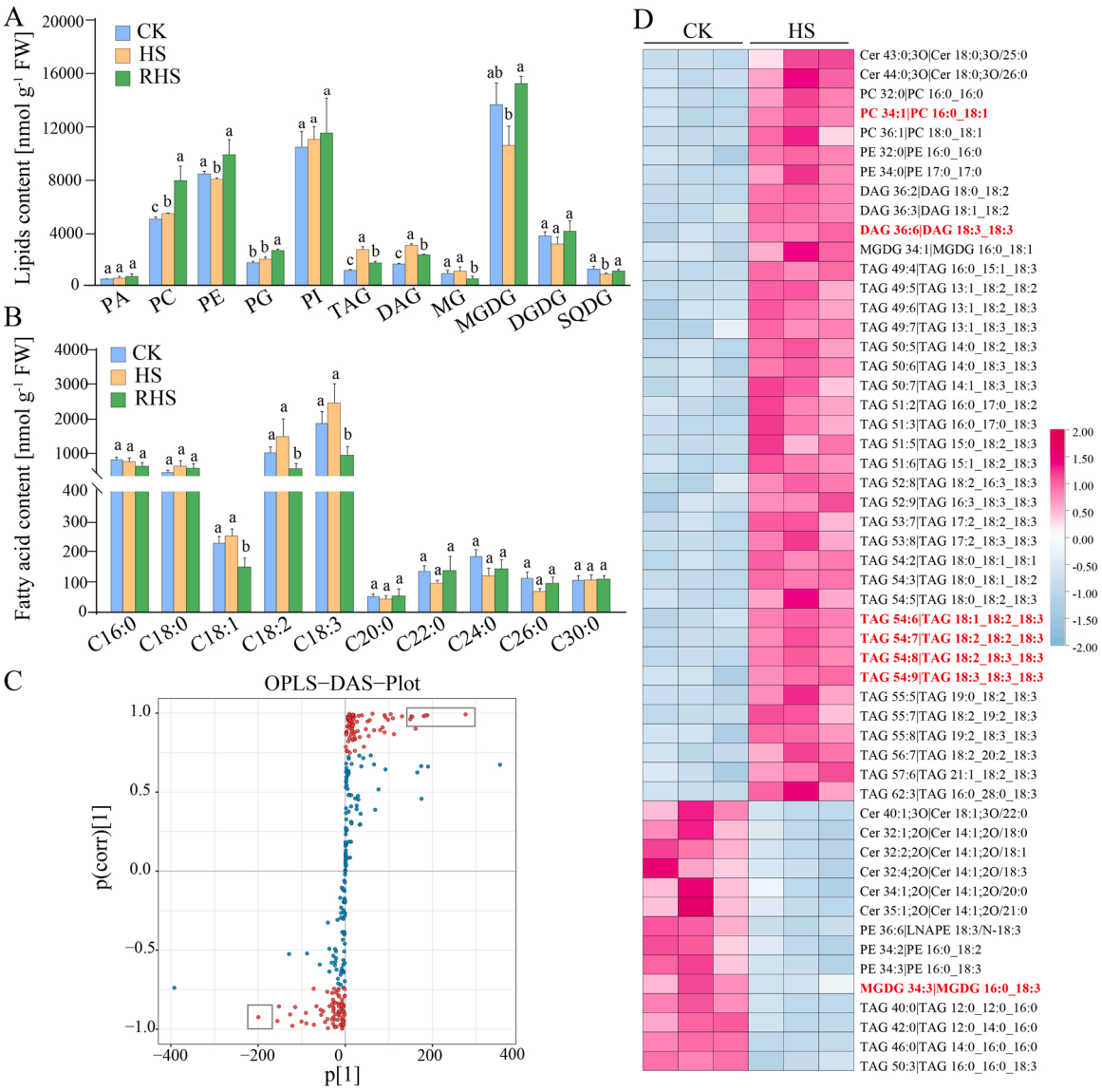


Fig. 3. UPLC-QTOF/MS-based lipidomics analysis. (A)The content of different lipid compounds in castor bean seedlings of CK, HS and RHS. The letters on the bar indicate the significant difference with a P-value < 0.05 using one-way ANOVA. (b)The content of free fatty acids in seedling from CK, HS and RHS. The letters on the bar indicate the significant difference with a P-value < 0.05 using one-way ANOVA. (C)Identification of heat-responsive lipid molecules based on orthogonal partial least square discriminant analysis (OPLS-DA). The red dots indicate the up-regulated (top right) and down-regulated lipid molecules (bottom left). The red dots in the box represent the most significant lipid molecules induced by heat stress. (D)Heatmaps of the content of differential lipid molecules between HS and CK. The color scale demonstrates log2 (molecule content) values. Abbreviations: Cer, Ceramide; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; FFA, free fatty acids; MGDG, Monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidyl glycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol.

castor bean seedlings (Fig. 3B).

Meanwhile, we analyzed the FA species esterified to different lipid compounds mentioned above. We found that PA, PC, PE, TAG, and DAG contained similar FA species, primarily consisting of C16:0, C18:2, and C18:3, while PG was mainly composed of C16:0 accounting for ~68% of total FAs (Fig. 4). MGDG and DGDG predominantly consisted of C18:3, accounting for 93% and 82% of total FAs, respectively. MG, on the other hand, mainly contained C18:3 (~72%), and SQDG was mainly composed of C16:0 (~41%) and C18:3 (~49%) (Fig. 4).

Multiple comparisons showed that heat treatment induced a significant increase in the content of TAG, DAG, and PC ($P < 0.05$, one-way ANOVA) and decrease in the content of MGDG, SQDG, and PE ($P < 0.05$, one-way ANOVA) compared with CK (Fig. 3A). The levels of PA, PG, and PI were slightly but not significantly increased after heat treatment (Fig. 3A), consistent with the results reported for Arabidopsis where PG, and PI were slightly affected by elevated temperature or light, but were significant accumulated under cold or darkness (Szymanski et al., 2014). When heat stress was subsequently relieved, the content of SQDG and PE was restored to levels comparable to those in CK, and TAG and DAG showed significant decreases relative to HS ($P < 0.05$, one-way ANOVA) but were still higher than CK seedlings (Fig. 3A), consistent with reports in Arabidopsis (Higashi et al., 2015; Mueller et al., 2015). Intriguingly, the level of PC exhibited continuous increase after recovery from HS, and the MGDG content recovered to a higher level in RHS than in either HS or CK (Fig. 3A). This indicates that TAGs and DAGs may function as temporary intermediate or 'buffer' of lipid turnover during heat stress (Troncoso-Ponce et al., 2013; Higashi et al., 2015). In addition, we observed that heat stress did not induce an obvious change in the content of total FFAs, but there was a significant decrease in the content of unsaturated FAs, including C18:1, C18:2, and C18:3 FAs, in

