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Genetic compensation of triacylglycerol biosynthesis in the green microalga *Chlamydomonas reinhardtii*

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SUMMARY

Genetic compensation has been proposed to explain phenotypic differences between gene knockouts and knockdowns in several metazoan and plant model systems. With the rapid development of reverse genetic tools such as CRISPR/Cas9 and RNAi in microalgae, it is increasingly important to assess whether genetic compensation affects the phenotype of engineered algal mutants. While exploring triacylglycerol (TAG) biosynthesis pathways in the model alga *Chlamydomonas reinhardtii*, it was discovered that knockout of certain genes catalyzing rate-limiting steps of TAG biosynthesis, type-2 diacylglycerol acyltransferase genes (*DGTTs*), triggered genetic compensation under abiotic stress conditions. Genetic compensation of a *DGTT1* null mutation by a related *PDAT* gene was observed regardless of the strain background or mutagenesis approach, for example, CRISPR/Cas 9 or insertional mutagenesis. However, no compensation was found in the *PDAT* knockout mutant. The effect of *PDAT* knockout was evaluated in a Δ*vtc1* mutant, in which *PDAT* was upregulated under stress, resulting in a 90% increase in TAG content. Knockout of *PDAT* in the Δ*vtc1* background induced a 12.8-fold upregulation of *DGTT1* and a 272.3% increase in TAG content in Δ*vtc1*/*pdat1* cells, while remaining viable. These data suggest that genetic compensation contributes to the genetic robustness of microalgal TAG biosynthetic pathways, maintaining lipid and redox homeostasis in the knockout mutants under abiotic stress. This work demonstrates examples of genetic compensation in microalgae, implies the physiological relevance of genetic compensation in TAG biosynthesis under stress, and provides guidance for future genetic engineering and mutant characterization efforts.

Keywords: genetic compensation, triacylglycerol biosynthesis, *Chlamydomonas reinhardtii*, CRISPR, gene knockout.

INTRODUCTION

Organisms have evolved various genetic buffering systems to maintain fitness in response to genetic perturbations, including functionally redundant genes (Tautz, 1992), protein feedback loops (Barabási & Oltvai, 2004) and acquisition of adaptive mutations (Teng et al., 2013). Such buffering or compensation systems ensure similar growth or developmental outcomes despite some genetic changes (El-Brolosy & Stainier, 2017). Genetic compensation is defined as the phenomenon whereby the effect of a deleterious mutation is buffered by the genome (Kontarakis & Stainier, 2020). Recently, genetic compensation through transcriptional adaptation, by which a homologous gene or genes are upregulated in response to an early nonsense mutation in a related gene, has been proposed as a mechanism for maintaining genetic robustness in a number of metazoans, including zebrafish, mice and nematodes, and to explain profound differences between gene knockouts and knockdowns at certain loci (El-Brolosy et al., 2019; Ma et al., 2019; Rossi et al., 2015; Serobyan et al., 2020). Likewise, comparison between gene knockouts and knockdowns in some model plant systems has revealed puzzling discrepancies that suggest transcriptional adaptation is acting to buffer the genome in these species as well (Braun et al., 2008; Chen et al., 2014; Gao et al., 2015; Rodriguez-Leal et al., 2019).

Increasingly, gene knockdown and knockout tools have been deployed in the green microalga *Chlamydomonas reinhardtii* (Chlamydomonas; Greiner et al., 2017; Li et al., 2019; Molnar et al., 2009; Rohr et al., 2004), but little

attention has been paid as to whether these tools might for some genes lead to very different outcomes and, more broadly, whether genetic compensation occurs in green algae (El-Brolosy & Stainier, 2017; Kontarakis & Stainier, 2020). Here we set out to address these questions as part of an ongoing study of lipid biosynthesis in Chlamydomonas, using triacylglycerol (TAG) biosynthesis pathways as examples.

Microalgae store TAG as a carbon- and energy-rich compound in cells, whose synthesis can be stimulated under various stress conditions (Hu et al., 2008; Lenka et al., 2016). Nutrient deprivation such as nitrogen (N) deprivation and phosphorus (P) deprivation are most commonly used stressors to induce TAG production. Under N deprivation, algal cell growth is almost completely arrested while P deprivation promotes TAG production with moderate cell growth (Iwai et al., 2014). Biosynthesis of TAG involves sequential acylation to the precursor glycerol-3-phosphate. Two pathways are involved in the last step of TAG biosynthesis. One pathway is a *de novo* acyl-CoA-dependent route mediated by diacylglycerol acyltransferase (DGAT; Ohlrogge & Browse, 1995). Three major types of DGATs including two structurally distinctive, membrane bound type-1 and type-2 DGATs and a soluble cytosolic type-3 DGAT have been identified in plants, algae and other eukaryotes (Cases et al., 1998; Lardizabal et al., 2001; Oelkers et al., 2002). The other TAG biosynthesis pathway is an acyl-CoA-independent route that requires membrane lipid breakdown and is mediated by a plastidic membrane-bound enzyme, phospholipid:-diacylglycerol acyltransferase (PDAT; Dahlqvist et al., 2000; Yoon et al., 2012).

The genome of *C. reinhardtii* harbors one type-1 *DGAT* gene (*DGAT1*), five type-2 *DGAT* genes (*DGTT1-5*) and one *PDAT* gene (Bagnato et al., 2017; Boyle et al., 2012; Deng et al., 2012; Miller et al., 2010; Yoon et al., 2012). RNA-Seq analysis previously revealed that the abundance of *PDAT*, *DGAT1* and *DGTT1* transcripts was upregulated, and that of *DGTT2* and *DGTT3* was moderate but constant, and the abundance of transcripts of *DGTT4* and *DGTT5* was low to undetectable in response to N deprivation (Boyle et al., 2012). Subsequent quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses corroborated these findings for *PDAT*, *DGAT1* and *DGTT1*, while *DGTT2-3* were found to be induced by N deprivation (Liu et al., 2016; Yoon et al., 2012). Similar results were found in response to P deprivation, where *PDAT*, *DGAT1* and *DGTT1-4* were upregulated during TAG accumulation (Iwai et al., 2014). Functional studies using *in vitro* enzyme assays and gene knockdowns have largely yielded results consistent with expectations based on these expression data. *DGTT1-3* and *PDAT* were shown to catalyze TAG biosynthesis *in vitro* (Liu et al., 2016; Yoon et al., 2012). Knockdown of *DGTT1*, *DGTT2* or *DGTT3* each resulted in a moderately reduced TAG (TAG-less) phenotype under persistent stress, while knockdown of *PDAT* resulted in a moderate TAG-less phenotype during both favorable growth and the early phase of stress induction (Liu et al., 2016; Yoon et al., 2012), suggesting *DGTT*s and *PDAT* are functionally related genes involved in TAG biosynthesis in Chlamydomonas. However, in some cases, analysis of gene knockdowns can be limited by off-target effects and low efficiency of gene silencing (Fedorov et al., 2006; Ma et al., 2006), so null mutants are needed to better understand gene function. Among Chlamydomonas TAG biosynthesis genes, so far only *pdat* mutants have been characterized, which had reduced TAG content under stress (Boyle et al., 2012). However, whether the *PDAT* insertion mutations analyzed were buffered by expression of related genes such as *DGTT*s is not known, and up to now no *DGTT* mutants have been analyzed. So the precise contributions of these genes to TAG accumulation remains obscure.

