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 *Dvtc1* mutant, in which *PDAT* was upregulated under stress, resulting in a 90% increase in TAG content. Knockout of *PDAT* in the *Dvtc1* background induced a 12.8-fold upregulation of *DGTT1* and a 272.3% increase in TAG content in *Dvtc1/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, 2017). Genetic compensation is 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., 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 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; Öhrrogge & 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 DGTTs 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 DGTTs 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 Δvtc1 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
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 ≤ 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

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).
TAG as that caused by the dgtt3 knockdown (Liu et al., 2016). In Chlamydomonas, DGTTs and PDAT are the main contributors of TAG biosynthesis. Sequence analysis revealed nucleotide identities between PDAT and DGT1, DGT2, and DGT3 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 knock-out mutants, the expression of TAG biosynthetic genes was examined, including that of DGT1, DGT2, DGT3, 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 DGT2, DGT3 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 DGT1, DGT3, 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 insertion 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 DGT1 allele carries a 1229-bp insertion corresponding to pHS_SaCas9 sequence (Cas9 expression vector) near the beginning of exon 3 of the coding region (Figures 2a and S2a) that results in premature stop codons (Figure S2b) and disrupts expression of the DGT1 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 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 (DGT2, DGT3 and PGD1) remained the same as in the control strain (Figure 2c). Taken together, genetic compensation of DGT1 seems to occur in Chlamydomonas.

| Gene 1 | Gene 2 | Identity matrix (genome) | Identity matrix (transcript) |
|-------|-------|-------------------------|----------------------------|
| PDAT  | DGT1  | 50.9%                   | 50.5%                      |
| PDAT  | DGT2  | 49.8%                   | 50.7%                      |
| PDAT  | DGT3  | 49.2%                   | 52.8%                      |
| DGT1  | DGT2  | 50.5%                   | 51.4%                      |
| DGT1  | DGT3  | 49.0%                   | 51.6%                      |
| DGT2  | DGT3  | 50.6%                   | 59.1%                      |

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

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mutants regardless of the strain background or early-framenesshift 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 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 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 Δvtc1 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 Δvtc1 mutant and in a VTC1-rescued strain (VTC1) under P-replete and P-depleted conditions. In Δvtc1 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...
under stress. When we analyzed the TAG biosynthetic genes in P-depleted Δvtc1 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 Δvtc1 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 Δvtc1 phenotype will require further study.

We predicted that for the Δvtc1 mutant, in which PDAT function seems critical, if PDAT expression is interrupted, genetic compensation by other TAG biosynthetic genes

Figure 4. Phenotype and gene expression of the VTC1 strain, Δvtc1 single mutant and Δvtc1/pdat double mutant. (a) Triacylglycerol (TAG) content of the Δvtc1 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, Δvtc1 strain and the Δvtc1/pdat strain grown was measured at 48 and 72 h under P deprivation. (c) Lipid droplets in VTC1, Δvtc1 and Δvtc1/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 Δvtc1/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).
Figure 5. Growth curves in cell density (a) and cell dry weight (b) of batch cultures of Chlamydomonas reinhardtii VTC1, Δvtc1 and Δvtc1/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 Δvtc1/pdat double mutant by crossing the Δvtc1 (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 Δvtc1/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 Δvtc1 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 Δvtc1/pdat cells was about 272.3% higher than the VTC1 control and 95% higher than the Δvtc1 mutant (Figure 4b). We found that under stress, DGTT1 transcripts were upregulated in Δvtc1/pdat cells compared with the control (by 12.4-fold), but no other TAG biosynthetic genes were transcriptionally upregulated (Figure 4d). Moreover, Δvtc1/pdat cells remained viable with moderate growth under P deprivation. Δvtc1/pdat cell density slightly increased from 0.69 × 10⁶ cells ml⁻¹ to 1.01 × 10⁶ cells ml⁻¹ between day 0 and day 4 (Figure 5a), while the per cell dry weight of the Δvtc1/pdat mutant about doubled compared with the control (VTC1; Figure 5b). By contrast, the growth rate and per cell dry weight of Δvtc1 cells were similar to that of the control (Figure 5). Therefore, our results suggest that upregulation of PDAT in the Δvtc1 mutant results in an increase in TAG accumulation, while the effect of PDAT disruption in the Δvtc1 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 have 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 (Falard 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 Δvtc1 mutant, in which PDAT is upregulated and likely critical for Δvtc1 cells to survive under stress (Figure 4). We expected that in the absence of compensation, the knockout of PDAT in the Δvtc1 background should result in a TAG-less phenotype. Intriguingly, the Δvtc1/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 Δvtc1/pdat double mutant, DGT1 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 Δvtc1/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′], Δvtc1 mutant strain CC5165 [Δvtc1; 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 Δvtc1/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 obtained from the Chlamydomonas Resource Center (http://www.chlamycollection.org). CC3403, the parent strain of CC5165, was used as the wild-type in CRISPR/Cas9 mutagenesis as previously described (Greiner et al., 2017). The Δvtc1/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^ΔΔCt 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 CRISPR/Cas9 mutagenesis

The Δ-dgt1 mutant was generated by the CRISPR/Cas9 method developed by Greiner et al. (2017). CC3403, the parental strain of the CC-dgt1 mutant, the SaCas9 expression vector (pHS_SaCas9, catalog # pPH187) and the sgRNA cloning vector (pCp6U.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’-actcgtcttaatcagggccccggcggcggg-3’ and 5’-aaactgcggcggccgcggctgatggaggacc-3’) and ligation into Esp3f-digested pCp6U.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 micro-liters from each well was transferred to a PCR tube and centrifuged at 10,000 × g for 10 min 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’-ATAATGCCCATCCGGAAAGT-3’) in a Bio-Rad T100 Thermal Cycler (Hercules, CA, USA) programmed to 98°C for 1 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 dgt1 mutant.

