Gene Expression in Plant Lipid Metabolism in Arabidopsis Seedlings

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Abstract

Events in plant lipid metabolism are important during seedling establishment. As it has not been experimentally verified whether lipid metabolism in 2- and 5-day-old Arabidopsis thaliana seedlings is diurnally-controlled, quantitative real-time PCR analysis was used to investigate the expression of target genes in acyl-lipid transfer, β-oxidation and triacylglycerol (TAG) synthesis and hydrolysis in wild-type Arabidopsis WS and Col-0. In both WS and Col-0, ACYL-COA-BINDING PROTEIN3 (ACBP3), DIACYLGlycerol ACYLTRANSFERASE1 (DGAT1) and DGAT3 showed diurnal control in 2- and 5-day-old seedlings. Also, COMATOSE (CTS) was diurnally regulated in 2-day-old seedlings and LONG-CHAIN ACYL-COA SYNTHETASE6 (LACS6) in 5-day-old seedlings in both WS and Col-0. Subsequently, the effect of CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) from the core clock system was examined using the cca1lhy mutant and CCA1-overexpressing (CCA1-OX) lines versus wild-type WS and Col-0, respectively. Results revealed differential gene expression in lipid metabolism between 2- and 5-day-old mutant and wild-type WS and Col-0, as well as between CCA1-OX and wild-type Col-0. Of the ACBP3, DGAT2, and DGAT3 displayed the most significant changes between cca1lhy and WS and between CCA1-OX and Col-0, consistent with previous reports that ACBP3 is greatly affected by light/dark cycling. Evidence of oil body retention in 4- and 5-day-old seedlings of the cca1lhy mutant in comparison to WS indicated the effect of cca1lhy on storage lipid reserve mobilization. Lipid profiling revealed differences in primary lipid metabolism, namely in TAG, fatty acid methyl ester and acyl-CoA content amongst wild-type and mutant Arabidopsis seedlings. Taken together, this study demonstrates that lipid metabolism is subject to diurnal regulation in the early stages of seedling development in Arabidopsis.

Introduction

In plant seeds, triacylglycerol (TAG) is the major storage lipid in oil bodies and functions as a critical energy reserve during germination and seedling establishment [1–3]. The biosynthesis of TAG occurs in the endoplasmic reticulum (ER) via the Kennedy pathway, and incorporates a series of membrane-bound enzymes [4–7]. Diacylglycerol acyltransferase (DGAT) and phospholipid-diacylglycerol acyltransferase (PDAT) catalyze the transacylation of diacylglycerol (DAG) to produce TAG [8,9]. In germinating Arabidopsis seeds and young seedlings, DGAT3 is more highly expressed than DGAT1, DGAT2, and PDAT1 [3]. During germination and early post-germinative growth, the fatty acids (FAs) released from stored TAGs are converted to sucrose (Suc), providing carbon and metabolic energy for seedling development [3,6].

Biochemical pathways in various subcellular locations participate in storage reserve mobilization [3,10,11]. Oil breakdown is initiated during lipolysis when TAG in oil bodies is hydrolyzed to free FA and glycerol [12]. It has been established in Arabidopsis that two TAG lipases, encoded by SUGAR DEPENDENT1 (SDP1) and SDP1-LIKE (SDP1L), are responsible for the majority of oil breakdown [13,14]. The released free FAs and/or acyl-CoA esters enter the β-oxidation pathway and are transported across the peroxisomal membrane by PEROXISOMAL ABC TRANSPORTER1 (PXAI)/PEROXISOME DEFICIENT3 (PED3)/COMATOSE (CTS) [15–17]. The conversion of FAs to fatty acyl-CoAs is activated by two peroxisomal long-chain acyl-CoA synthetases (LACS6 and LACS7) [18]. The three core enzymes in the β-oxidation pathway consist of acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT) [3]. ACX, which catalyzes the conversion of CoA thiolase (KAT) [3]. ACX, which catalyzes the conversion of acyl-CoA to acetyl-CoA, is a key enzyme in the β-oxidation pathway.

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which catalyzes thiolytic cleavage in the last step of β-oxidation [10], is expressed during germination [22]. The Arabidopsis kat2 mutant is defective in storage oil breakdown and is dependent on exogenous Suc during seedling establishment [22]. After β-oxidation, acetyl-CoA is converted to either citrate for respiration, or soluble sugars through the glyoxylate cycle and gluconeogenesis to support metabolism and growth [10].

During plant lipid metabolism, lipids and their acyl-CoA derivatives are transported between different subcellular compartments [10,23]. Acyl-CoA-binding proteins (ACBPs) are candidates for such transfer because recombinant ACBPs have been demonstrated to bind acyl-CoA esters and phospholipids in vitro [24–32]. Arabidopsis ACBPs have been shown to mediate heavy metal stress tolerance [28,33], plant defense [34], drought tolerance [35], and freezing tolerance [26,29,36]. Both ACBP1 and ACBP2 are expressed during seedling establishment [33,37] while ACBP3 is highly expressed in germinating seedlings [38]. Some ACBPs have been reported to display diurnal expression [30,31,38,39]. In 4-week-old Arabidopsis Col-0 rosettes, the expression of ACBP4 and ACBP5 was higher in the light period [30], while ACBP4 and ACBP5 accumulation lagged behind, with peak expression at the end of the subjective day [39]. In contrast, ACBP3 was induced in the dark in 4-week-old Col-0 rosettes [30,31] and 2- to 3-week-old ACBP3pro::GUS transformants [39].

In Arabidopsis, the clock regulatory circuit comprises a series of interlinked transcriptional feedback loops [40,41]. The core clock loop consists of an evening-phased pseudorepressor rhythm TIMING OF CAB EXPRESSION1 (TOC1) and two morning-expressed MYB transcription factors CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which are reciprocally regulated [42]. CCA1 and LHY are DNA-binding proteins suppressing TOC1 expression by binding to its 5'-flanking region [42–44]. As CCA1 and LHY act synergistically [45], the ca1lhy double mutant [44,46–49] was included in this study to address the diurnal regulation of ACBPs and other genes in lipid metabolism in early developing Arabidopsis seedlings. As our previous studies on diurnal control of ACBPs were conducted using 2- to 4-week-old rosettes [30,31,38], we were interested to investigate if any ACBPs are diurnally regulated earlier in development.