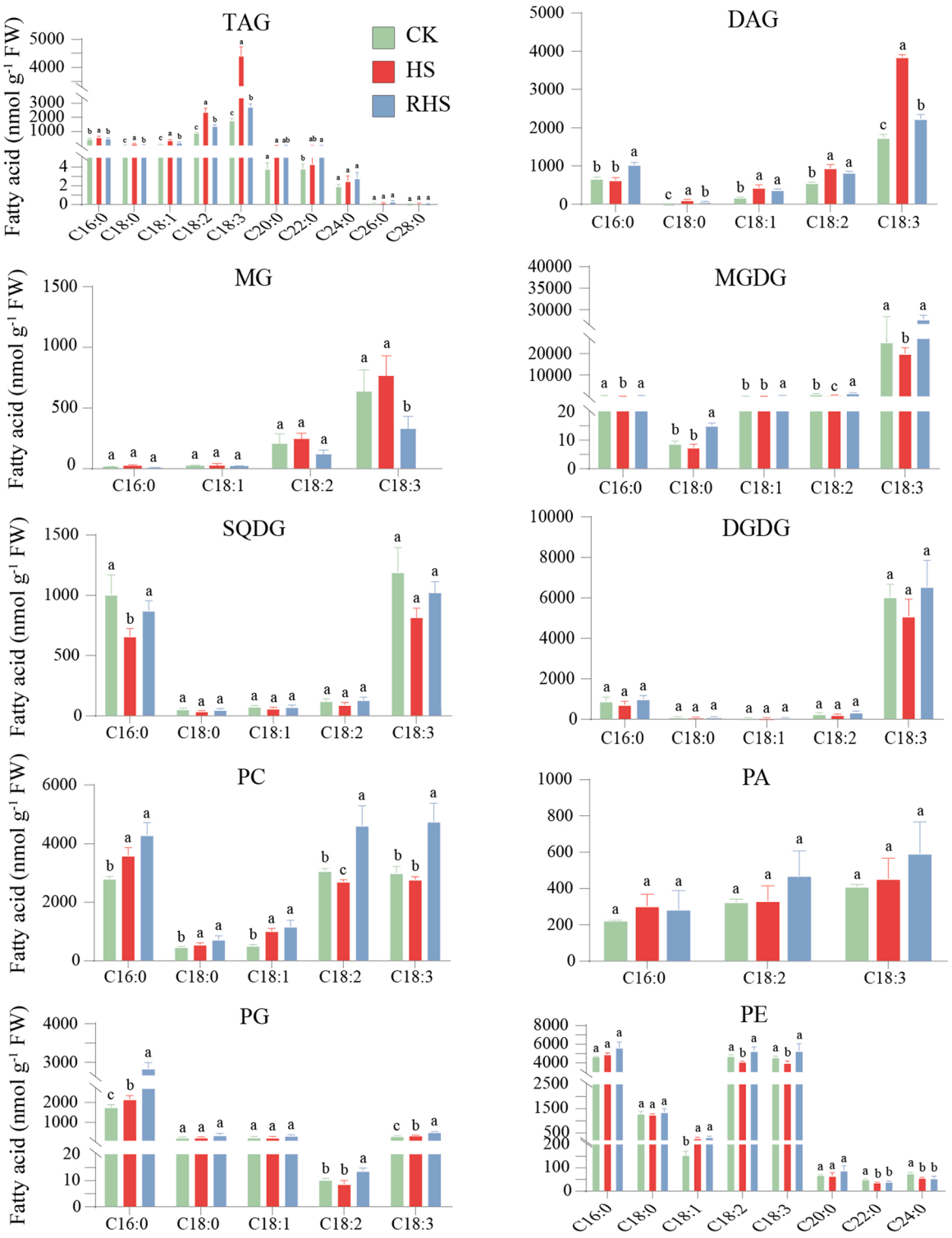


Fig. 4. The content of different fatty acids esterified to different lipid compounds in CK, HS and RHS. The letters on the bar indicate the significant difference with a *P*-value < 0.05 using one-way ANOVA. Abbreviations: DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; MG, Monoacylglycerol; MGDG, Monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidyl glycerol; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol.

the seedlings from the RHS relative to the HS and CK groups ($P < 0.05$, one-way ANOVA) (Fig. 3B). Overall, we revealed a large number of lipid compounds and their dynamic changes under heat stress and during recovery.

3.3. The accumulation of predominantly polyunsaturated TAG species under heat stress

We employed partial least squares-discriminant analysis (PLS-DA) to identify differential lipid molecules under heat stress in castor bean seedlings, based on the criteria of VIP value ≥ 1 and FDR ≤ 0.05. The

results revealed 39 up-regulated and 15 down-regulated lipids under heat stress (Fig. 3C and D and Table S5). Notably, there was a dramatic accumulation of DAGs and TAGs, which increased 2- to 43-fold under heat stress, especially DAG36:6, TAG54:6, TAG54:7, TAG54:8, and TAG54:9. The same lipid molecules, especially TAG54:9, were likewise substantially induced under heat stress in Arabidopsis seedlings (Higashi et al., 2015; Mueller et al., 2015), suggesting they could be used as potential markers of response to heat acclimation. Interestingly, all up-regulated TAGs and DAGs bore polyunsaturated FAs such as C18:2 and C18:3 at the *sn*-2 and/or *sn*-3 position, while other up-regulated lipid molecules, such as PC, MGDG, and PE, contained either saturated or monounsaturated FAs (Fig. 3D). This suggests that polyunsaturated FAs were preferentially and efficiently esterified into DAGs or TAGs under heat stress. Correspondingly, we found that the contents of polyunsaturated PE (e.g., PE36:6, PE34:2, and PE34:3) and MGDG (MGDG34:3) and saturated TAGs were significantly down-regulated (VIP value ≥ 1 and FDR ≤ 0.05, Fig. 3D). Changes in FA species in other lipid compounds show a similar trend. We found that the content of polyunsaturated FAs (e.g., C18:2 and C18:3) had a substantial increase in TAGs and DAGs and decrease in MGDG and PE under heat stress ($P < 0.05$, one-way ANOVA, Fig. 4).

After recovery from heat stress, 19 up-regulated and 12 down-regulated lipid molecules were identified in the RHS group compared with the HS group (Table S6). Specifically, polyunsaturated MGDG (e.g., MGDG36:5, MGDG36:4, MGDG36:3, and MGDG36:2), PC (PC36:4, PC36:5, and PC36:6), and PE (PE36:5) were significantly up-regulated (VIP value ≥ 1 and FDR ≤ 0.05) and even restored to higher levels relative to CK (Table S6), while unsaturated FA, TAG, DAG, and MG molecules exhibited significantly reduced abundances in RHS relative to HS (Table S6). Meanwhile, the content of total polyunsaturated FAs, especially C18:3, in MGDG, PC, and PE were restored in RHS and reached higher levels relative to CK, yet the contents of TAG and DAG were substantially reduced in RHS seedling relative to HS (Fig. 4). Overall, these results show the turnover of polyunsaturated FAs in different lipid molecules, especially TAGs and MGDGs, under heat stress and during recovery in castor bean seedlings.