In this work we analyzed TAG accumulation in strains bearing null mutations in several genes including *DGTT1-3* and *PDAT*, and found phenotypes that contradicted known knockdown phenotypes in these genes. Our data showed genetic compensation through upregulation of related TAG biosynthetic genes for *dgtt1* and *dgtt2* mutants resulted in a higher TAG content under stress, which is contrary to the TAG-less phenotype in the respective knockdown lines. Interestingly, no genetic compensation was found in the *pdat* mutant. However, when evaluated in the Δ*vtc1* background (defective for Vacuolar Transporter Chaperone 1, a component of the polyphosphate polymerase complex), where *PDAT* expression and function are likely critical for storing excess energy as TAG under stress, the *PDAT* knockout resulted in compensation by upregulation of *DGTT1* and a higher TAG content. Our data demonstrate genetic compensation exists in microalgae, and imply the physiological importance of genetic compensation in TAG biosynthesis under stress.

RESULTS

Genetic compensation is present in microalgae

Type-2 DGATs are believed to be major contributors for TAG biosynthesis in algae, but previous knockdown of Chlamydomonas type-2 *DGAT* genes *DGTT1*, *DGTT2* and *DGTT3* caused only relatively small (~10–20%) suppression of TAG accumulation (Liu et al., 2016). We set out to determine whether null mutations in these genes might have larger effects, so we obtained *dgtt1*, *dgtt2* and *dgtt3* CLiP library knockout mutants generated by the Jonikas group (Li et al., 2016; Li et al., 2019) and analyzed their TAG accumulation. Each mutant strain carries a CIB1 cassette insertion that disrupts the respective *DGTT* gene. We further examined the genotype of these mutants and found the *dgtt1* mutant (LMJ.RY0402.223444) insertion is in the middle of intron 1, the *dgtt2* mutant (LMJ.RY0402.213587) insertion is at the junction between exon 4 and intron 4, and the *dgtt3*

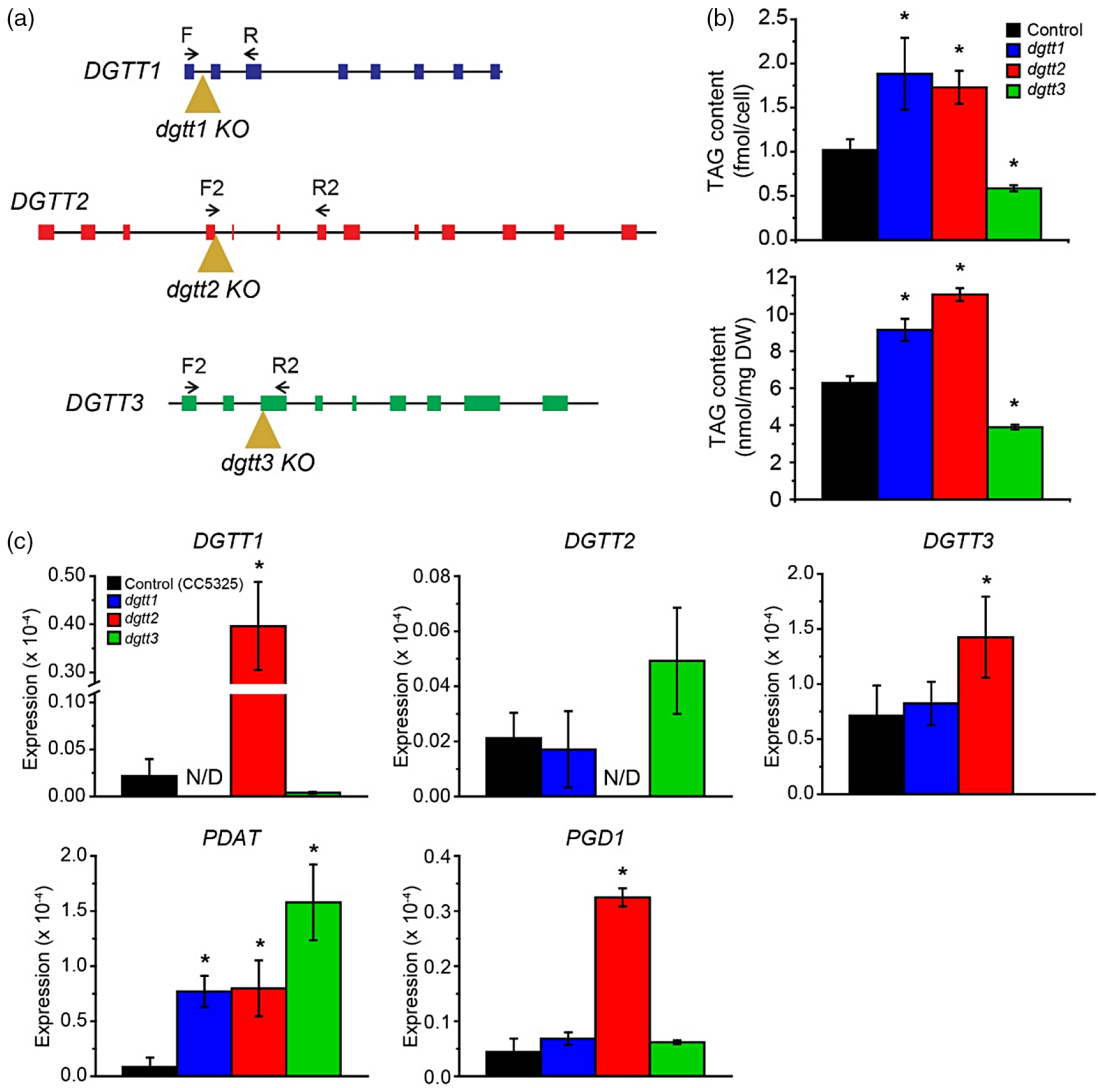

Figure 1. Genetic structure, phenotype and gene expression of the *dgtt1*, *dgtt2*, *dgtt3* knockout mutants obtained from the CLiP library. (a) Locations of the insertion sites of the CIB1 cassette (yellow triangles) and of the primer pairs (black arrows) used to detect gene expression in the *DGTT1*, *DGTT2* and *DGTT3* genes are presented. (b) Triacylglycerol (TAG) content of the *dgtt1* (blue), *dgtt2* (red), *dgtt3* (green) mutants and the parent strain CC5325 (black) grown was measured at 72 h under P deprivation. (c) Relative transcriptional level of selected TAG biosynthesis genes (*DGTT1*, *DGTT2*, *DGTT3*, *PDAT* and *PGD1*) in the *dgtt1* (blue), *dgtt2* (red), *dgtt3* (green) mutants and the parent strain CC5325 (black) was measured at 24 h under P deprivation. Transcripts of *DGTT1* in the *dgtt1* mutant and *DGTT2* in the *dgtt2* mutant were non-detectable (N/D). Data represent mean ± standard deviation (SD) from three biological repeats. An asterisk indicates significance by Student's *t*-test (*P*-value ≤0.05).

mutant (LMJ.RY0402.223565) insertion is at the beginning of exon 3 (Figures 1a and S1). No detectable transcripts of *DGTT1*, *DGTT2* and *DGTT3* were found in the respective mutants (Figure 1c), suggesting all the mutants are null.