Many ACBPs are stress responsive [26,28,29,32–36] and harmony between external environmental signals and the internal clock can improve plant fitness and survival [50,51]. For example, CCA1 regulation of defense genes allows plants to anticipate infection at dawn and better time responses to balance growth and defense [52]. This would be pertinent to ACBP3 which has been reported to play a role in plant defense [34,36]. Furthermore, the clock is also known to control events in primary metabolism [53–56], for example CCA1 affects chlorophyll synthesis and biomass production, leading to starch metabolism and growth vigour [57]. Other examples include the regulation of CCA1 by glutamate (Glu) and Glu-derived metabolites and CCA1 control of nitrogen (N)-assimilatory genes [58]. Indeed, defective clock regulation reduced starch turnover and caused irregular leaf growth during the day [59]. High throughput analysis of several circadian microarray experiments revealed that about one-third of the genes expressed in 9-day-old seedlings are influenced by the biological clock [60]. As it has not been experimentally verified whether lipid metabolism in 2- and 5-day-old seedlings is diurnally-controlled, we initiated investigations on the expression of ACBPs and lipid metabolism genes in wild-type WS and Col-0 versus their ca1lhy and CCA1-OX derivatives, and subsequently demonstrated that lipid metabolism is diurnally affected even at the early stages in seedling development.

Materials and Methods

Plant materials and growth conditions

Wild-type Arabidopsis (Arabidopsis thaliana) consisted of ecotypes WS (Columbia) and Col-0. Arabidopsis wild-type and mutant cca1-11 lhy-21 (Columbia) [61] seeds were purchased from the Arabidopsis Biological Resource Center (ABRC), CCA1-OX (35S::CCA1) was provided by Professor E.M. Tobin [62]. Seeds of each genotype were harvested at the same time from plants grown under the same conditions in the growth chamber (16 h light, 270 μmol m–2 s–1 and 8 h dark) at 22°C. Seeds were stored in a desiccator in the dark at room temperature. For germination assays, quantitative real-time PCR (qRT-PCR) and lipid analysis, seeds were surface-sterilized and germinated in half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich) containing 1% (w/v) agar with 20 mM sucrose [20], to encourage more rapid seedling growth [63]. Following 4 days of 4°C treatment in the dark, plates were incubated in the tissue culture room (12 h light, 250 μmol m–2 s–1 and 12 h dark) at 22°C. For germination assays, freshly-harvested and after-ripening (harvested 3–6 months prior to use) seeds were tested and seeds were scored as germinated when radicle protrusion occurred. For Nile Red staining, seeds were grown on 1% (w/v) water-based agar.

qRT-PCR analysis

Seeds germinated from after-ripening seeds (harvested 3–6 months prior to use) were used for qRT-PCR analysis. Two sets of 2- and 5-day-old seedlings from each genotype were prepared in opposing 12-h-light/12-h-dark regimes according to Baudry et al. (2010) [64]. Samples were collected from both sets. Eight time points per day were selected [65]. For each time point, 500–600 2-day-old seedlings or 30–40 5-day-old seedlings were pooled for RNA isolation.

RNA, prepared using a RNasy Isolation Kit (Qiagen), was treated with DNase and reverse-transcribed to cDNA according to the procedure supplied by the cDNA Synthesis Kit (Invitrogen). The expression of IPP2 (isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase 2), which is not affected by diurnal or circadian regulation in Arabidopsis seedlings, was used as an internal control [64,66]. The expression of genes in lipid metabolism was detected in qRT-PCR using gene-specific primers as listed in Table S1. StepOne Plus (Applied Biosystems) and FastStart Universal SYBR Green Master (Roche) were utilized in qRT-PCR. Conditions for qRT-PCR were: 95°C, 10 min, followed by 40 cycles of 95°C, 15 s and 60°C, 1 min. The relative ratio of threshold cycle (Ct) values between the IPP2 gene and the specific gene was calculated. For quantification to calculate 2–ΔΔCt, three technical replicates at each time point were used. Data in Figures 1–6 and S1 represents a mean value of six repeats from two independent biological samples. Genes which displayed a 2-fold or greater value at peak expression over its lowest expression level in wild-type WS or Col-0, in both two biological repeats, were deemed to be diurnally regulated.

Confocal microscopy

One-day-old imbibed seeds and seedlings (aged 2 to 5 days) grown under 12-h-light/12-h-dark cycles were stained with an aqueous solution of Nile Red (Sigma) to visually detect neutral lipids [23,67–70]. Images were obtained with a 65× objective by confocal laser scanning microscopy using a Zeiss LSM 710 system equipped with argon and HeNe lasers as excitation sources. Fluorescence was excited at 514 nm and collected with a 539–653 nm filter.
TAG extraction and mass spectrometry (MS) profiling

Dry seeds and seedlings germinated from after-ripening seeds (harvested 3–6 months prior to use) were used for lipid analysis. Dry seeds and 1- to 5-day-old seedlings germinated under 12-h-light/12-h-dark cycles were collected for lipid analysis. Seed TAGs were extracted following Bligh and Dyer (1959) [71]; seeds were heated for 10 min at 95°C in 1 ml of isopropanol and...
homogenized using a mortar and pestle. The homogenate was centrifuged, supernatant collected, and the pellet re-extracted. The molecular species of TAGs were analysed by electrospray ionisation triple quadrupole mass spectrometry (API 4000 QTRAP; Applied Biosystems). The profiling samples were prepared by combining 50 µl of the total lipid extract with 950 µl of isopropanol/methanol/50 mM ammonium acetate/dichloromethane (4:3:2:1). TAGs [M+NH4]^+ were measured.

Figure 2. Expression pattern of the ACBP gene family in CCA1-OX and wild-type Col-0 as investigated by qRT-PCR. Expression of ACBPs in 2- and 5-day-old seedlings of wild-type Col-0 (open circle) and CCA1-OX (closed rhombus) germinated under 12-h-light/12-h-dark cycles. Relative gene expression level on the Y axis was normalized against IPP2. Each time point represents a mean value of six repeats from two independent biological samples ± SE. White boxes, subjective day; black boxes, subjective night.