3.4. TAGs accumulated predominantly in the cytosol

Considering that TAGs can be synthesized and stored either in the cytosol or in chloroplasts, we wondered where the heat-induced TAGs were stored. Usually, TAGs are synthesized and packaged into oil bodies that are formed by a phospholipid monolayer and proteins such as oleosins, caleosins, and steroleosins (Shimada and Hara-Nishimura, 2010). The oil bodies can be visualized using Nile red, a fluorescent, lipophilic dye that provides an effective means to monitor the location of TAGs. Using a fluorescence microscope, we observed that a large number of lipid droplets (red fluorescence) were produced in protoplasts obtained from heat-treated leaves, while only a few lipid droplets were observed in protoplasts obtained from CK (see Fig. 5). After recovery from heat stress, the number of lipid droplets were obviously reduced relative to HS, but were still higher than CK (see Fig. 5), consistent with the changes of TAG content (Fig. 3A). Furthermore, we found that there was no obvious colocalization between the fluorescence of lipid droplets (red) and the autofluorescence of the chloroplasts (green). It is suggested that heat-induced lipid droplets were most likely stored in the cytosol rather than in chloroplastic compartments, consistent with previous observations in Arabidopsis (Mueller et al., 2015). Nevertheless, emerging evidence also suggests that TAGs can accumulate in plastid lipid bodies (plastoglobuli) during drought stress (Eymery and Rey, 1999), nitrogen starvation (Gaude et al., 2007), moderate heat stress (Zhang et al., 2010), and senescence (Lippold et al., 2012) in plants. It seems to have different mechanisms to drive TAG accumulation under different abiotic stresses.

3.5. Transcriptomic analyzes revealed heat-responsive genes in castor bean seedling

As shown in Figs. 1 and 6A, TAG can be synthesized *de novo* via the Kennedy pathway or acyl-CoA-independent pathway. A crucial question is where TAGs and polyunsaturated FAs are derived under heat stress. To determine this, we performed transcriptomic analysis on seedlings from CK, HS, and RHS with three biological replicates. We obtained 37.6 million clean reads for each sample, with an average of 92.5% mapping

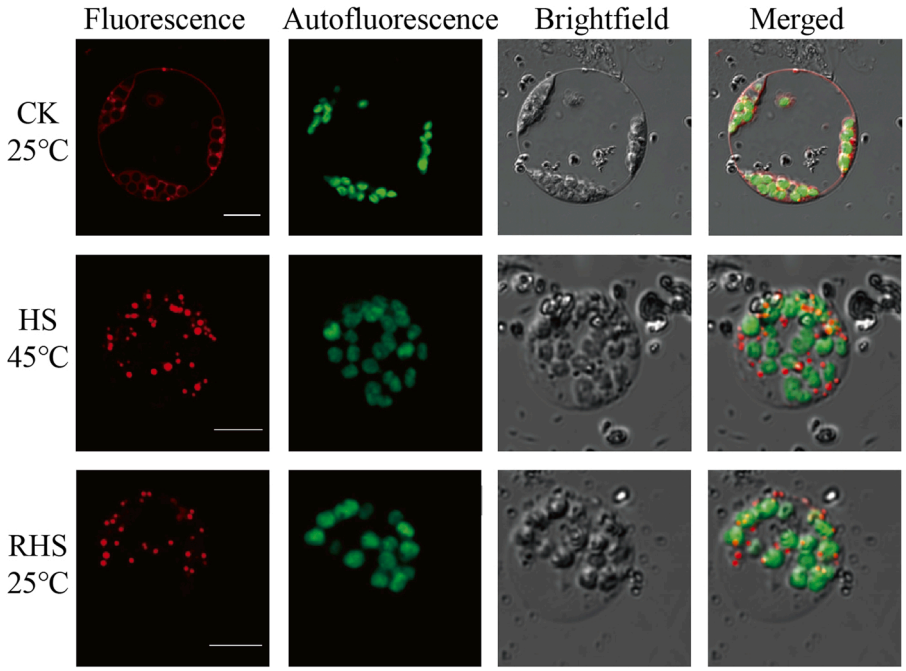


Fig. 5. Localization of lipid droplets in castor bean protoplasts from CK, HS and RHS leaf of four-week-old seedling. Chloroplast autofluorescence (green) and lipid droplets stained with Nile Red (red) were visualized under a fluorescence microscope. The bar in the images indicates 10 μm.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