The TAG biosynthesis was comparatively studied in the *dgtt1*, *dgtt2* and *dgtt3* mutants and their parent strain (CC5325; control) under P deprivation, which was reported to induce TAG accumulation in *C. reinhardtii* while maintaining moderate cell growth (Molnar et al., 2009). Surprisingly, the *dgtt1* and *dgtt2* knockout mutants had significantly higher TAG content than the control strain (*t*-test, $P \leq 0.05$; Figure 1b) either on a per cell basis or per dry weight (DW) basis. The TAG content of *dgtt1* and *dgtt2* mutant cells increased by about 90% and 70% per cell, respectively, and by 45% and 76% per unit dry weight, compared with control cells at 72 h under P deprivation (Figure 1b). These results are in stark contrast to those for the knockdown strains, which had reduced TAG content (Liu et al., 2016), and they suggest a compensation mechanism exists in Chlamydomonas that affects other TAG genes/enzymes in the *dgtt1* and *dgtt2* mutant. By contrast, the *dgtt3* knockout mutant contained ~40% less TAG per cell and per dry weight compared with the control strain (Figure 1b), and notably it had about twice the decrease in

Table 1 Pair-wise sequence similarity between TAG biosynthetic genes calculated by LALIGN program

Gene 1	Gene 2	Identity matrix (genome)	Identity matrix (transcript)
PDAT	*DGTT1*	50.9%	50.5%
PDAT	*DGTT2*	49.8%	50.7%
PDAT	*DGTT3*	49.2%	52.8%
DGTT1	*DGTT2*	50.5%	51.4%
DGTT1	*DGTT3*	49.0%	51.6%
DGTT2	*DGTT3*	50.6%	59.1%

TAG as that caused by the *dgtt3* knockdown (Liu et al., 2016). In Chlamydomonas, *DGTT*s and *PDAT* are the main contributors of TAG biosynthesis. Sequence analysis revealed nucleotide identities between *PDAT* and *DGTT1*, *DGTT2*, and *DGTT3* were 50.9%, 49.8% and 49.2%, respectively (Table 1), all above the 40% sequence identity threshold needed to induce genetic compensation by genes with high sequence similarity in previous studies (Ma et al., 2019).

To explore possible genetic compensation in the knockout mutants, the expression of TAG biosynthetic genes was examined, including that of *DGTT1*, *DGTT2*, *DGTT3*, *PDAT* and *PGD1* (encodes an MGDG-specific lipase and presumably functions with PDAT in membrane recycling; Li et al., 2012; Liu et al., 2016; Yoon et al., 2012). In the *dgtt1* mutant, the expression of *DGTT2*, *DGTT3* and *PGD1* remained unchanged, but the expression of *PDAT* was upregulated by ninefold compared with the control at 24 h under P deprivation (Figure 1c). In the *dgtt2* mutant, the expression of all tested genes was upregulated; compared with the control, the expression of *DGTT1*, *DGTT3*, *PDAT* and *PGD1* in the *dgtt2* mutant was increased by 19-fold, 2.6-fold, ninefold and 7.6-fold, respectively, at 24 h under P deprivation (Figure 1c), correlating with higher TAG accumulation in the *dgtt2* mutant. In the *dgtt3* knockout strain, the expression level of *PDAT* was significantly higher than for the control (*t*-test, $P \leq 0.05$; Figure 1c), but that increase did not lead to a functional compensation for TAG accumulation (Figure 1b).

Our findings suggest that a mutation leading to a premature stop codon in two different type-2 *DGAT* genes likely activates a genetic compensatory network by upregulation of homologous genes in Chlamydomonas. To determine whether this genetic compensation was specific to insertional mutants in the CLiP library genetic background, or whether it might be a more general phenomenon of null mutants with premature stop codons, we used CRISPR/Cas9 to generate a *dgtt1* mutant in a different strain background (*C. reinhardtii* CC3403). In the resulting mutant (*CC-dgtt1*), the *DGTT1* allele carries a 1229-bp insertion corresponding to pHS_SaCas9 sequence (Cas9 expression vector) near the beginning of exon 3 of the coding region

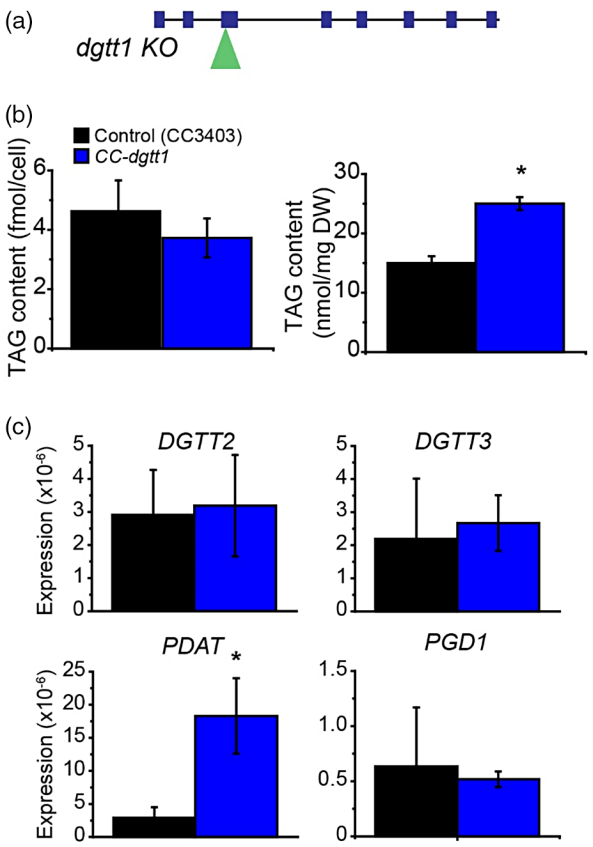

Figure 2. Genetic structure, phenotype and gene expression of the CRISPR-derived *dgtt1* knockout mutant. (a) The locations of an *AphIII* cassette insertion (green triangle) generated by CRISPR in the *DGTT1* gene are presented. (b) Triacylglycerol (TAG) content of the *CC-dgtt1* mutant (blue) and the parent strain CC3403 (black) grown was measured at 72 h under P deprivation. (c) Relative transcriptional level of selected TAG biosynthesis genes (*DGTT2*, *DGTT3*, *PDAT* and *PGD1*) in the *dgtt1* mutant (blue) and the parent strain CC3403 (black) was measured at 48 h under P deprivation. Data represent mean ± standard deviation (SD) from three biological repeats. An asterisk indicates significance by Student's *t*-test (*P*-value ≤0.05).

(Figures 2a and S2a) that results in premature stop codons (Figure S2b) and disrupts expression of the *DGTT1* gene. The TAG content of *CC-dgtt1* was comparable to that of the parent strain on a per cell basis, and 66% higher than the wild-type per unit dry weight under P deprivation (Figure 2b). Moreover, this phenotype was maintained under N deprivation (Figure S3). The small difference in TAG content of *dgtt1* and *CC-dgtt1* mutants (Figures 1b and 2b) is likely due to different strain backgrounds: the parent strain of *dgtt1* is CC5325 (*cw15*; mt^-), while that for *CC-dgtt1* is CC3403 (*arg7*; *cw15*; mt^-). Similar to the situation for the *dgtt1* mutant from the CLiP library, qRT-PCR analyses revealed that *PDAT* expression was upregulated by 6.3-fold in the *CC-dgtt1* mutant, while other TAG biosynthesis genes (*DGTT2*, *DGTT3* and *PGD1*) remained the same as in the control strain (Figure 2c). Taken together, genetic compensation of *DGTT* seems to occur in Chlamydomonas

mutants regardless of the strain background or early-frameshift mutant allele.