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following Li et al. (2014) [72] and were defined by the presence of one acyl fragment and the mass/charge of the ion formed from the intact lipid (neutral loss profiling). This allows identification of TAG acyl species and the total acyl carbons and total number of acyl double bonds in the other two chains. TAGs were quantified after background subtraction, smoothing, integration, isotope deconvolution and comparison of sample peaks with those of the internal standard (using LipidView, Applied Biosystems). The mass spectral responses of various TAG species are variable, owing to differential ionization of individual molecular TAG species. The data were normalized to the internal standard tri-17:0 (Nu-Chek Prep, USA). Fatty acid methyl esters (FAMEs) were obtained by transmethylation [73] and analyzed by gas chromatography-flame ionization detector (GC-FID) [74].

Acyl-CoA profiling

Five-day-old seedlings germinated under 12-h-light/12-h-dark cycles were subjected to qRT-PCR to compare relative gene expression on the Y axis was normalized against [64,66]. As a positive control, the expression of core clock genes TOC1 and GIGANTEA (GI) between the cca1lhy mutant and wild-type WS (Figure S1A) and between CCA1-OX and wild-type Col-0 (Figure S1B) were compared. TOC1 showed peak expression at Zeitgeber time 9 (ZT9) in 2-day-old Col-0 seedlings (P < 0.05; Student’s t test), ZT12 in 2-day-old WS and 5-day-old Col-0 seedlings (P < 0.05; Student’s t test), and ZT18 in 5-day-old WS seedlings (P < 0.05; Student’s t test) (Figure S1). Peak GI expression occurred during the subjective day, at ZT6 in 2- and 5-day-old WS (P < 0.01; Student’s t test) and 2-day-old Col-0 seedlings and at ZT9 in 5-day-old Col-0 seedlings (P < 0.01; Student’s t test) (Figure S1). Fluctuations in expression of TOC1 and GI in the wild types were generally not apparent in the cca1lhy mutant and CCA1-OX (Figure S1), suggesting that both the cca1lhy mutant and CCA1-OX are arrhythmic lines as previous reported [44,62].

In 2-day-old WS seedlings, obvious fluctuation in expression was not observed in both ACPB1 and ACPB2 except at ZT3, when ACPB1 peaked in cca1lhy (P < 0.05; Student’s t test) (Figure 1). In 5-day-old seedlings, both ACPB1 and ACPB2 mRNAs peaked in WS at ZT15 while this pattern was not evident in cca1lhy (P < 0.05; Student’s t test) (Figure 1). ACPB3 expression peaked at ZT12 in 2- and 5-day-old WS and at ZT24 in 2- and 5-day-old cca1lhy (P < 0.05; Student’s t test) (Figure 1). In 2-day-old but not 5-day-old cca1lhy, ACPB4 mRNA generally showed higher expression than WS (P < 0.05; Student’s t test) (Figure 1). In 2-day-old seedlings, ACPB5 expression was higher at ZT6 in cca1lhy than in WS (P < 0.05; Student’s t test) (Figure 1). In 5-day-old WS seedlings, ACPB5 showed higher expression than the cca1lhy mutant from ZT18 to ZT24 (P < 0.05; Student’s t test) but differences between them were not significant for ACPB6 (Figure 1). In 2-day-old seedlings, ACPB6 expression peaked at ZT6 and showed lowest expression at ZT12 in WS (P < 0.001; Student’s t test); such fluctuation was absent in cca1lhy (Figure 1).

Similar to WS, wild-type Col-0 showed more obvious fluctuation in ACPB1 and ACPB2 expression in 5-day-old rather than 2-day-old seedlings (Figure 1), again peaking at ZT15 at day 5 (Figure 1), while the expression pattern of ACPB1 and ACPB2 in CCA1-OX was rather similar to the wild type (Figure 2). In comparison to Col-0, peak ACPB3 expression at ZT12 and ZT24 was greater in 2-day-old CCA1-OX (P < 0.05; Student’s t test) (Figure 2). In 5-day-old CCA1-OX, ACPB3 showed lower expression than Col-0 from ZT3 to ZT9 but expression significantly increased between ZT15 to ZT24 (P < 0.05; Student’s t test) (Figure 2). In 2- and 5-day-old Col-0, ACPB4 did not show obvious diurnal regulation, while in CCA1-OX its expression deviated from Col-0 with greatest differences between them at ZT9 on day 2 (P < 0.05; Student’s t test; Figure 2). In 2-day-old Col-0, both ACPB5 and ACPB6 mRNAs did not show obvious diurnal expression, while ACPB5 expression was enhanced in 5-day-old CCA1-OX at ZT15 and ACPB6 generally showed higher expression in 2- and 5-day-old CCA1-OX (P < 0.05; Student’s t test) in comparison to Col-0 (Figure 2).
Comparative gene expression in lipid metabolism between cca1lhy and wild-type seedlings

When the expression of genes in lipolysis and β-oxidation in 2- and 5-day-old seedlings were analyzed by qRT-PCR, SDP1 was down-regulated at ZT3 and ZT6, and up-regulated at ZT21 and ZT24 in cca1lhy versus WS (Figure 3). In cca1lhy, CTS showed lower expression than WS at ZT15 (P<0.001; Student’s t test) especially in 2-day-old seedlings (Figure 3), but LACS6 did not demonstrate obvious changes. However, in 2-day-old cca1lhy, LACS7 showed higher expression than WS at ZT6 and ZT9 (P<0.05; Student’s t test), but lower expression at ZT18-24 in 5-day-old seedlings (Figure 3). ACX1 mRNA expression was somewhat reduced in cca1lhy in comparison to WS especially at ZT3 (P<0.01; Student’s t test) at days 2 and 5 (Figure 3). In 2-day-old WS, ACX2, MFP2 and KAT2 mRNAs all showed the lowest expression at ZT12 (Figure 3). However, the expression of ACX2 was higher in 2-day-old cca1lhy at most time points in comparison to WS (Figure 3). MFP2 and KAT2 expression was generally lower (P<0.05; Student’s t test) in 5-day-old cca1lhy at most time points in comparison to WS, although most MFP2 and KAT2 values were higher at day 2 (P<0.05; Student’s t test; Figure 3). Hence, the genes involved in storage reserve mobilization, such as ACX2, MFP2, and KAT2 seemed more highly-expressed in cca1lhy than WS at day 2 (Figure 3). At day 5, ACX2, MFP2, and KAT2 were generally down-regulated during the subjective night in cca1lhy in comparison to WS (Figure 3).