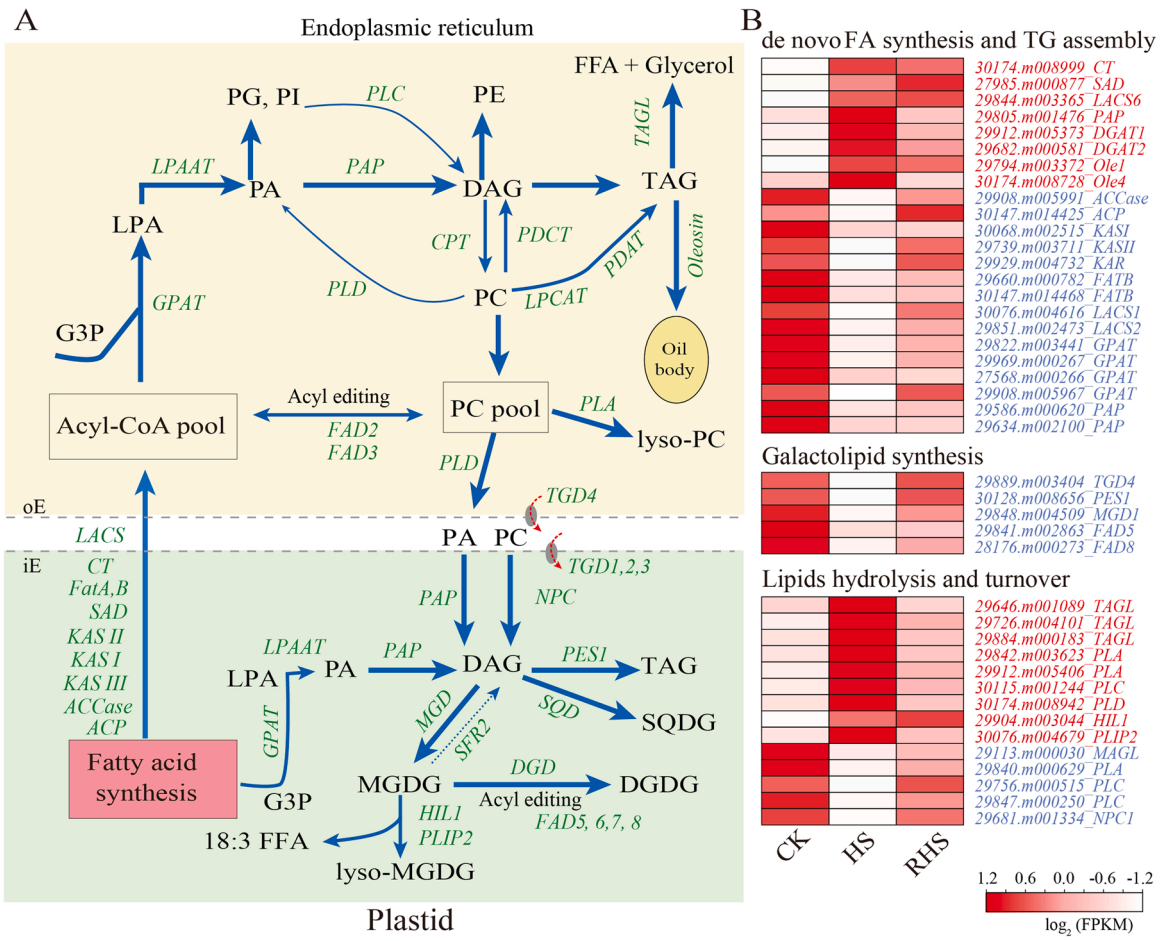


Fig. 6. Lipids metabolism and related gene expression in castor bean seedlings. (A)Network of genes involved in the fatty acid, triacylglycerols and galactolipids synthesis as well as lipids hydrolysis and trafficking. Main reaction step (black font) and key genes (green font) in these pathways were displayed. The abbreviations and functional description of all genes were listed in Table S10. (B)The heatmap shows the expression level of differentially expressed genes between HS and CK involved into the process of lipids metabolism. Genes with red or blue color indicate up-regulated or down-regulated genes under heat stress in castor bean seedling. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

onto the castor bean genome (Table S7). There was good replicability between two samples from the same group, suggesting that our data were reliable and can be used for subsequent analysis (Fig. S2). We detected 18,591 genes in castor bean seedlings with a FPKM value > 1 in at least one sample. Differential expression analysis identified 3163 up-regulated and 3172 down-regulated genes in HS relative to CK (Table S8). Interestingly, these up-regulated genes were mainly enriched in GO terms including response to abscisic acid, response to heat, heat acclimation, and temperature compensation of the circadian clock (Fig. S3A), consistent with known heat response mechanisms in plants (Liu et al., 2015). We noted that lipid catabolic process, lipid storage, and triglyceride lipases were also significantly enriched (Fig. S3A), supported by our observations from lipidomic analysis. Down-regulated genes, on the other hand, were primarily involved in chloroplast and thylakoid membrane metabolism (Fig. S3B), suggesting that the chloroplast membrane may be sensitive to heat stress.

Heat shock proteins (HSP) are well known to have important roles in thermotolerance by preventing protein denaturation and aggregation (Nishad and Nandi, 2021). Among these heat-responsive genes, we found that 34 genes encoding HSP exhibited dramatic increase under heat stress in castor bean seedlings (Table S9). For example, the gene *HSP101* (30138.m003984) increased ~20-fold under heat stress and its homolog in Arabidopsis was found to be involved in basal and acquired thermotolerance (Hong and Vierling, 2000). Meanwhile, we identified 415 heat-responsive genes encoding transcription factor (TF), including 233 up-regulated and 182 down-regulated TFs (Table S9). Specially, 8 genes encoding heat shock transcription factors (HSFs) were significantly up-regulated under heat stress (Table S9), which were known master regulators of the heat shock response (Ohama et al., 2017; Nishad and Nandi, 2021). These heat-responsive HSPs and HSFs as well as other TFs may contribute to thermotolerance of castor bean seedling.

3.6. *The molecular basis of TAG accumulation under heat stress*

We focused our attention on lipid metabolism and remodeling under heat stress in castor bean seedlings. Thus, we inspected the castor bean genome and identified 135 genes coding for enzymes involved in FA synthesis, glycerolipid and phospholipid metabolism in the ER and plastids, oil body formation, and lipid hydrolysis (Fig. 6A and Table S10). Of them, 17 were up-regulated and 25 were down-regulated under heat stress in castor bean seedlings (Fig. 6B). We found that key genes involved in *de novo* FA synthesis, such as *ACCase*, 3-Ketoacyl-ACP synthase I (*KAS I*), 3-Ketoacyl-ACP Reductase (*KAR*), Palmitoyl-acyl carrier protein thioesterase (*FATB*), and *LACS1*, were significantly down-regulated under heat stress ($P < 0.05$, Fig. 6B). Considering the transcriptional dynamics during heat stress, we performed qRT-PCR to investigate the expression pattern of these FA synthesis-related genes in 4 h, 8 h and 12 h heat-treated castor bean seedlings under light condition. As a result, we found genes encoding *ACCase*, *KAS I*, *KAS II*, *KAS III* and *KAR* were significantly repressed during heat stress (Fig. S4). Studies in Arabidopsis have revealed that FA synthesis-related genes were down-regulated under heat stress and light condition, but were