Next, we tested whether upregulation of *PDAT* in *dgtt* mutants resulted in any changes at the translational level. Western blot analysis showed PDAT expression levels were 2.12-fold, 1.57-fold and 3.37-fold higher in the CC-*dgtt1*, *dgtt2* and *dgtt3* mutants, respectively, than the control at 24 h under P deprivation (Figure S4), agreeing with changes at the transcript level. We further hypothesize that in the *dgtt* mutants upregulation of PDAT may result in increased membrane lipid recycling for TAG biosynthesis. In corroboration, analysis of the membrane lipid profiles showed that in the *cc-dgtt1* mutant, in which only *PDAT* was upregulated, PG and SQDG levels were significantly decreased by 40% and 53%, respectively, compared with the control at 72 h under P deprivation (*t*-test, $P < 0.05$; Figure S5a). However, different scenarios were observed for the *dgtt2* and *dgtt3* mutants, in which expression of multiple TAG biosynthetic genes was affected (Figure 1). In the *dgtt2* mutant, there were no changes in membrane lipid profiles compared with the control (Figure S5b), likely because the effect of *PDAT* upregulation was buffered by changes in other TAG biosynthetic genes (*DGTT1*, *DGTT3* and *PGD1*). In the *dgtt3* mutant, MGDG level was higher, PI level was lower while other membrane lipids remained unchanged (Figure S5b).

Compensation of *DGTT* knockouts is not reciprocal in the *pdat* mutant

Because *C. reinhardtii* cells are capable of compensating defects of *DGTT1* and *DGTT2* mutations by upregulating related genes such as *PDAT*, we asked whether the mechanism is reciprocal in the *pdat* mutant. To this end, we assessed TAG accumulation and the expression of the *DGTT1-3* genes in a *pdat* knockout mutant (CC4502) previously described in Boyle et al. (2012). Unlike the *dgtt1 and dgtt2* mutants, the *pdat* mutant had reduced TAG levels (Figure 3), similar to the *pdat* knockdown lines (Yoon et al., 2012). The relative TAG content of the *pdat* knockout mutant was ~30% lower than that of the control strain at 48 h and 72 h under P deprivation (Figure 3a), similar to the decrease in TAG content previously reported for this strain in response to N deprivation (Boyle et al., 2012). In P-deprived *pdat* cells, the expression of *DGTT1-3* were the same as or slightly lower than in the control (Figure 3b). These data suggest no genetic compensation of TAG biosynthesis occurs in the *pdat* mutant.

Compensation by upregulation of *DGTT1* in a *Δvtc1/pdat1* double mutant

Our previous work suggests *PDAT* primarily regulates TAG biosynthesis under favorable, nutrient-replete conditions (Yoon et al., 2012), but not under persistent nutrient-deficient stress conditions (Liu et al., 2016). A possible

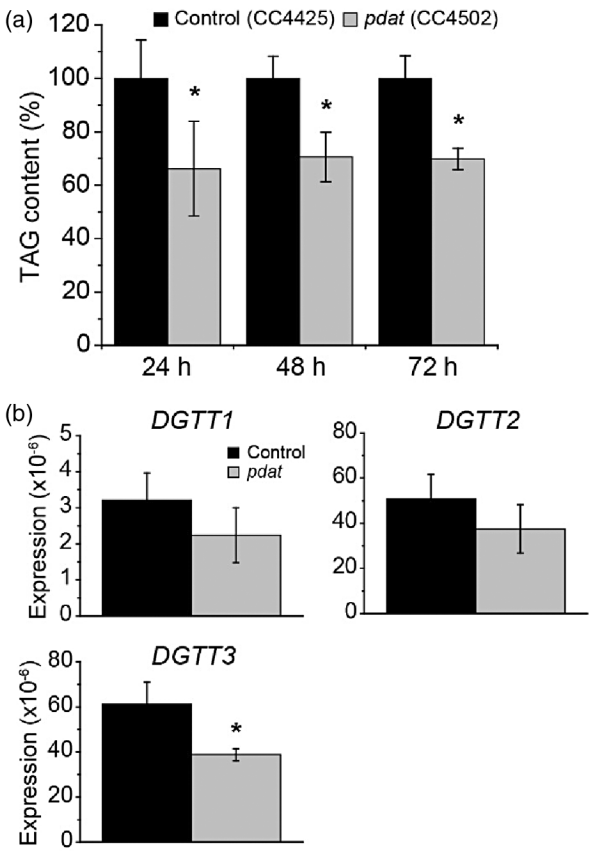

Figure 3. Phenotype and gene expression of the *pdat* mutant (CC4502). (a) Relative concentrations of triacylglycerol (TAG) from the *pdat* knockout strain CC4502 (gray) and the control CC4425 (black) grown under 24-h, 48-h, 72-h and 96-h P deprivation were shown. (b) Relative transcriptional level of selected TAG biosynthesis genes (*DGTT1, DGTT2, DGTT3*) in the *pdat* mutant (gray) and the wild-type (black) was measured at 24 h under P deprivation. Data represent mean ± standard deviation (SD) from three biological repeats. An asterisk indicates significance by Student's *t*-test (*P*-value ≤ 0.05).

explanation for lack of genetic compensation in the *pdat* mutant is thus the relatively poor function of *PDAT* under stress conditions, in which *DGTTs* are highly expressed and more important for TAG biosynthesis. Previous work showed P metabolism, particularly P uptake, was impaired in a Δ*vtc1* mutant, in which the vacuolar transport chaperon (VTC) complex was disrupted (Aksoy et al., 2014; Plouviez et al., 2021; Sanz-Luque et al., 2020; Schmollinger et al., 2021). As such, in this Δ*vtc1* mutant we reasoned that *PDAT* expression and function may be critical to recycle phospholipids and balance P metabolism under stress. To test this idea, we analyzed TAG biosynthesis in the Δ*vtc1* mutant and in a *VTC1*-rescued strain (*VTC1*) under P-replete and P-depleted conditions. In Δ*vtc1* cells TAG content was 90% higher than the control under P-deprived conditions but not under P-replete conditions (Figure 4a,b), suggesting loss of *VTC1* had induced TAG biosynthesis