Given that TAG synthesis plays a role during seedling establishment [3], DGAT1, DGAT2, DGAT3 and PDAT1

Figure 4. Comparison of gene expression of lipid metabolism between CCA1-OX and wild-type Col-0 as investigated by qRT-PCR. Expression of CTS, SDP1, LAC6, LACS7, ACX1, ACX2, MFP2 and KAT2 in 2- and 5-day-old seedlings of wild-type Col-0 (open circle) and CCA1-OX (closed rhombus) germinated under 12-h-light/12-h-dark cycles. Relative gene expression level on the Y axis was normalized against IPP2. Each time point represents a mean value of six repeats from two independent biological samples ± SE. White boxes, subjective day; black boxes, subjective night. doi:10.1371/journal.pone.0107372.g004

Figure 5. Comparison in expression of genes involved in TAG synthesis in the cca1lhy mutant and wild-type WS as investigated by qRT-PCR. Expression of DGAT1, DGAT2, DGAT3 and PDAT1 in 2- and 5-day-old seedlings of the wild type (open circle) and the cca1lhy mutant (closed rhombus) germinated under 12-h-light/12-h-dark cycles. Relative gene expression on the Y axis was normalized against IPP2. Each time point represents a mean value of six repeats from two independent biological samples ± SE. White boxes, subjective day; black boxes, subjective night. doi:10.1371/journal.pone.0107372.g005
expression was analyzed in 2- and 5-day-old seedlings (Figure 5). Differences in DGAT1, DGAT3 and PDAT1 expression were observed between the 2- and/or 5-day-old cca1lhy and WS (Figure 5). Variation of DGAT1 expression was higher in 2-day-old WS than in cca1lhy, with the lowest expression at ZT24 (P<0.01; Student’s t test; Figure 5). DGAT1 showed pronounced fluctuation in 5-day-old WS but not cca1lhy (Figure 5). In contrast, DGAT1 and DGAT3 expression in 5-day-old cca1lhy seemed less affected than WS by light/dark cycling whilst there was no significant difference between the genotypes in DGAT2 expression in both 2- and 5-day-old seedlings (Figure 5). Five-day-old WS showed peak DGAT1 expression at ZT9 with a second peak at ZT18 (Figure 5). Peak DGAT3 expression occurred at ZT18 (Figure 5). Absence of these peaks in the mutant suggests that diurnal control of DGAT1 and DGAT3 was quashed. Interestingly, PDAT1 expression showed greater fluctuation in 5-day-old cca1lhy than WS but such differences were less obvious in 2-day-old seedlings (Figure 5). These findings provide evidence for diurnal control of TAG synthesis in DGAT1, DGAT3, and PDAT1 expression in 2- and 5-day-old seedlings.

Figure 6. Comparison in expression of genes involved in TAG synthesis in CCA1-OX and wild-type Col-0 as investigated by qRT-PCR. Expression of DGAT1, DGAT2, DGAT3 and PDAT1 in 2- and 5-day-old seedlings of wild-type Col-0 (open circle) and CCA1-OX (closed rhombus) germinated under 12-h-light/12-h-dark cycles. Relative gene expression level on the Y axis was normalized against IPP2. Each time point represents a mean value of six repeats from two independent biological samples ± SE. White boxes, subjective day; black boxes, subjective night. doi:10.1371/journal.pone.0107372.g006

Comparative gene expression in lipid metabolism between CCA1-OX and wild-type seedlings

To further investigate the role of CCA1 in lipid metabolism, the expression of CTS, SDP1, LACS6, LACS7, ACX1, ACX2, MFP2 and KAT2 in 2- and 5-day-old seedlings germinated under 12-h-light/12-h-dark cycles of wild-type Col-0 and CCA1-OX was analyzed by qRT-PCR (Figure 4). In 2-day-old Col-0, CTS and LACS7 expression peaked at ZT9 and ZT21, respectively, while LACS6 peaked at ZT15 and the lowest expression of SDP1, CTS, LACS6, LACS7, ACX1, ACX2, MFP2 and KAT2 appeared at ZT12 or ZT24 (Figure 4). Genes involved in storage reserve mobilization in CCA1-OX were generally down-regulated in 2-day-old seedlings and up-regulated in 5-day-old seedlings in comparison to Col-0 (Figure 4). In particular, in 2-day-old CCA1-OX, SDP1 expression was generally lower than Col-0 from ZT6 to ZT15, LACS6 from ZT15 to ZT21, ACX1 from ZT15 to ZT24, MFP2 from ZT3 to ZT21 and KAT2 from ZT3 to ZT21 (Figure 4). Also, loss in diurnal regulation was evident in 2-day-old CCA1-OX for CTS, in contrast to Col-0 which peaked at ZT9 and showed lowest expression at ZT12 (P<0.05; Student’s t test)
Regulation of Plant Lipid Metabolism in Seedlings

Data mining of microarray analysis specific to 9-day-old seedlings

Covington et al. (2008) [60] have integrated information from multiple circadian microarray experiments using 9-day-old Arabidopsis seedlings to evaluate the circadian transcriptome. Genes that were expressed (Exp) and those under circadian control (Cir) are summarized (Table S2) in a total of nine datasets, including four original datasets: namely Covington [78], Edwards [79], Michael 1 and Michael 2 [55]; three of which the original Covington (C) and Edwards (E) datasets were combined in three different ways: CECE, CCEE, and EECC; and finally two combined datasets: C+E intersection and C+E union [60]. Mining their summarized data (Additional Data File 2 in [60]), we identified ACBPs and the genes associated with lipolysis, \( \beta \)-oxidation and TAG synthesis that were expressed, as well as those that showed clock regulation (Table S2), and the expression pattern of the selected genes in the normalized CCE dataset is presented in Figure S2. ACBPs did not show any obvious clock regulation in 9-day-old seedlings (Table S2) while ACBP2 and ACBP5 showed some fluctuations (Figure S2). The lipolysis gene SDP1 demonstrated clock regulation in all nine datasets (Table S2), peaking at the subjective day (Figure S2). Of the \( \beta \)-oxidation genes, CTS indicated clock regulation only in the Covington dataset (Table S2); while LAC6 displayed clock regulation in seven datasets except for the original Covington and Edwards datasets (Table S2), peaking at the late subjective day (Figure S2). LAC5 and MFP2 did not exhibit any evidence of clock regulation in all nine datasets (Table S2). ACX1, peaking at the subjective night or from the late subjective night to the early subjective day (Figure S2) and KAT2, peaking from the late subjective night to the early subjective day (Figure S2), showed clock regulation in most datasets except in the original Edwards and Covington datasets, respectively (Table S2); whilst ACX2 demonstrated such regulation only in Michael 1 and Michael 2 (Table S2). Of the TAG synthesis genes, DGAT1 (but not DGAT2) showed clock regulation in all nine datasets (Table S2), peaking at the late subjective day (Figure S2). PDAT1 was clock-regulated in six datasets except the original Covington, Michael 1, and Michael 2 (Table S2), peaking at the subjective day (Figure S2). In general, genes under clock control showed high expression in the subjective day or at day-night transition.