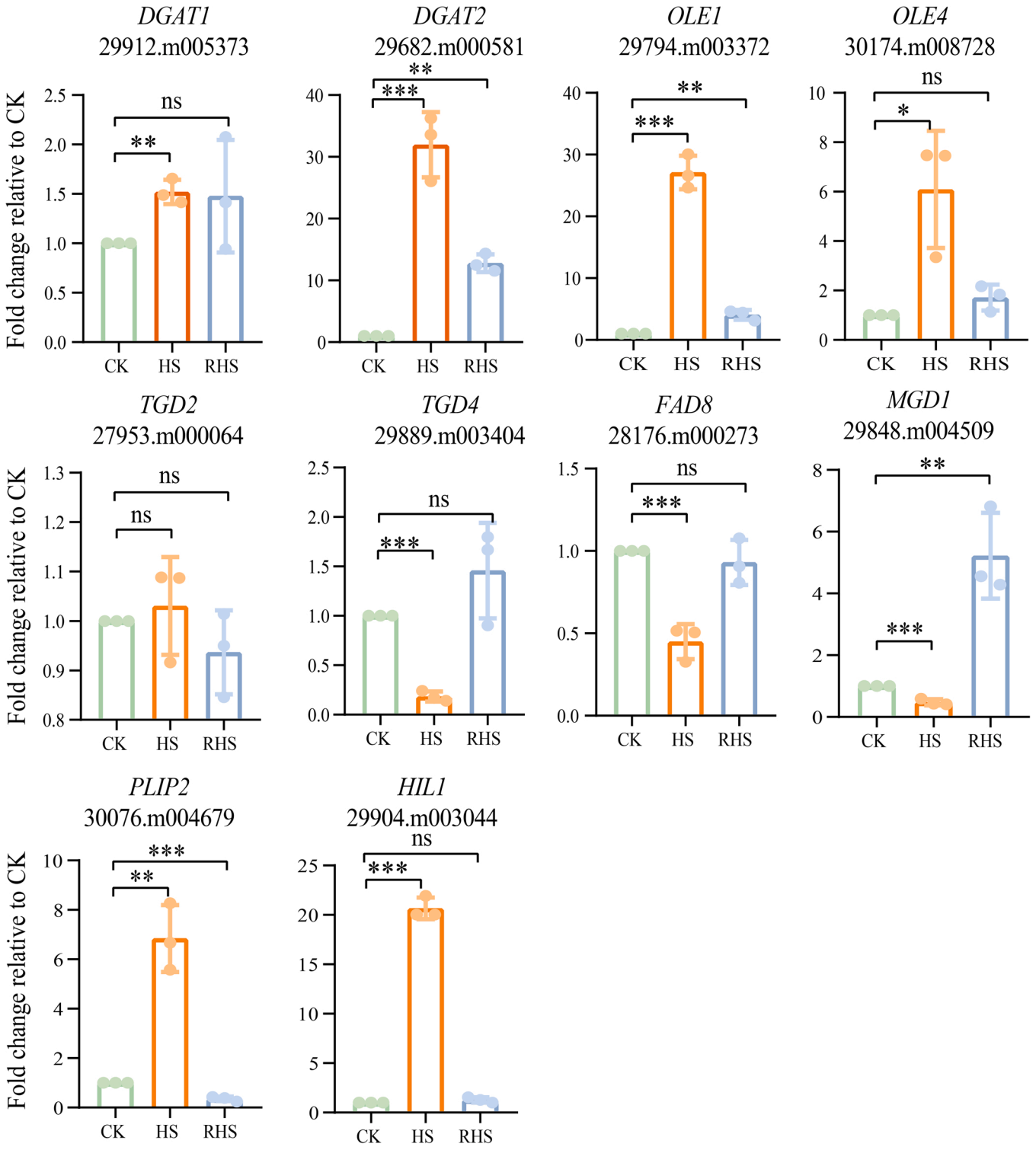


Fig. 7. Quantitative real-time PCR analysis of ten genes we selected. Error bars depict standard error of the mean based on three biological replicates. The expression level of genes in CK was normalized to 1. Asterisks indicate the statistical significance between two sample (two-tailed unpaired t tests; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns: no significant). Abbreviations: DGAT, diacylglycerol acyltransferase; FAD, fatty acid desaturase; *HIL1*, HEAT INDUCIBLE LIPASE 1; MGD, monogalactosyl diacylglycerol synthase; OLE, oleosin; *PLIP2*, PLASTID LIPASE2; TGD, trigalactosyldiacylglycerol;.

activated under darkness (Szymanski et al., 2014; Higashi et al., 2015). Similarly, genes encoding GPATs and PAPs in the Kennedy pathway exhibited lower expression levels in HS seedlings compared to CK seedlings (Fig. 6B and Table S10). These results show that the accumulation of TAGs does not derive from *de novo* FA and TAG synthesis. Interestingly, we found that genes encoding DGATs (including RcDGAT1 and RcDGAT2), which represent the last and rate-limiting step for TAG assembly in the Kennedy pathway, were substantially up-regulated under heat stress, along with two oleosin genes involved in oil body formation (OLE1: 29794.m003372 and OLE4: 30174.m008728) (Fig. 6B and Table S10). However, genes encoding phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), lysophophatidylcholine

acyltransferase (LPCAT), and PDAT in the acyl-CoA-independent pathway had no significant change in their expression levels under heat stress (Fig. 6B and Table S10).

Meanwhile, we investigated the expression level of a gene encoding plastoglobule-localized PHYTYL ESTER SYNTHASE (PES), which can catalyze the conversion of DAGs into TAGs in chloroplasts in Arabidopsis (Lippold et al., 2012). The homologous gene *RcPES1* (30128.m008656) in castor bean was substantially down-regulated under heat stress, suggesting that it is not involved in the response to heat stress. Additionally, evidence showed that the Arabidopsis gene *SENSITIVE TO FREEZING2* (*SFR2*) can catalyze the conversion of MGDGs into oligogalactolipids and DAGs, which are then converted to TAGs during freezing (Moellering et al., 2010). Also, recent studies in Arabidopsis show that severe heat stress (45 ℃, 90 min) can activate SFR2 to participate in the MGDG remodeling (Mueller et al., 2017). However, the homologous gene *RcSFR2* (29889.m003347) was not induced by heat stress (45 ℃, 12 h) in castor bean seedling. Overall, these results show that DGAT in the Kennedy pathway may contribute substantially to the accumulation of polyunsaturated TAGs in the cytosol under heat stress in castor bean seedlings. However, we cannot ignore the effects of PDAT activity on TAG synthesis, as demonstrated in Arabidopsis where *PDAT1* mutant seedlings were more sensitive to heat stress despite its expression not being induced by heat stress (Mueller et al., 2017).

Since FAs used for TAG synthesis are not derived from *de novo* FA synthesis, we speculate that they may be generated through the release of other lipid molecules, as suggested by the changing FAs composition in different lipid molecules, especially MGDG. Transcriptomic analysis indicated that many genes encoding diverse lipases were substantially induced under heat stress in castor bean seedlings (Fig. 6 and Table S10), and these might be involved in lipids and FAs turnover. For example, genes encoding triacylglycerol lipase (TAGL) are involved in the degradation of TAG or other structural lipids to release FFAs (Sandoval and Herrera-López, 2018). It is suggested that TAG accumulation was accompanied by its degradation, which may be required for the change of FAs composition in TAG. Similarly, genes encoding phospholipases such as PLA, PLC, and PLD can hydrolyze phospholipids and release unsaturated FAs involved in lipid turnover (Dong et al., 2012). Additionally, the gene *HIL1* (HEAT INDUCIBLE LIPASE1) and *PLIP2* (PLASTID LIPASE2), which is thought to function in the degradation of MGDG to produce C18:3-FFA in Arabidopsis (Higashi et al., 2018; Wang et al., 2018), were substantially induced by heat stress in castor bean seedling. This result showed that lipid turnover may play an important role in the production of FFAs used for TAG synthesis, although it remains unclear how these FFAs were transported and exported to ER.

Under heat stress, we also observed a substantial decrease in MGDG content, which may be closely related to the significant decrease in the expression of *MGD1* (29848.m004509, monogalactosyl diacylglycerol synthase 1) (Fig. 6B). Studies in Arabidopsis revealed that MGD1 is responsible for the biosynthesis of MGDG (Shimojima et al., 1997; Jarvis et al., 2000) and is significantly repressed under heat stress (Higashi et al., 2015). Additionally, genes encoding trigalactosyldiacylglycerols (TGDs), especially *TGD4* (29889.m003404), which is involved in lipid transfer from the ER to the chloroplast (Xu et al., 2008), were down-regulated under heat stress (Fig. 6B). The transcriptional repression of *RcMGD1* and *RcTGDs* may be closely linked to the reduction of MGDG content. Meanwhile, we observed that the *FAD8* gene, encoding a chloroplast-localized desaturase for the production of C16:3 and C18:3 (Gibson et al., 1994; McConn et al., 1994), was down-regulated under heat stress, resulting in the further reduction of polyunsaturated FAs in chloroplast lipids. Plastidic *FAD* mutants were shown to be more tolerant to heat stress (Kunst et al., 1989; Murakami et al., 2000; Routaboul et al., 2012).