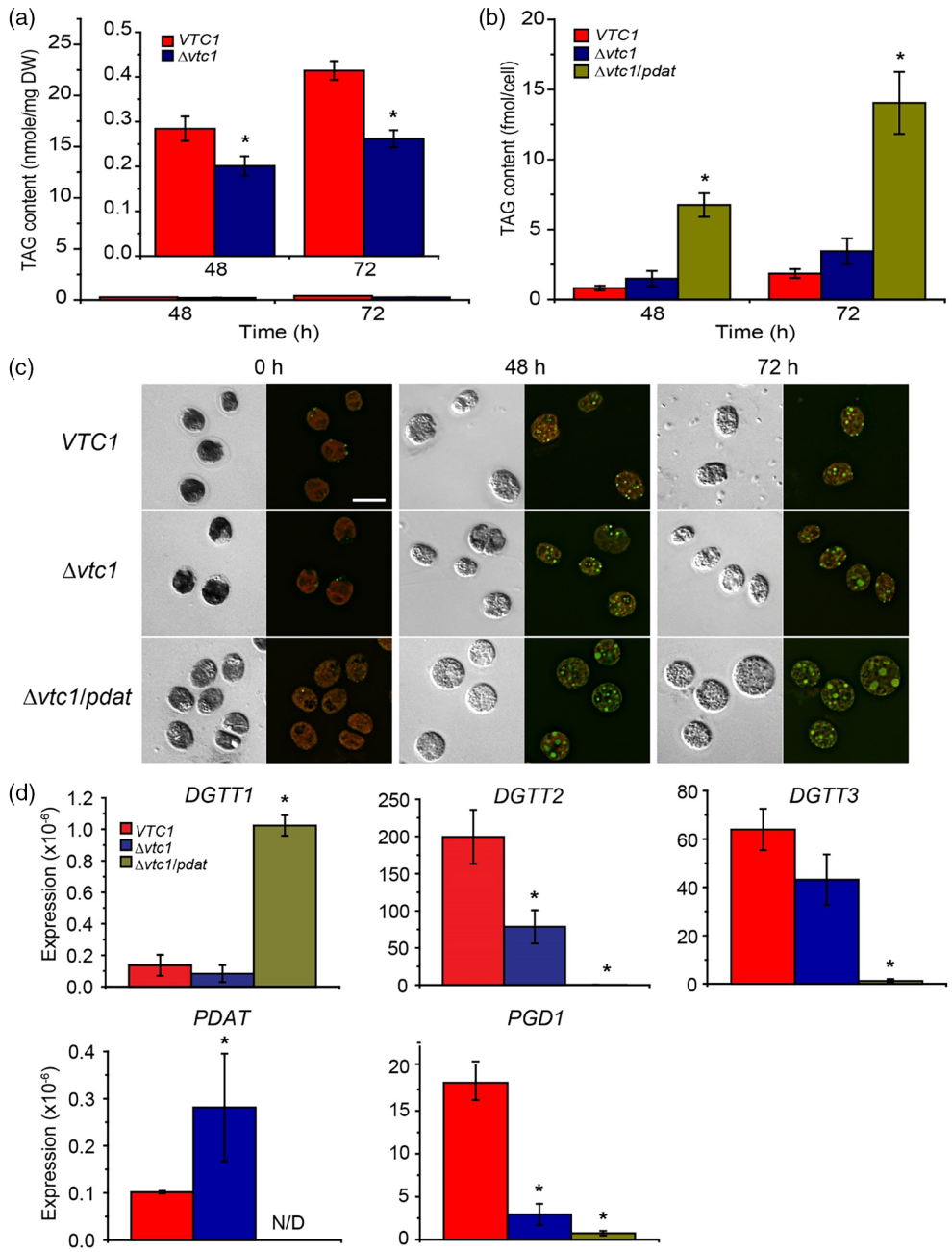

Figure 4. Phenotype and gene expression of the *VTC1* strain, Δ*vtc1* single mutant and Δ*vtc1*/*pdat* double mutant. (a) Triacylglycerol (TAG) content of the Δ*vtc1* strain CC5165 and the *VTC1* rescue strain CC5166 grown was measured at 48 and 72 h under P replete. (b) TAG content of the *VTC1* strain, Δ*vtc1* strain and the Δ*vtc1*/*pdat* strain grown was measured at 48 and 72 h under P deprivation. (c) Lipid droplets in *VTC1*, Δ*vtc1* and Δ*vtc1*/*pdat* cells grown under P deprivation were shown by Nile red staining. DIC images and fluorescent images in two channels (green: lipid droplets; red: chlorophyll autofluorescence) are shown. Scale bar: 10 μm. (d) Relative transcriptional level of selected genes (*DGTT1*, *DGTT2*, *DGTT3*, *PDAT* and *PGD1*) was measured at 24 h under P deprivation. Transcripts of *PDAT* in the Δ*vtc1*/*pdat* double mutant were non-detectable (N/D). Data represent mean ± standard deviation (SD) from three biological repeats. An asterisk indicates significance by Student's *t*-test (*P*-value ≤0.05).

under stress. When we analyzed the TAG biosynthetic genes in P-depleted Δ*vtc1* cells, only the *PDAT* gene showed an increase in expression (2.8-fold; Figure 4d) while none of the *DGTT* or *PGD* genes were upregulated, suggesting a role of *PDAT* in controlling TAG biosynthesis under stress in the Δ*vtc1* mutant. It seems likely that the effects of *VTC1* knockout might extend beyond those on P and lipid metabolism, and a more complete understanding of the Δ*vtc1* phenotype will require further study.

We predicted that for the Δ*vtc1* mutant, in which *PDAT* function seems critical, if *PDAT* expression is interrupted, genetic compensation by other TAG biosynthetic genes

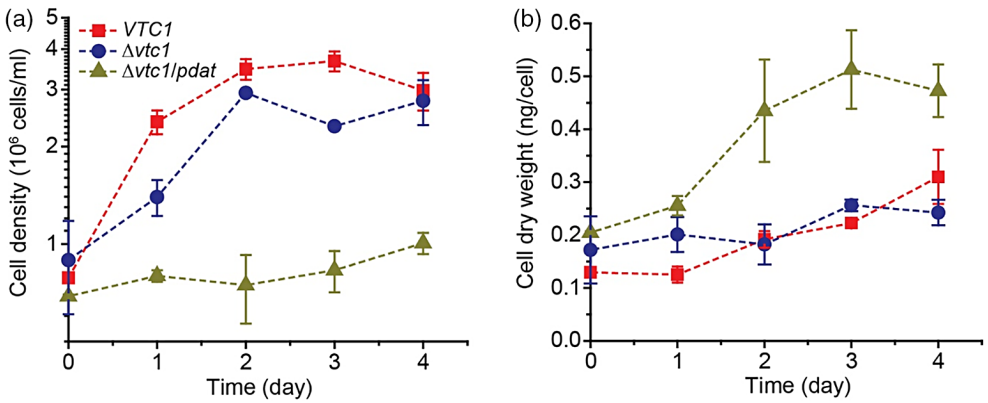

Figure 5. Growth curves in cell density (a) and cell dry weight (b) of batch cultures of *Chlamydomonas reinhardtii VTC1*, Δ*vtc1* and Δ*vtc1*/*pdat1* strains under P deprivation. Values are mean ± standard deviation (SD) from three biological repeats.

may be necessary for the resulting mutant to survive under stress. To test this idea, we generated a Δ*vtc1*/*pdat* double mutant by crossing the Δ*vtc1* (CC5165) and the *pdat* (CC4502) single mutant strains. Nile red was first applied to visualize cellular lipid droplets rich in TAG in the mutant. The Δ*vtc1*/*pdat* double mutant cells were larger under P deprivation and, after 48 h, the number and size of their lipid droplets appeared to be greater than the control and Δ*vtc1* mutant (Figure 4c). Quantitative measurement of TAG in the mutant was consistent with the observation by lipid droplet staining (Figure 4b). After 48 h of P deprivation, the TAG content of Δ*vtc1*/*pdat* cells was about 272.3% higher than the *VTC1* control and 95% higher than the Δ*vtc1* mutant (Figure 4b). We found that under stress, *DGTT1* transcripts were upregulated in Δ*vtc1*/*pdat* cells compared with the control (by 12.4-fold), but no other TAG biosynthetic genes were transcriptionally upregulated (Figure 4d). Moreover, Δ*vtc1*/*pdat* cells remained viable with moderate growth under P deprivation. Δ*vtc1*/*pdat* cell density slightly increased from 0.69×10^6 cells ml^{-1} to 1.01×10^6 cells ml^{-1} between day 0 and day 4 (Figure 5a), while the per cell dry weight of the Δ*vtc1*/*pdat* mutant about doubled compared with the control (*VTC1*; Figure 5b). By contrast, the growth rate and per cell dry weight of Δ*vtc1* cells were similar to that of the control (Figure 5). Therefore, our results suggest that upregulation of *PDAT* in the Δ*vtc1* mutant results in an increase in TAG accumulation, while the effect of *PDAT* disruption in the Δ*vtc1* background is buffered by upregulation of *DGTT1*. In the double mutant, overexpression of *DGTT1*, a rate-limiting gene for TAG biosynthesis, likely compensates for downregulation of *DGTT2* and *DGTT3* (Figure 4d).