The cca1 lhy mutant retains oil bodies

It has been reported that oil body accumulation is a phenotype observed in Arabidopsis mutants abrogated in the mobilization of storage reserves [13,16,18,20–22]. Freshly-harvested and after-ripening seeds of cca1 lhy and WS, germinated under 12-h-light/12-h-dark cycles on water-based agar, were stained with Nile Red during seedling growth from days 1 to 5 after imbibition (Figure 7). Confocal laser microscopy revealed the presence of red-stained spherical inclusions representing oil body accumulation in cca1 lhy from days 1 to 5 in comparison to WS (Figure 7). In cca1 lhy, the oil bodies were evident at day 3 in samples derived from freshly-harvested and after-ripening seeds (Figure 7) although seedling establishment would have been completed by then [20]. In WS, the number of oil bodies had substantially declined by day 5 and very few were evident by days 4 and 5 (Figure 7). When the germination frequency was investigated using freshly-harvested seeds of the cca1 lhy mutant and wild-type WS cultured under 12-

![Figure 7. The cca1 lhy mutant showed oil body retention in comparison to wild-type WS. Confocal laser microscopy of Nile Red stained oil bodies of the radicle from 1-day-old imbibed seeds (Day 1) and hypocotyl epidermis from 2- to 5-day-old seedlings (Day 2 to Day 5) of the cca1 lhy mutant and wild-type WS germinated under 12-h-light/12-h-dark cycles on water-based agar. Samples from freshly-harvested and after-ripening seeds of the cca1 lhy mutant and wild-type WS are shown. Scale bar = 10 μm.](image)
h-light/12-h-dark cycles on half-strength MS medium containing 20 mM sucrose, there were no significant differences between them except at day 1 when the wild type germinated better (Figure S3A). However, after-ripening (Figure S3B) seeds of the cca1lhy mutant were slightly impaired in germination in comparison to the wild type over a 7-day observation period.

TAG accumulates in 5-day-old cca1lhy seedlings

When 4- and 5-day-old cca1lhy seedlings showed oil body accumulation in contrast to the wild type (Figure 7), we were prompted to investigate whether this coincided with changes in TAG content. Dry seed and seedling samples collected at regular intervals, 1 to 5 days after imbibition under 12-h-light/12-h-dark cycles, were analyzed for TAG content by electrospray ionization–tandem mass spectrometry mass spectrometry (ESI-MS/MS) (Figure 8). CCA1-OX dry seeds showed significantly higher TAG content than wild-type Col-0 (Figure 8A), while cca1lhy seemed to contain slightly lower TAG than WS (Figure 8A), but this difference was not statistically significant (Figure 8A). At day 1 after imbibition, there were no apparent differences in TAG content amongst cca1lhy, CCA1-OX, and their respective wild types (Figure 8B). At days 2 and 3 after imbibition, only CCA1-OX indicated an elevated TAG content in comparison to Col-0 (Figure 8C–D), and this was maintained to day 4 (Figure 8E). The increase of TAG in cca1lhy over WS appeared at days 4 and 5 after imbibition (Figure 8E–F). The cca1lhy mutant showed highest TAG content amongst all the genotypes on days 4 to 5 (Figure 8E–F). In contrast, by day 5 there were no apparent differences between CCA1-OX and Col-0 (Figure 8F).

Total FAs accumulate in 5-day-old cca1lhy seedlings

Changes in FA composition were identified between cca1lhy and WS (Figure 9), as well as CCA1-OX and Col-0 (Figure 10), using GC-FID analysis of FAMEs on dry seeds and seedlings grown under 12-h-light/12-h-dark cycles. In dry seeds and 1- to 3-day-old seedlings, there were no significant changes in fatty acid content between cca1lhy and WS (Figure 9A–D). However, cca1lhy showed an increase in fatty acids at days 4 to 5 after imbibition in comparison to WS (Figure 9E–F).

Interestingly, in comparison to Col-0, CCA1-OX showed some compositional changes i.e. higher 18:2 and 18:3 fatty acid content in dry seeds (Figure 10A), similar fatty acid content in 1-day-old seedlings (Figure 10B), higher 18:2, 18:3, and 20:1 fatty acid content in 2-day-old seedlings (Figure 10C), increased 16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, and 22:1 fatty acid content in 3-day-old seedlings (Figure 10D), higher 18:1, 18:2, 18:3, and 20:1 fatty acid content in 4-day-old seedlings (Figure 10E), but similar fatty acid content in 5-day-old seedlings (Figure 10F).

Acyl-CoA profiling highlighted differences in acyl-CoA composition between cca1lhy, CCA1-OX, and wild-type seedlings

During storage reserve mobilization in post-germination development, fatty acids from oil bodies are activated to acyl-CoAs before they enter peroxisomal FA β-oxidation [19]. To determine the acyl-CoA compositional changes amongst cca1lhy, CCA1-OX, and wild-type Arabidopsis, the acyl-CoA pool was profiled in 5-day-old seedlings germinated under 12-h-light/12-h-dark cycles. Some acyl-CoA esters (16:0, 16:3, 24:0, and 26:0) accumulated in cca1lhy in comparison to wild-type WS (Figure S4); whilst others, e.g. 18:2, 22:1, and 28:1-CoA, decreased in cca1lhy in comparison to WS (Figure S4). When compared to wild-type Col-0, 16:3, 18:3, 20:0, 20:1, 22:1, 24:1 and 26:1-CoA were reduced in CCA1-OX (Figure S4). Only 24:0 and 30:0-CoA accumulated in CCA1-OX in comparison to Col-0 (Figure S4).