Subsequently, we selected six up-regulated genes (*DGAT1*, *DGAT2*, *OLE1*, *OLE4, PLIP2*, and *HIL1*) and four down-regulated genes (*TGD2*, *TGD4*, *FAD8*, and *MGD1*) for qRT-PCR validation. All up-regulated genes and three of the four down-regulated genes were confirmed by qRT-PCR, the one exception being *TGD2*, whose expression did not change under heat stress (see Fig. 7). This result indicates a general consistency between transcriptomics and qRT-PCR. Overall, our results suggest that heat-induced changes in transcription levels coordinate changes in lipids metabolism—in other words, lipid turnover under heat stress may be controlled by heat-responsive genes.

4. Conclusions

We performed an integrated analysis of lipidomics and transcriptomics in castor bean seedlings under heat stress and during recovery and identified a large number of heat-responsive lipid molecules and genes. We found that TAGs synthesis can be significantly induced by heat stress and stored in the cytosol, but they decrease during recovery. TAGs may be transient intermediates for the recycling of FAs released from other lipids, especially chloroplast lipids such as MGDG. Transcriptomic analysis identified many genes related to heat-responsive lipids that coordinate lipid remodeling. Our findings add insights to the understanding of lipid turnover under heat stress in castor bean and other plant species, while offering potential applications in manipulating specific lipid molecules, especially TAGs, to improve plant heat tolerance in the future.

Funding

This work was supported by National Natural Science Foundation of China, China (31970341), the Youth Innovation Promotion Association of CAS, China (2020389 to W.X.), and Yunnan Young & Elite Talents Project, China (YNWR-QNBJ-2020-286 to W.X).

CRediT authorship contribution statement

Yanyu Zhang: Methodology, Data curation, Formal analysis, Verification, Software, Visualization, Writing – original draft. **Yelan Li**: Methodology, Investigation, Verification, Software, Visualization. **Bing Han**: Investigation, Data curation, Resources, Formal analysis, **Aizhong Liu:** Conceptualization, Supervision, Project administration, Writing – review & editing. **Wei Xu**: Conceptualization, Funding acquisition, Supervision, Project administration, Visualization, Writing – original draft, Writing – review & editing. All authors read and contributed to the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data of RNA-Seq reads in this study have been deposited in NCBI Sequence Read Archive (SRA) under BioProject accession PRJNA803603.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.114702.

References

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11, R106. https://doi.org/10.1186/gb-2010-11-10-r106.

Balazadeh, S., 2021. A 'hot' cocktail: the multiple layers of thermomemory in plants. Curr. Opin. Plant Biol. 65, 102147 https://doi.org/10.1016/j.pbi.2021.102147.

Broun, P., Somerville, C., 1997. Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic Arabidopsis plants that express a fatty acyl hydroxylase