DISCUSSION

Genetic compensation and, more specifically, transcriptional adaptation through upregulation of related genes are proposed as an underlying mechanism to explain discrepancies between knockdown and knockout phenotypes in several model systems including zebrafish and mouse (El-Brolosy et al., 2019; Ma et al., 2019; Rossi et al., 2015), *Caenorhabditis elegans* (Serobyan et al., 2020), Arabidopsis (Braun et al., 2008; Chen et al., 2014; Gao et al., 2015) and tomato (Rodriguez-Leal et al., 2019). One mechanism for this phenomenon involves the nonsense-mediated mRNA decay pathway that is activated by early nonsense mutations (El-Brolosy et al., 2019; Ma et al., 2019). However, it is not known whether this phenomenon exists across eukaryotes, including microalgae (El-Brolosy & Stainier, 2017; Kontarakis & Stainier, 2020). Here, we demonstrate that compensation by related genes buffers the effect of deleterious mutations in Chlamydomonas lipid biosynthesis genes, in a process that resembles transcriptional adaptation. Knockouts of *DGTT1* triggered upregulation of the *PDAT* gene regardless of the strain background, mutagenesis approach or stress conditions (P or N deprivation). In the *dgtt1*, *CC-dgtt1* and *dgtt2* mutant, the TAG-less phenotype found in respective knockdown lines was not observed, in which about 70% downregulation of *DGTT1* and *DGTT2* resulted in a decrease of TAG by 30.6% and 21.5%, respectively (Liu et al., 2016). Instead, these mutants produced more TAG than the control, likely due to compensation by upregulation of homologous genes. In the *dgtt3* mutant, however, TAG content was reduced despite compensation by upregulation of *PDAT* and *DGTT2* (Figure 1). It is plausible that *DGTT3* is a critical gene for TAG biosynthesis in Chlamydomonas. It is also possible that knockout of *DGTT3* triggers unknown mechanisms that counteract the effect of *PDAT* upregulation, or other regulatory mechanisms at the translational or post-translation level play a role here, similar to the case for *Brassica napus* DGAT1 (Caldo et al., 2018).

It is interesting to note in these *dgtt* mutants, upregulation of *PDAT* was observed at the transcriptional and translational levels (Figures 1, 2 and S4). Because Chlamydomonas

PDAT has been reported to use membrane lipids such as phospholipids and SQDG for TAG biosynthesis (Yoon et al., 2012), it would be interesting to test whether upregulation of *PDAT* in the *dgtt* mutants may impact membrane lipid metabolism. In the *cc-dgtt1* mutant, upregulation of PDAT coincided with a decrease in PG and SQDG while TAG level remained the same, suggesting that in this mutant *PDAT* upregulation compensates for TAG biosynthesis through membrane lipid recycling.

Recent advances in reverse genetic tools including CRISPR, TALEN and zinc finger nuclease (ZFN) genome editing methods have greatly accelerated progress in generating knockout mutants in traditional and new algal model systems, including Chlamydomonas (Greiner et al., 2017; Park et al., 2020), Nannochloropsis (Poliner et al., 2018; Wang et al., 2016), *Volvox carteri* (Ortega-Escalante et al., 2019) and Ectocarpus (Badis et al., 2021). Moreover, there is a genome-wide insertional mutant pool, the CLiP library (Li et al., 2019), for Chlamydomonas. Such tools and mutant libraries dramatically facilitate analysis of gene function. However, as we demonstrated here, genetic compensation by related genes might, in some cases, offset the effects of a null mutation, confounding genetic analysis. Likewise, previous efforts to screen and characterize TAG-less Chlamydomonas mutants from the CLiP library failed to identify any *DGTT* knockout mutants (XB Li, personal communication), possibly due to genetic compensation, while knockdown lines of *DGTTs* clearly showed a reduced-TAG phenotype (Liu et al., 2016). Therefore, when studying gene function using null mutants, preferably multiple mutant strains targeting the same gene should be obtained by a targeted genome editing method (e.g. CRISPR) or from the CLiP library (because some might activate genetic compensation while others might not) (El-Brolosy et al., 2019; Ma et al., 2019), and these mutants should be carefully evaluated for their genotypic and phenotypic traits. If genetic compensation is observed in the mutants, alternative methods such as knockdown should be used to confirm the respective gene function. Ideally, deletion mutants should be analyzed as they will not trigger transcriptional adaptation through the nonsense-mediated mRNA decay pathway (El-Brolosy et al., 2019; Ma et al., 2019), though to date a relatively small number of deletion mutants have been generated in Chlamydomonas due to current technical limitations. In Chlamydomonas, previous CRISPR work showed that homology directed repair was 10 times less likely with Cas9 compared with ZFN, and CRISPR mutations tend to create insertions (Greiner et al., 2017). In the future, CRISPR systems may be modified to use with small phosphothioate-protected double-stranded homology directed repair donors to mitigate this problem (Greiner et al., 2017).

In Chlamydomonas, biosynthesis of TAG has been implicated in the protection of the photosynthetic electron transport chain from over-reduction during stress conditions, and TAG accumulation is suggested to be essential for cells to survive under stress (Du et al., 2018; Li et al., 2012). Our initial experiments raised two interesting questions. First, does genetic compensation of TAG biosynthesis, as appears to occur in the *dgtt1* and *dgtt2* mutants, play an essential survival role under persistent stress conditions and, second, might genetic compensation occur for other mutated TAG biosynthetic genes? To address these questions, we first examined the *pdat* mutant. Unlike the *dgtt1* and *dgtt2* mutants, it has a similar TAG-less phenotype and TAG biosynthetic gene expression profiles when stressed as the knockdown lines (Figure 3; Boyle et al., 2012; Yoon et al., 2012), suggesting no genetic compensation in the *pdat* mutant. This outcome may be due to the poor function of *PDAT* in stress conditions, under which *DGTTs* are primarily responsible for TAG biosynthesis (Liu et al., 2016; Yoon et al., 2012). Consistent with this notion is a recent *PDAT* analysis in the green lineage showing that *PDAT* plays a less important role in algae than in plants (Falarz et al., 2020). Disruption of *AtPDAT1* in *Arabidopsis thaliana* led to an over 50% decrease in oil accumulation in growing tissues, and significant gametophytic and growth defects (Fan et al., 2013a; Fan et al., 2013b), whereas knockout of Chlamydomonas *PDAT* reduced TAG content by only 25–30%, and *pdat* cells were indistinguishable in gross morphology and growth compared with the wild-type (Figure 3a; Boyle et al., 2012).