Discussion

Comparative expression of ACBPs and lipid metabolism genes in Arabidopsis seedlings

In plants, it has been reported that the mRNAs of many metabolic enzymes are clock-regulated [53–56]. For example, the expression of genes involved in chlorophyll biosynthesis peaked at late dark; those of the electron transport photosystems peaked in the light; starch synthesis genes were highly expressed at early light...
or during the day in contrast to starch degradation genes which peaked at late light; and most genes related to nitrogen and sulfate assimilation peaked at the subjective night or at early light [53,56,80–83]. Various enzymes involved in plant lipid biosynthesis including β-ketoacyl-CoA synthase 16 (KCS16; At4g34250), acyl-CoA desaturase-like 2 (ADS2; At2g31360), sphingolipid Δ8-desaturase 2 (SLD2; At2g46210), UDP-Glc:sterol glucosyltransferase (UGT80A2; At3g07020), CDP-DAG synthase 1 (CDS1; At1g62430), and lecithine cholesterol acyltransferase-like protein (At1g27480) are known to be transcriptionally regulated by the biological clock [53].

In this study, the comparative expression of ACBPs and lipid metabolism genes was investigated in 2- and 5-day-old Arabidopsis seedlings. Consistent with previous reports that TOC1 peaks at ZT12 [43] and GI peaks during the subjective day in 7-day-old WS seedlings [84], our investigation on WS and Col-0 seedlings revealed that TOC1 expression also peaked at ZT12 in 2-day-old WS and 5-day-old Col-0, while GI peaked during the subjective day in both 2- and 5-day-old WS and Col-0 (Figure S1). The expression of ACBPs and lipid metabolism genes was observed herein to align with the expression of TOC1 and GI and they too showed variation in expression between the cca1lhy mutant and wild-type WS (Figure S1A) and between CCA1-OX and wild-type Col-0 (Figure S1B). At days 2 and 5, ACBP3 showed more obvious diurnal regulation in wild-type WS (Figure 1) than Col-0 (Figure 2). In 5-day-old wild-type WS and Col-0 seedlings, a similar diurnal expression pattern was observed for ACBP1 and ACBP2 (Figures 1, 2 and S5). Fluctuation of ACBP6 expression was more obvious in 2-day-old wild-type WS (Figures 1 and S5). As candidates for acyl-lipid transfer, fluctuation in expression of ACBPs suggests that it may diurnally affect acyl-lipid metabolism in seedlings. When we compared the expression pattern between WS and cca1lhy, our results revealed that the expression pattern of all ACBP mRNAs in cca1lhy slightly deviated from the wild type (Figure 1). Moreover, the diurnal expression pattern of ACBPs in wild-type Col-0 seedlings germinated under 12-h-light/12-h-dark cycles showed some differences from results conducted on 4-week-old rosettes under 16-h-light/8-h-dark [30]: both ACBP1 and ACBP2 showed peak expression at ZT15 in 5-day-old seedlings but not in rosettes; both ACBP4 and ACBP5 showed peak expression at ZT9 in rosettes but not in 2- and 5-day-old seedlings. Nevertheless, similarity was noted in ACBP3 peaks at ZT24 in 2-day-old seedlings and 4-week-old rosettes. Indeed, ACBP3 expression displayed the most contrast between the cca1lhy mutant and WS (Figure 1) and between CCA1-OX and Col-0 (Figure 2), consistent with our previous reports that ACBP3 mRNA is most affected by light/dark cycling [30,31] and that the 5′-flanking region of ACBP3 is responsive to dark/light [38]. This finding relating ACBP3 to CCA1 supports a previous report that CCA1 regulates plant defense [52]; ACBP3 has been shown to play a role in the plant defense response [34,38]. Furthermore, variations in the gene expression patterns of ACBPs in seedlings and rosettes indicate that diurnal regulation may alter at various stages in plant development.

![Figure 9. Fatty acid profiling of WS and cca1lhy seeds and seedlings during germination and early post-germinative growth. (A) Major fatty acid (FA) content of WS and cca1lhy dry seeds. (B)-(F) Major FA content of WS and cca1lhy seedlings at Day 1 (B), Day 2 (C), Day 3 (D), Day 4 (E), and Day 5 (F) cultured under 12-h-light/12-h-dark cycles on half-strength MS medium supplemented with 20 mM sucrose. White bar, wild-type WS; light gray bar, the cca1lhy mutant. Data in (A) represents a mean value of three repeats ± SD per seed, each measurement contains 20 dry seeds. Data in (B)-(F) represents a mean value of three repeats ± SD per mg fresh weight (FW) of seedlings, per measurement contains 96–122 mg seedlings. Student’s t test for *, P<0.05; **, P<0.01. doi:10.1371/journal.pone.0107372.g009](https://www.plosone.org/doi/10.1371/journal.pone.0107372.g009)
Besides ACBPs, genes related to storage reserve mobilization showed diurnal fluctuation in expression in wild-type seedlings (Figures 3, 4 and S5). In some cases peak expression differed between WS and Col-0. For example in 2-day-old WS, peak expression of CTS was observed at ZT15 (Figure 3) in contrast to ZT9 in Col-0 (Figure 4). Nevertheless, in both 2-day-old wild-type WS and Col-0 seedlings, a similar expression pattern was observed for SDP1 with lowest expression at ZT24 (Figures 3–4). In 2-day-old WS seedlings, lowest expression at ZT12 was noted for ACX2, MFP2 and KAT2 (Figure 3) while in 2-day-old Col-0 seedlings two troughs at ZT12 and ZT24 were observed for LACS6, ACX2 and KAT2 (Figure 4). Genes involved in storage reserve mobilization somewhat showed reduced expression at day-night transition (ZT12 and ZT24) in 2-day-old wild-type seedlings but this pattern became less obvious in older (5-day-old) seedlings (Figures 3–4). Such fluctuations suggest that β-oxidation may be subject to diurnal regulation in these young Arabidopsis seedlings (Figure S5).