cDNA from castor bean. Plant Physiol. 113, 933–942. https://doi.org/10.1104/pp.113.3.933.
Brown, A.P., Kroon, J.T., Swarbreck, D., Febrer, M., Larson, T.R., Graham, I.A., Caccamo, M., Slabas, A.R., 2012. Tissue-specific whole transcriptome sequencing in castor, directed at understanding triacylglycerol lipid biosynthetic pathways. Plos One 7, e30100. https://doi.org/10.1371/journal.pone.0030100.
Brzezinka, K., Altmann, S., Bäurle, I., 2019. BRUSHY1/TONSOKU/MGOUN3 is required for heat stress memory. Plant Cell Environ. 42, 771–781. https://doi.org/10.1111/pce.13365.
Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I., Browse, J., 2008. Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotechnol. J. 6, 819–831. https://doi.org/10.1111/j.1467-7652.2008.00361.x.
Burgos, A., Szymanski, J., Seiwert, B., Degenkolbe, T., Hannah, M.A., Giavalisco, P., Willmitzer, L., 2011. Analysis of short-term changes in the *Arabidopsis thaliana* glycerolipidome in response to temperature and light. Plant J. 66, 656–668. https://doi.org/10.1111/j.1365-313X.2011.04531.x.
César, A.S., Batalha, M.O., 2010. Biodiesel production from castor oil in Brazil: a difficult reality. Energy Policy 38, 4031–4039. https://doi.org/10.1016/j.enpol.2010.03.027.
Chan, A.P., Crabtree, J., Zhao, Q., Lorenzi, H., Orvis, J., Puiu, D., Melake-Berhan, A., Jones, K.M., Redman, J., Chen, G., Cahoon, E.B., Gedil, M., Stanke, M., Haas, B.J., Wortman, J.R., Fraser-Liggett, C.M., Ravel, J., Rabinowicz, P.D., 2010. Draft genome sequence of the oilseed species Ricinus communis. Nat. Biotechnol. 28, 951–956. https://doi.org/10.1038/nbt.1674.
deVries, J., Ischebeck, T., 2020. Ties between stress and lipid droplets pre-date seeds. Trends Plant Sci. 25, 1203–1214. https://doi.org/10.1016/j.tplants.2020.07.017.
Deryng, D., Conway, D., Ramankutty, N., Price, J., Warren, R., 2014. Global crop yield response to extreme heat stress under multiple climate change futures. Environ. Res. Lett. 9, 1–13 https://iopscience.iop.org/article/10.1088/1748-9326/9/3/034011.
Dong, W., Lv, H., Xia, G., Wang, M., 2012. Does diacylglycerol serve as a signaling molecule in plants? Plant Signal. Behav. 7, 472–475. https://doi.org/10.4161/psb.19644.
Eymery, F., Rey, P., 1999. Immunocytolocalization of CDSP 32 and CDSP 34, two chloroplastic drought-induced stress proteins in *Solanum tuberosum* plants. Plant Physiol. Biochem. 37, 305–312. https://doi.org/10.1016/S0981-9428(99)80029-1.
Fahy, E., Subramaniam, S., Murphy, R.C., Nishijima, M., Raetz, C.R., Shimizu, T., Spener, F., van Meer, G., Wakelam, M.J., Dennis, E.A., 2009. Update of the LIPID MAPS comprehensive classification system for lipids. J. Lipid Res. 50, S9–S14. https://doi.org/10.1194/jlr.R800095-JLR200.
Falcone, D.L., Ogas, J.P., Somerville, C.R., 2004. Regulation of membrane fatty acid composition by temperature in mutants of Arabidopsis with alterations in membrane lipid composition. BMC Plant Biol. 4, 17. https://doi.org/10.1186/1471-2229-4-17.
Gaude, N., Bréhélin, C., Tischendorf, G., Kessler, F., Dörmann, P., 2007. Nitrogen deficiency in Arabidopsis affects galactolipid composition and gene expression and results in accumulation of fatty acid phytyl esters. Plant J. 49, 729–739. https://doi.org/10.1111/j.1365-313X.2006.02992.x.
Gibson, S., Arondel, V., Iba, K., Somerville, C., 1994. Cloning of a temperature-regulated gene encoding a chloroplast ω-3 desaturase from *Arabidopsis thaliana*. Plant Physiol. 106, 1615–1621. https://doi.org/10.1104/pp.106.4.1615.
He, K., Cao, X., Deng, X., 2021. Histone methylation in epigenetic regulation and temperature responses. Curr. Opin. Plant Biol. 61, 102001 https://doi.org/10.1016/j.pbi.2021.102001.
Higashi, Y., Okazaki, Y., Myouga, F., Shinozaki, K., Saito, K., 2015. Landscape of the lipidome and transcriptome under heat stress in *Arabidopsis thaliana*. Sci. Rep. 5, 10533. https://doi.org/10.1038/srep10533.
Higashi, Y., Okazaki, Y., Takano, K., Myouga, F., Shinozaki, K., Knoch, E., Fukushima, A., Saito, K., 2018. Heat inducible LIPASE1 remodels chloroplastic monogalactosyldiacylglycerol by liberating α-linolenic acid in arabidopsis leaves under heat stress. Plant Cell 30, 1887–1905. https://doi.org/10.1105/tpc.18.00347.
Hölzl, G., Dörmann, P., 2019. Chloroplast lipids and their biosynthesis. Annu. Rev. Plant Biol. 70, 51–81. https://doi.org/10.1146/annurev-arplant-050718-100202.
Hong, S.W., Vierling, E., 2000. Mutants of Arabidopsis thaliana defective in the acquisition of tolerance to high temperature stress. Proc. Natl. Acad. Sci. USA 97, 4392–4397. https://doi.org/10.1073/pnas.97.8.4392.
Jarvis, P., Dörmann, P., Peto, C.A., Lutes, J., Benning, C., Chory, J., 2000. Galactolipid deficiency and abnormal chloroplast development in the Arabidopsis MGD synthase 1 mutant. Proc. Natl. Acad. Sci. USA 97, 8175–8179. https://doi.org/10.1073/pnas.100132197.
Kim, H.U., Lee, K.R., Go, Y.S., Jung, J.H., Suh, M.C., Kim, J.B., 2011. Endoplasmic reticulum-located PDAT1-2 from castor bean enhances hydroxy fatty acid accumulation in transgenic plants. Plant Cell Physiol. 52, 983–993. https://doi.org/10.1093/pcp/pcr051.
Kumar, P.V., Ramakrishna, Y.S., Rao, B.V.R., Victor, U.S., Srivastava, N.N., Rao, A., 1997. Influence of moisture, thermal and photoperiodic regimes on the productivity of castor beans (*Ricinus communis* L.). Agric. For. Meteorol. 88, 279–289. https://doi.org/10.1016/S0168-1923(97)00019-1.
Kunst, L., Browse, J., Somerville, C., 1989. Enhanced thermal tolerance in a mutant of Arabidopsis deficient in palmitic acid unsaturation. Plant Physiol. 91, 401–408. https://doi.org/10.1104/pp.91.1.401.
Lämke, J., Bäurle, I., 2017. Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. Genome Biol. 18, 124. https://doi.org/10.1186/s13059-017-1263-6.
Lämke, J., Brzezinka, K., Altmann, S., Bäurle, I., 2016. A hit-and-run heat shock factor governs sustained histone methylation and transcriptional stress memory. EMBO J. 35, 162–175. https://doi.org/10.15252/embj.201592593.
Lee, A.G., 2000. Membrane lipids: it's only a phase. Curr. Biol. 10, 377–380. https://doi.org/10.1016/s0960-9822(00)00477-2.
Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D., Baud, S., Bird, D., Debono, A., Durrett, T.P., Franke, R.B., Graham, I.A., Katayama, K., Kelly, A.A., Larson, T., Markham, J.E., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K.M., Wada, H., Welti, R., Xu, C., Zallot, R., Ohlrogge, J., 2013. Acyl-lipid metabolism. Arabidopsis Book 11, e0161. https://doi.org/10.1199/table0161.
Lippold, F., vom, Dorp, K., Abraham, M., Hölzl, G., Wewer, V., Yilmaz, J.L., Lager, I., Montandon, C., Besagni, C., Kessler, F., Stymne, S., Dörmann, P., 2012. Fatty acid phytyl ester synthesis in chloroplasts of Arabidopsis. Plant Cell 24, 2001–2014. https://doi.org/10.1105/tpc.112.095588.
Liu, J., Feng, L., Gu, X., Deng, X., Qiu, Q., Li, Q., Zhang, Y., Wang, M., Deng, Y., Wang, E., He, Y., Bäurle, I., Li, J., Cao, X., He, Z., 2019. An H3K27me3 demethylase-HSFA2 regulatory loop orchestrates transgenerational thermomemory in Arabidopsis. Cell Res. 29, 379–390. https://doi.org/10.1038/s41422-019-0145-8.
Liu, J., Feng, L., Li, J., He, Z., 2015. Genetic and epigenetic control of plant heat responses. Front. Plant Sci. 6, 267. https://doi.org/10.3389/fpls.2015.00267.
Lobell, D.B., Schlenker, W., Costa-Roberts, J., 2011. Climate trends and global crop production since 1980. Science 333, 616–620. https://doi.org/10.1126/science.1204531.
Los, D.A., Murata, N., 2004. Membrane fluidity and its roles in the perception of environmental signals. Biochim. Biophys. Acta 1666, 142–157. https://doi.org/10.1016/j.bbamem.2004.08.002.
Lu, J., Xu, Y., Wang, J., Singer, S.D., Chen, G., 2020. The role of triacylglycerol in plant stress response. Plants 9 (4), 472. https://doi.org/10.3390/plants9040472.
McConn, M., Hugly, S., Browse, J., Somerville, C., 1994. A mutation at the fad8 locus of Arabidopsis identifiesa second chloroplast ω-3 desaturase. Plant Physiol. 106, 1609–1614. https://doi.org/10.1104/pp.106.4.1609.
Moellering, E.R., Muthan, B., Benning, C., 2010. Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. Science 330, 226–228. https://doi.org/10.1126/science.1191803.
Moshkin, V.A., 1986 Castor. Amerind Publishing Co. Pvt. Ltd, New Delhi.
Mueller, S.P., Krause, D.M., Mueller, M.J., Fekete, A., 2015. Accumulation of extra-chloroplastic triacylglycerols in Arabidopsis seedlings during heat acclimation. J. Exp. Bot. 66, 4517–4526. https://doi.org/10.1093/jxb/erv226.
Mueller, S.P., Unger, M., Guender, L., Fekete, A., Mueller, M.J., 2017. Phospholipid: diacylglycerol acyltransferase-mediated triacylglyerol synthesis augments basal thermotolerance. Plant Physiol. 175, 486–497. https://doi.org/10.1104/pp.17.00861.
Murakami, Y., Tsuyama, M., Kobayashi, Y., Kodama, H., Iba, K., 2000. Trienoic fatty acids and plant tolerance of high temperature. Science 287, 476–479. https://doi.org/10.1126/science.287.5452.476.
Nishad, A., Nandi, A.K., 2021. Recent advances in plant thermomemory. Plant Cell Rep. 40, 19–27. https://doi.org/10.1007/s00299-020-02604-1.
Ogunniyi, D.S., 2006. Castor oil: a vital industrial raw material. Bioresour. Technol. 97, 1086–1091. https://doi.org/10.1016/j.biortech.2005.03.028.
Ohama, N., Sato, H., Shinazaki, K., Yamaguchi-Shinozaki, K., 2017. Transcriptional regulatory network of plant heat stress response. Trends Plant Sci. 22, 53–65. https://doi.org/10.1016/j.tplants.2016.08.015.
Okazaki, Y., Saito, K., 2018. Plant lipidomics using UPLC-QTOF-MS. Methods Mol. Biol. 1778, 157–169. https://doi.org/10.1007/978-1-4939-7819-9_11.
Pertea, M., Kim, D., Pertea, G.M., Leek, J.T., Salzberg, S.L., 2016. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and ballgown. Nat. Protoc. 11, 1650–1667. https://doi.org/10.1038/nprot.2016.095.
Routaboul, J.M., Skidmore, C., Wallis, J.G., Browse, J., 2012. Arabidopsis mutants reveal that short- and long-term thermotolerance have different requirements for trienoic fatty acids. J. Exp. Bot. 63, 1435–1443. https://doi.org/10.1093/jxb/err381.
Sandoval, G., Herrera-López, E.J., 2018. Lipase, phospholipase, and esterase biosensors. Methods Mol. Biol. 1835, 391–425. https://doi.org/10.1007/978-1-4939-8672-9_22.
Sausen, T.L., Rosa, L.M.G., 2010. Growth and limitations to carbon assimilation in *Ricinus communis* (Euphorbiaceae) under soil water stress conditions. Acta Bot. Bras. 24, 648–654. https://doi.org/10.1590/S0102-33062010000300008.
Shimada, T.L., Hara-Nishimura, I., 2010. Oil-body-membrane proteins and their physiological functions in plants. Biol. Pharm. Bull. 33, 360–363. https://doi.org/10.1248/bpb.33.360.
Shimojima, M., Ohta, H., Iwamatsu, A., Masuda, T., Shioi, Y., Takamiya, K., 1997. Cloning of the gene for monogalactosyldiacylglycerol synthase and its evolutionary origin. Proc. Natl. Acad. Sci. USA 94, 333–337.
Song, P., Jia, Q., Xiao, X., Tang, Y., Liu, C., Li, W., Li, T., Li, L., Chen, H., Zhang, W., Zhang, Q., 2021. HSP70-3 interacts with phospholipase Dδ and participates in heat stress defense. Plant Physiol. 185, 1148–1165. https://doi.org/10.1093/plphys/kiaa083.
Szymanski, J., Brotman, Y., Willmitzer, L., Cuadros-Inostroza, Á., 2014. Linking gene expression and membrane lipid composition of Arabidopsis. Plant Cell 26, 915–928. https://doi.org/10.1105/tpc.113.118919.
Troncoso-Ponce, M.A., Cao, X., Yang, Z., Ohlrogge, J.B., 2013. Lipid turnover during senescence. Plant Sci. 205–206, 13–19. https://doi.org/10.1016/j.plantsci.2013.01.004.
Van de Loo, F.J., Broun, P., Turner, S., Somerville, C., 1995. An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. Proc. Natl. Acad. Sci. USA 92, 6743–6747. https://doi.org/10.1073/pnas.92.15.6743.
Vanhercke, T., Divi, U.K., El Tahchy, A., Liu, Q., Mitchell, M., Taylor, M.C., Eastmond, P. J., Bryant, F., Mechanicos, A., Blundell, C., Zhi, Y., Belide, S., Shrestha, P., Zhou, X. R., Ral, J.P., White, R.G., Green, A., Singh, S.P., Petrie, J.R., 2017. Step changes in