We evaluated the effect of the *PDAT* knockout in a Δ*vtc1* mutant, in which *PDAT* is upregulated and likely critical for Δ*vtc1* cells to survive under stress (Figure 4). We expected that in the absence of compensation, the knockout of *PDAT* in the Δ*vtc1* background should result in a TAG-less phenotype. Intriguingly, the Δ*vtc1*/*pdat* mutant exhibited a much higher TAG content than the *VTC1* control, likely because of a large upregulation of *DGTT1* (Figure 4). So while no genetic compensation was observed in the *pdat* mutant, in the Δ*vtc1*/*pdat* double mutant, *DGTT1* was highly overexpressed, and more TAG was produced under P deprivation. Under stress conditions, these cells continue to capture light energy through photosynthesis and, if electron acceptors become over-reduced due to the slow growth, cytotoxic reactive oxygen species are produced that lead to cell death (Li et al., 2008). TAG biosynthesis is likely needed for Δ*vtc1*/*pdat* cells to survive during stress because TAG is the most reduced electron sink in algae (Hu et al., 2008). In line with our findings, it was previously reported that a Chlamydomonas mutant defective for the MGDG-specific lipase PGD1 accumulated less TAG than the wild-type while its cells lost viability under stress, suggesting TAG might play a role as an electron and energy sink under stress conditions (Du et al., 2018; Li et al., 2012). Together, these data suggest that under stress conditions, loss of TAG biosynthetic gene function might

be buffered by upregulation of a related gene or genes through transcriptional adaptation in algae.

In summary, we have demonstrated that genetic compensation contributes to genetic robustness in microalgal TAG biosynthesis, maintaining TAG biosynthesis and redox homeostasis in knockout mutants under abiotic stress. This work demonstrates an example of genetic compensation in microalgae, implies the physiological relevance of genetic compensation in TAG biosynthesis under stress, and provides guidance for future genetic engineering and mutant characterization efforts. The exact mechanisms inducing genetic compensation in the Chlamydomonas mutants are not understood and warrant further investigation. Because transcriptional adaptation in other systems is triggered by mutant mRNA decay and involves Upf3a and COMPASS components (El-Brolosy et al., 2019; Ma et al., 2019), testing the involvement of these components in algal genetic compensation is a logical next step.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

Chlamydomonas reinhardtii LMJ.RY0402.223444, LMJ.RY0402.213587, LMJ.RY0402.223565 and their parent strain CC5325 (*cw15*; mt^-) (Li et al., 2016), CC4502 (*pdat1-1*; mt^+) and its parent strain CC4425 [D66] (*cw15*; mt^+) (Boyle et al., 2012), CC3403 (*arg7*; *cw15*; mt^-), Δ*vtc1* mutant strain CC5165 (Δ*vtc1*; mt^-) and the *VTC1* rescue strain CC5166 (*ars76::VTC1*) (Aksoy et al., 2014) were obtained from the Chlamydomonas Resource Center (http://www.chlamycollection.org). CC3403 was used as the wild-type in CRISPR/Cas9 mutagenesis as previously described (Greiner et al., 2017). The Δ*vtc1*/*pdat* double mutant was derived from a cross between CC5165 and CC4502 (methods described below). All strains were maintained in Tris-acetate-phosphate (TAP) medium containing paromomycin as necessary. P deprivation was imposed by transferring *C. reinhardtii* cells to P-free TAP, in which potassium phosphate was replaced by 1.5 mM KCl (Li et al., 2010). Ammonium chloride was omitted to generate N-free TAP. All batch cultures were grown under continuous light illumination at 60 μmol photons m^{-2} sec^{-1}, shaking at 150 rpm, and room temperature.

Gene expression measurement by qRT-PCR

Total RNA was extracted and purified using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using Protoscript II First Strand cDNA synthesis kit (NEB). The expression of target genes was determined by qRT-PCR in Applied Biosystems 7500 using the $2^{-\Delta\Delta CT}$ method normalized by the expression of 18s rRNA. Details are described in the Materials and Methods S1. The qRT-PCR analysis for each targeted gene was performed twice, with three biological replicates and three technical replicates for each sample. The Primer sequences used in qRT-PCR are listed in Table S1. The use of different parent/control strains resulted in different expression level of *DGTTs* in the control/parent strain across different experiments. Nevertheless, in all experiments we tested the respective parent/control strain with the mutant strains side by side in biological triplicates and compared expression results only within the same strain background.

Generation of *Chlamydomonas reinhardtii* mutants by CRIPR/Cas9 mutagenesis

The CC-*dgtt1* mutant was generated by the CRISPR/Cas9 method developed by Greiner et al. (2017). CC3403, the parent strain of the *CC-dgtt1* mutant, the SaCas9 expression vector (pHS_SaCas9, catalog # pPH187) and the sgRNA cloning vector (pCrU6.4-SaCloning-aphVIII, catalog # pPH339) that expressed customized sgRNA targeting *DGTT1* were obtained from the Chlamydomonas Resource Center (http://www.chlamycollection.org). The customized targeting vector was made by annealing oligonucleotides (5′-acttGGTCTTAATCAGGCGGGCGCCG-3′ and 5′-aaacCGGCGCCCGCCTGATTAAGACC-3′) and ligating into *Esp3I*-digested pCrU6.4-SaCloning-aphVIII. This targeting vector and pHS_SaCas9 were co-transformed by electroporation into CC3403 (Park et al., 2020; Serobyan et al., 2020). Paromomycin-resistant transformants were selected and screened by PCR for insertions at the *DGTT1* locus as follows. Paromomycin-resistant colonies were transferred to wells of a 96-well plate each containing 180 μl of TAP containing 100 μg ml^{-1} $_L$-arginine and 10 μg ml^{-1} paromomycin. The plates were grown under room light (2–4 μmol photons m^{-2} sec^{-1} for 10–12 h day^{-1}) at room temperature for 7–12 days. Forty microliters from each well was transferred to a PCR tube and centrifuged for 10 min at 2000 ***g*** at room temperature. The pellet was resuspended in 20 μl of Dilution Buffer from the Phire Plant Direct PCR Master Mix Kit (ThermoFisher Scientific, Waltham, MA, USA) and incubated for 5 min at room temperature. Samples were centrifuged for 10 min at 2000 ***g*** at room temperature, then 15 μl of supernatant was removed to another PCR tube with 60 μl distilled water to dilute the sample prior to PCR. The PCR reaction consisted of 2.5 μl distilled water, 5 μl 2 × Phire Plant Direct PCR Master Mix, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM) and 0.5 μl DNA extract solution. Reactions included oligonucleotides CrDGTT1F (5′-CTCTGCTCATCGGCACATTG-3′) and CrDGTT1R (5′-ATATGCCACTTGCGGAAGGT-3′) in a Bio-Rad T100 Thermal Cycler (Hercules, CA, USA) programmed to 98°C for 5 min, followed by 35 cycles of 98°C for 5 sec, 65°C for 5 sec, 72°C for 60 sec, and a final extension step at 72°C for 30 sec. Reaction products larger than that expected for the wild-type *DGTT1* were sequenced to identify the *dgtt1* mutant.