Loss of diurnal regulation of CTS was observed in both 2-day-old cca1lhy and CCA1-OX seedlings (Figures 3–4). ACX2, MFP2 and KAT2 were generally up-regulated in 2-day-old cca1lhy and MFP2 and KAT2 mildly down-regulated during the subjective night at day 5 in comparison to WS (Figure 3). In contrast, ACX1, MFP2 and KAT2 were generally down-regulated in 2-day-old CCA1-OX seedlings and up-regulated at day 5 in comparison to Col-0 (Figure 4). Taken together, these results suggest that the expression of genes in storage reserve mobilization is altered in both cca1lhy and CCA1-OX seedlings. ACX1, MFP2 and KAT2 have corresponding mutants previously reported to show an oil body retention phenotype [20–22]. Such down-regulation may explain for the oil body accumulation phenotype we observed in the 4- and 5-day-old cca1lhy mutants (Figure 7) arising from a reduction in lipid catabolism.

Genes of TAG synthesis as well as ACBPs and genes involved in storage reserve mobilization showed fluctuation in expression in wild-type seedlings (Figures 5, 6 and S5). When comparing the expression pattern in the wild types versus the cca1lhy mutant or CCA1-OX, both DGAT1 and DGAT3 showed different patterns between 5-day-old cca1lhy and WS (Figure 5) as well as CCA1-OX and Col-0 (Figure 6), suggesting that both genes play important roles in seedling establishment and TAG recycling during storage reserve mobilization, in good agreement with the results of Hernández et al. (2012) [3]. The lack of diurnal fluctuation in the expression of DGAT1 and DGAT3 in 5-day-old cca1lhy in comparison to WS (Figure 5) and the increased fluctuation of PDAT1 expression in cca1lhy (Figure 5) may also account for the significant lipid changes in TAG (Figure 8) and FAs (Figure 9) at day 5 (and day 4). In summary, the qRT-PCR results have indicated that both TAG synthesis and its hydrolysis is affected in the cca1lhy mutant.

Our data mined from microarray analysis [60] suggested that SDP1, LACS6, ACX1, KAT2, DGAT1, and PDAT1 are circadian regulated in at least six datasets (Table S2). Our qRT-PCR is consistent with Covington et al. (2008) [60] in that the
expression of some genes in lipid metabolism is diurnally-regulated. We had observed a diurnal expression pattern of target genes in WS and Col-0 and differences between 2- and 5-day-old cca1lhy and WS seedlings, as well as between CCA1-OX and Col-0 (Figures 3, 4, 5, 6 and S5).

However, day 2 is important and significant changes in transcription patterns of genes involved in storage reserve mobilization have been reported [63]. Therefore, variation in gene expression within day 2 might partially be ascribed to developmental rather than diurnal regulation. Moreover, for many metabolic genes, cycling of transcripts need not correlate to changes in maximal catalytic activity or protein level. For example, the mRNAs of many genes in starch degradation oscillate, but their protein levels are kept constant in Arabidopsis [80,82,83,85]. Hence, we caution that our transcriptomic analysis merely provides evidence of diurnal regulation of lipid metabolism at the level of transcription.

Comparison between the cca1lhy mutant and WS at germination

Except for day 1, the germination rate of freshly-harvested seeds after stratification did not significantly differ between the cca1lhy mutant and wild-type WS (Figure S3A), consistent with a previous report [86] which concluded that circadian clock genes coordinate environmental signalling affecting dormancy release in plants. Herein, we used 4-day stratification at 4°C to eliminate the effect of dormancy and subsequently investigated the relationship between the biological clock and lipid metabolism from germination to seedling establishment. Our data showed only minor changes from days 1 to 7 between the cca1lhy mutant and wild-type WS in germination rate for after-ripening seeds (Figure S3B). An oil body retention phenotype was evident in seedlings germinated from either freshly-harvested seeds or after-ripening seeds of the cca1lhy mutant (Figure 7), suggesting that seed germination and storage reserve mobilization are regulated independently as previously shown [87]. In after-ripening seeds, the germination frequencies between the cca1lhy mutant and the wild type further diversified (Figure S3B), in contrast to the hypersensitive dominant phenotype that freshly-harvested seeds of the cca1lhy mutant showed a higher frequency of germination than the wild type and exhibited germination hypersensitivity on cold treatments of 1, 2, and 3 days [86]. Our results indicate that dormant seed and non-dormant seed germination, though differentially regulated, are subject to diurnal control and establish a link between clock regulation and lipid metabolism in Arabidopsis seedlings.

Lipid profiling indicates lipid metabolism is altered in cca1lhy and CCA1-OX seedlings

In plants, storage reserve mobilization that occurs during seed germination and early seedling establishment is known to be a dynamic process [10,11]. A process which we demonstrate is intimately linked to internal plant biological clock. Herein, we showed an oil body accumulation phenotype in the cca1lhy mutant, indicating that lipid metabolism in Arabidopsis seedlings is affected by clock components (CCA1 and LHY). Direct evidence for the influence of the clock in metabolism can be obtained from measurements of metabolites in clock mutants [83]. The Arabidopsis arrhythmic mutant pprr5prr7ppr9 had elevated levels of citric acid cycle intermediates and other metabolites, including amino acids [88]. With regard to lipids, the amounts of linoleic and linolenic acids showed temperature-related fluctuations in cotton seedlings [89]. It has also been shown that only 18:1 FA, but not other fatty acids, oscillates under diurnal cycles in Arabidopsis, with higher levels accumulated in the light rather than the dark [90]. Besides FA, phosphatidylcholine was recently reported to oscillate diurnally and affects florigen-mediated flowering [91].