leaf oil accumulation via iterative metabolic engineering. Metab. Eng. 39, 237–246. https://doi.org/10.1016/j.ymben.2016.12.007.

Venegas-Calerón, M., Sánchez, R., Salas, J.J., Garcés, R., Martínez-Force, E., 2016. Molecular and biochemical characterization of the OLE-1 high-oleic castor seed (*Ricinus communis* L.) mutant. Planta 244, 245–258. https://doi.org/10.1007/s00425-016-2508-4.

Wang, K., Guo, Q., Froehlich, J.E., Hersh, H.L., Zienkiewicz, A., Howe, G.A., Benning, C., 2018. Two abscisic acid-responsive plastid lipase genes involved in jasmonic acid biosynthesis in *Arabidopsis thaliana*. Plant Cell 30, 1006–1022. https://doi.org/10.1105/tpc.18.00250.

Wang, X., Cai, J., Liu, F.L., Jin, M., Yu, H.X., Jiang, D., Wollenweber, B., Dai, T.B., Cao, W.X., 2012. Pre-anthesis high temperature acclimation alleviates the negative effects of post-anthesis heat stress on stem stored carbohydrates remobilization and grain starch accumulation in wheat. J. Cereal Sci. 55, 331–336. https://doi.org/10.1016/j.jcs.2012.01.004.

Xu, C., Fan, J., Cornish, A.J., Benning, C., 2008. Lipid trafficking between the endoplasmic reticulum and the plastid in Arabidopsis requires the extraplastidic TGD4 protein. Plant Cell 20, 2190–2204. https://doi.org/10.1105/tpc.108.061176.

Xu, W., Wu, D., Yang, T., Sun, C., Wang, Z., Han, B., Wu, S., Yu, A., Chapman, M.A., Muraguri, S., Tan, Q., Wang, W., Bao, Z., Liu, A., Li, D.Z., 2021. Genomic insights into the origin, domestication and genetic basis of agronomic traits of castor bean. Genome Biol. 22, 113. https://doi.org/10.1186/s13059-021-02333-y.

Yang, Y., Benning, C., 2018. Functions of triacylglycerols during plant development and stress. Curr. Opin. Biotechnol. 49, 191–198. https://doi.org/10.1016/j.copbio.2017.09.003.

Yoo, S.D., Cho, Y.H., Sheen, J., 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572. https://doi.org/10.1038/nprot.2007.199.

Zhai, Z., Jung, H.I., Vatamaniuk, O.K., 2009. Isolation of protoplasts from tissues of 14-day-old seedlings of Arabidopsis thaliana. J. Vis. Exp. 1149. https://doi.org/10.3791/1149.

Zhang, R., Wise, R.R., Struck, K.R., Sharkey, T.D., 2010. Moderate heat stress of Arabidopsis thaliana leaves causes chloroplast swelling and plastoglobule formation. Photosynth. Res. 105, 123–134. https://doi.org/10.1007/s11120-010-9572-6.

Zhao, J., Lu, Z., Wang, L., Jin, B., 2020. Plant responses to heat stress: physiology, transcription, noncoding RNAs, and epigenetics. Int. J. Mol. Sci. 22, 117. https://doi.org/10.3390/ijms22010117.