Generation of *Chlamydomonas reinhardtii* mutants by crossing

The Δ*vtc1*/*pdat* double mutant was generated by crossing as described previously by Goodenough et al. (1976) with some modification. Cultures of Chlamydomonas were grown in M medium under light–dark cycle (13 h light: 11 h dark) until reaching the exponential phase, and then switched to N-free M medium under continuous light at 170 rpm shaking for overnight to induce gamate formation. Gamate cells were harvested by centrifugation at 3000 ***g*** and room temperature for 5 min, and washed with N-free M medium twice before being resuspended in fresh N-free medium. Equal numbers of gamate cells of the opposite mating types were mixed together with the addition of 150 mM db-cAMP and incubated under light for up to 2 h. A 200-μl portion of gamates mixture was spread on a TAP medium plate containing 2% agar every 30 min of the 2-h incubation. The plates were exposed to light overnight and then placed in the dark for 5–7 days to allow zygote formation. At the end of the incubation period, poorly adhered vegetative cells were gently scraped off from the plate surface by a razor blade and killed by the vapor of chloroform (30–60 sec exposure). The plates were exposed to light overnight to allow zygotes to germinate before being incubated under light–

dark cycles (13 h light: 11 h dark) to allow colonies to grow. Once zygotic colonies became visible, they were picked and resuspended in 200 μl TAP medium, and subsequently plated on a TAP medium containing 1.5% agar and 15 μg ml^{-1} paromomycin. The plates were incubated under continuous light to allow the paromomycin-resistant mutant candidates to grow. The crossed candidates were subsequently screened by colony PCR for a co-occurrence with the deletion of the *VTC1* gene and the presence of the *pdat1-1* mutation (Boyle et al., 2012) in the genome. Primers CrVTC1-pF1 and CrVTC1-stopR1 (sequences in Table S1) were used for determination of the presence (yielding a 1148-bp PCR product) or absence (yielding no PCR products) of the *VTC1* gene. Primers CrPDAT1-1-F2 and RIM5-2 (sequences in Table S1; Boyle et al., 2012) were used to determine the presence (~1-kb PCR product) or absence (no PCR product) of the *AphIII* insertion in the *PDAT* gene.

TAG content measurement

Total lipid from *C. reinhardtii* cells was extracted using chloroform:methanol (2:1, v/v) as previously described (Yoon et al., 2012). The lipid extracts were dried under a gentle stream of nitrogen gas and re-dissolved in chloroform. Total lipid was resolved by thin-layer chromatography (TLC) on a silica gel 60 F_{254} plates (EMD Millipore) using hexane:t-butyl methyl ether: acetic acid (80:20:2, v/v/v) solvent mixture as a mobile phase to develop TAG or using chloroform:methanol:acetic acid:water (25:4:0.7:0.3) solvent mixture as a mobile phase to develop polar lipids. For visualization, the developed TLC plates were sprayed with 8% (w/v) H_3PO_4 solution containing 10% (w/v) copper (II) sulfate pentahydrate, and then charred at 180°C for 3 min. The relative intensity of signals corresponding to TAG can be measured using ImageJ software (NIH). The absolute quantification of TAG can be calculated by the sum of fatty acid fractions composed of TAG using gas chromatography–mass spectrometry (GC–MS) as previously described (Li et al., 2010; Wang et al., 2017). TAG and polar lipid species fractions in the lipid extracts on a developed TLC plate were visualized by I_2 vapor and isolated by scrapping off the silica gel from the TLC plate. Fatty acids of the isolated TAG fractions were converted to fatty acid methyl esters (FAMEs) with 1% H_2SO_4 in methanol at 85°C for 1.5 h, dissolved in hexane, and then profiled using TSQ 8000 Triple GC–MS System (Thermo Scientific, Waltham, MA, USA). FAME standards (Sigma-Aldrich, St. Louis, MO, USA) and heptadecanoic acid (C17:0; Sigma) were used as the external and internal standards, respectively, for fatty acid analysis. The quantification for TAG was calculated by the sum of fatty acid amount based on GC–MS analysis.

Fluorescent microscopy

Nile red was used to stain lipid droplets in live Chlamydomonas cells. Prior to staining, cells were harvested and washed with phosphate-buffered saline (PBS). Cells were incubated with 200 μl 0.1 μg ml^{-1} Nile red solutions (diluted in 1 × PBS from a 1 mg ml^{-1} stock solution prepared in acetone) for 5 min in the dark.

Stained cell in suspension was placed on a coverslip, dried and mounted with Slow Fade Diamond (Invitrogen). The specimen was imaged using wide-field fluorescence microscopy with a Zeiss Axio Observer Z1 microscope and Hamamatsu Orca-R2 charge-coupled device camera. Zeiss filter sets 10 (beam pass excitation 450–490 nm; beam pass emission 515–565 nm) and 15 (beam pass excitation 540–552 nm; long pass emission 590 nm) were used to detect Nile red staining (the green fluorescence) and chlorophyll autofluorescence (the red fluorescence), respectively. The images were analyzed with Volocity 6.2.1 software (PerkinElmer, Waltham, MA, USA). The fluorescence channels were deconvolved using the restoration function of the Volocity software.

Protein level measurement by Western blot

Total proteins (whole cell extracts) from *C. reinharditii* cells were extracted as described previously (Yoon et al., 2012). The protein concentration of the whole cell extract was determined using Bio-Rad protein assay reagent; 30 μg of whole cell protein extract of each sample was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Nitrocellulose membrane was blocked by incubating in the blocking agent (1 × PBS containing 5% dry skim milk) overnight at room temperature. Rabbit anti-CrPDAT antibody (Agrisera, Vännäs, Sweden) at a dilution of 1:500 and mouse anti-α-Tubulin antibody (Sigma) at a dilution of 1:4000 in the blocking agent were used as primary antibody reagents for detection of PDAT and α-Tubulin proteins, respectively. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) (Cell Signaling Technology, Danvers, MA, USA) and HRP-conjugated goat anti-mouse IgG (H + L) (Bio-Rad, Hercules, CA, USA) at a titer of 1:2000 and 1:3000, respectively, were used as the secondary antibodies. PBST reagent (1 × PBS containing 0.1% Tween-20) was used to wash the nitrocellulose membrane after primary and secondary antibodies incubation. The signal of target proteins was developed using Clarity Western ECL substrate (Bio-Rad) and detected by ChemiDoc Imaging System (Bio-Rad). The intensity of the target protein was determined using ImageJ software (NIH). The abundance of PDAT protein was normalized by the abundance of α-Tubulin protein on the Western blot image.

Statistical analysis

Statistical analysis was performed by Student's *t*-test with two-tailed distribution. A *P*-value ≤ 0.05 was the criteria of the significance.

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AUTHOR CONTRIBUTIONS

YL and YYL conceived the project and analyses, and YL supervised the study. YYL performed the *dgtt1-3*, *pdat*, Δ*vtc1* and double knockout mutants experiments and data analysis. RP and SMM performed the CRISPR mutant generation experiment and data analyses. YL and YYL wrote the manuscript, with input and approval from RP and SMM.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and the supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Primer sequences used in this study.

Figure S1. Locations of the CIB1 cassette insertion in the *dgtt1*, *dgtt2* and *dgtt3* strains.

Figure S2. Location of the pHS SaCas9 plasmid fragment in *DGTT1* CRISPR/Cas9 mutant.

Figure S3. TAG content of the *CC-dgtt1* mutant and the parent strain CC3403.

Figure S4. Relative level of PDAT protein in the *dgtt1*, *dgtt2* and *dgtt3* mutants.

Figure S5. Polar lipids contents in the *dgtt1*, *dgtt2* and *dgtt3* mutants.

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