Generation of Chlamydomonas reinhardtii mutants by crossing

The Δvtc1/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 gamete formation. Gamete 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 gamete cells of the opposite mating types were mixed together with the addition of 150 μM db-CAMP and incubated under light for up to 2 h. A 200-μl portion of gametes 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. Oncezygotic colonies became visible, they were picked and resuspended in 200 μl TAP medium, and subsequently plated on a TAPmedium containing 1.5% agar and 15 μg ml⁻¹ paromomycin. The plates were incubated under continuous light to allow theparomomycin-resistant mutant candidates to grow. The crossedcandidates were subsequently screened by colony PCR for a cooccurrence 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-bpPCR product) or absence (yielding no PCR products) of the VTC1gene. Primers CrPDA1-1-F2 and RIM5-2 (sequences in Table S1;Boyle et al., 2012) were used to determine the presence (~1-kbPCR product) or absence (no PCR product) of the AphII insertionin 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 F254 plates (EMD Millipore) using hexane:t-butyl methyl ether:acetic acid:water (25:4:0.7:0.3) solvent mixture as a mobile phase to develop TAG or using chloroform:methanol:acetic acid:water (80:20:2, v/v/v/v) solvent mixture as a mobile phase to develop polar lipids. For visualization, the developed TLC plates were sprayed with 8% (w/v) H₃PO₄ solution containing 10% (w/v) copper (II) sulfapentahydrate, and then charred at 180 °C for 3 min. The relativeintensity of signals corresponding to TAG can be measured byusing ImageJ software (NIH). The absolute quantification of TAGcan be calculated by the sum of fatty acid fractions composed ofTAG using gas chromatography–mass spectrometry (GC–MS) aspreviously described (Li et al., 2010; Wang et al., 2017). TAG andpolar lipid species fractions in the lipid extracts on a developedTLC plate were visualized by I₃ vapor and isolated by scrapingoff the silica gel from the TLC plate. Fatty acids of the isolatedTAG fractions were converted to fatty acid methyl esters (FAMEs)with 1% H₂SO₄ in methanol at 85 °C for 1.5 h, dissolved in hexane, andthen profiled using TQ 8000 Triple GC–MS System (ThermoScientific, Waltham, MA, USA). FAME standards (Sigma-Aldrich,St. Louis, MO, USA) and heptadecanoic acid (C₁₇:0; Sigma) wereused as the external and internal standards, respectively, for fattyacids analysis. The quantification for TAG was calculated by thesum 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 withphosphate-buffered saline (PBS). Cells were incubated with 200 μl0.1 μg ml⁻¹ Nile red solutions (diluted in 1 x PBS from a 1 mg ml⁻¹stock solution prepared in acetone) for 5 min in the dark.

Stained cell in suspension was placed on a coverslip, dried andmounted with Slow Fade Diamond (Invitrogen). The specimenwas imaged using wide-field fluorescence microscopy with aZeiss Axio Observer Z1 microscope and Hamamatsu Orca-R2charge-coupled device camera. Zeiss filter sets 10 (beam passexcitation 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) andchlorophyll autofluorescence (the red fluorescence), respectively.The images were analyzed with Volocity 6.2.1 software(PerkinElmer, Waltham, MA, USA). The fluorescence channelswere deconvolved using the restoration function of the Volocitysoftware.

Protein level measurement by Western blot

Total proteins (whole cell extracts) from C. reinhardtii cells wereextracted as described previously (Yoon et al., 2012). The proteinconcentration of the whole cell extract was determined using BioRad protein assay reagent; 30 μg of whole cell protein extract ofeach sample was resolved by sodium dodecyl sulfate-polyacrylamidegel electrophoresis and transferred to nitrocellulose membrane.Nitrocellulose membrane was blocked by incubating in theblocking agent (1 x PBS containing 5% dry skim milk) overnight atroom 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 asprimary antibody reagents for detection of PDAT and α-Tubulinproteins, respectively. Horseradish peroxidase (HRP)-conjugated goatanti-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 dilution of 1:2000 and 1:3000, respectively,were used as the secondary antibodies. PBST reagent (1 x PBS containings0.1% Tween-20) was used to wash the nitrocellulose membrane afterprimary and secondary antibodies incubation. The signal oftarget 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 ImageJsoftware (NIH). The abundance of PDAT protein was normalized bythe abundance of α-Tubulin protein on the Western blot image.

Statistical analysis

Statistical analysis was performed by Student’s t-test with twotailed distribution. A P-value ≤ 0.05 was the criteria of thesignificance.

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AUTHOR CONTRIBUTIONS

YL and YYL conceived the project and analyses, and YLsupervised the study. YYL performed the dgtt1-3, pdat, Δvtc1 and double knockout mutants experiments and dataanalysis. RP and SMM performed the CRISPR mutant generationexperiment 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 pH5 SaCas9 plasmid fragment in DGGT1 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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