Previous reports have revealed that the accumulation of 20:1-CoA is characteristic of the storage reserve mobilization related mutants, ctt/pttal, lac6lac7, aoxlax2, mfp2 and kat2 [3,16,19,20–22]. In this study, although a slight down-regulation of various β-oxidation key enzymes including ACX1, MFP2, and KAT2, was observed in 5-day-old cca1lhy (Figure 3), 20:1-CoA did not accumulate (Figure S4), suggesting that TAG hydrolysis may be more affected by diurnal regulation than β-oxidation. However, some acyl-CoAs (16:0, 16:3, 24:0, and 26:0) accumulated significantly in 5-day-old cca1lhy in comparison to WS (Figure S4), while an 18:1-CoA increase was reported in the 5-day-old aoxlax2, mfp2 and kat2 mutants [3,20,22] and 18:2-CoA accumulation in the 5-day-old aoxlax2 mutant [3,20]. Elevation in different kinds of acyl-CoA species might indicate variation in transport mechanisms during storage reserve mobilization. Other than β-oxidation, it has been reported that the N-end rule pathway can regulate seed oil mobilization as was proven using mutants in PROTEOLYSIS6 and ARGINYL-TRNA:PROTEIN ARGINYLTTRANSFERASE [70]. The data represented herein was expressed as % fresh weight and minor changes in lipid content may be attributed to differences in the water content between cca1lhy and WS. For example, lower amounts of 30:0-CoA in the cca1lhy mutant may suggest lower epidermal waxes and higher water loss in the mutant.

Variation in TAG content between CCA1-OX and Col-0 exists in dry seeds (Figure 8A) and was observed in 2- to 4-day-old seedlings (Figure 8C–E), but no significant differences were detected in 1- and 5-day-old seedlings (Figure 8B, F). It appears that differences in 18:2 and 18:3 FAs between dry seeds of CCA1-OX and Col-0 (Figure 10A) attributed to the TAG content increases in 2- to 4-day-old seedlings (Figure 8C–E) with 18:2 being the major FA present (Figure 10). Meanwhile, a general elevation in the expression of β-oxidation key enzymes (ACX1, ACX2, and KAT2) in 5-day-old CCA1-OX (Figure 4), suggests that most acyl-CoA utilization correspondingly increased, concomitant with a decrease in many acyl-CoA species in CCA1-OX. Only 24:0 and 30:0-CoAs accumulated in CCA1-OX, perhaps indicative that other proteins are involved in lipid transport during storage reserve mobilization (Figure S4). Given the predominance of 24:0 and 30:0 acyl chains in the formation of sphingolipids and waxes as observed for 24:0 and 30:0 derivatives in the wax-deficient eceriferum mutants [92–96], the major changes noted in 24:0 and 30:0-CoAs in CCA1-OX may have contributed to the high levels of C24-OH in the formation of sphingolipids and waxes. Interestingly, it has been reported that KCS16 and SL2D2 are transcriptionally regulated by the biological clock [53], and the extent of diurnal regulation in wax and sphingolipid biosynthesis remains to be further determined.

Supporting Information

Figure S1 Expression of TOCI and GI in 2- and 5-day-old seedlings germinated under 12-h-light/12-h-dark cycles. (A) Comparison in expression between TOCI and GI in the cca1lhy mutant (closed rhombus) and wild-type WS (open circle) as investigated by qRT-PCR. (B) Comparison in expression between TOCI and GI in CCA1-OX (closed rhombus) and wild-type Col-0 (open circle) as investigated by qRT-PCR. Relative gene expression level on the Y axis was normalized against IPP2.
Each time point represents a mean value of six repeats from two independent biological samples ± SE. White boxes, subjective day; black boxes, subjective night. (TIF)

**Figure S2** The expression pattern of ACBP1 and lipid metabolism genes in 9-day-old Arabidopsis seedlings. The expression pattern was sieved out from the normalized CCEE dataset from the Additional Data File 1 of Covington et al. (2008). White boxes, subjective day; black boxes, subjective night. (TIF)

**Figure S3** Germination frequencies of WS and the cca1lhy mutant under 12-h-light/12-h-dark cycles on half-strength MS medium supplemented with 20 mM sucrose. (A) Freshly-harvested seeds of the cca1lhy mutant (closed rhombus) and wild-type WS (open circle). (B) After-ripening seeds of the cca1lhy mutant (closed rhombus) and wild-type WS (open circle) were harvested 3–6 months prior to the assay. Values are mean ± SD of measurements made on four separate batches of 50–100 seeds. Student’s *t* test for *, *P*<0.01; **, *P*<0.01; ***, *P*<0.001. (TIF)

**Figure S4** Acyl-CoA profiling of the cca1lhy mutant and CCA1-OX in comparison to wild-type Arabidopsis. Acyl-CoA content of 5-day-old seedlings from the CCA1-OX mutant, CCA1-OX, WS and Col-0 germinated under 12-h-light/12-h-dark cycles on half-strength MS medium supplemented with 20 mM sucrose. (A) Freshly-harvested seeds of the cca1lhy mutant (closed rhombus) and wild-type WS (open circle) were harvested 3–6 months prior to the assay. Values are mean ± SD of measurements made on four separate batches of 50–100 seeds. Student’s *t* test for *, *P*<0.01; **, *P*<0.01; ***. White boxes, subjective day; black boxes, subjective night. (DOC)

**Figure S5** Diurnal regulation of the major lipid metabolic pathways in germinating Arabidopsis seedlings. Target genes in acyl-lipid transfer (ACBP1, ACBP2, ACBP3, ACBP4, ACBP5 and ACBP6), lipolysis (SDP1), β-oxidation (DGAT1, DGAT2, DGAT3 and PDAT1) and TAG synthesis (DGAT1) at Day 2 (A) and Day 5 (B) are represented in italics. Genes which displayed diurnal regulation in wild-type WS in qRT-PCR are colour-coded in orange; those diurnally-regulated in wild-type Col-0 are in blue; and those diurnally-regulated in both WS and Col-0 are in green. (TIF)

**Table S1** Gene-specific primers for qRT-PCR used in this study. (DOC)

**Table S2** The expressed and circadian pattern of lipid metabolism genes in 9-day-old Arabidopsis seedlings mined from circadian microarray data sets. Expressed and circadian pattern of lipid metabolism genes sieved out from Additional Data File 2 in Covington et al. (2008). Exp represents expressed; Cir represents circadian. (DOC)

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**Author Contributions**

Conceived and designed the experiments: ASH RPH MLC. Performed the experiments: ASH RPH LVM PL. Analyzed the data: ASH RPH LVM MLC. Contributed reagents/materials/analysis tools: JAN MLC. Wrote the paper: ASH RPH MLC. Coordinated the project: JAN MLC.

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