

LIP4 Is Involved in Triacylglycerol Degradation in *Chlamydomonas reinhardtii*

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COVER PAGE

Title: CrLIP4 Is Involved in Triacylglycerol Degradation in *Chlamydomonas reinhardtii*

Running head: Role of CrLIP4 in Triacylglycerol Degradation in *Chlamydomonas*

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Footnotes:

The amino acid sequence of Chlamydomonas CrLIP4 was obtained from The Plant Genomics Resource (https://phytozome.jgi.doe.gov/pz/portal.html) accession number Cre17.g699100.t1.1. Sequences of Arabidopsis proteins were obtained from TAIR; SDP1: AT5G04040.1, and SDP1L: AT3G57140. Sequences of other proteins were obtained from National Center for Biotechnology Information (NCBI); LiSDP1: ARQ20718.1, TGL1: XP_002184517.1, Tgl4p: NP_013015.1, Tgl5p: NP_014724.1, Tgl3p: NP_014044.1, and ATGL: NP_065109.1.

Abstract:

Degradation of the storage compound triacylglycerol (TAG) is a crucial process in response to environmental stimuli. Failing to respond properly may be detrimental for survival. In microalgae, this process is important for re-growth when conditions become favorable after cells have experienced stresses. Mobilization of TAG is initiated by actions of lipases causing the release of glycerol and free fatty acids, which can be further broken down for energy production or recycled to synthesize membrane lipids. Although key enzymes in the process, TAG lipases remain to be characterized in the model green alga *Chlamydomonas reinhardtii*. Here we describe the functional analysis of a putative TAG lipase, i.e. CrLIP4, which shares 44% amino acid identity with the major TAG lipase in *Arabidopsis* (SUGAR DEPENDENT1 - SDP1). The *CrLIP4* transcript level was down regulated during nitrogen deprivation (ND) when TAG accumulates, but was upregulated during nitrogen recovery (NR) when TAG was degraded. Both artificial microRNA knockdown and insertional knockout mutants showed a delay in TAG mobilization during NR. The difference in TAG degradation was more pronounced when the cultures were incubated without acetate in the dark. Furthermore, the *crlip4* knock-out mutant over-accumulated TAG during optimal growth conditions. Taken together, the results suggest to us that CrLIP4 likely acts as a TAG lipase and plays a major role in TAG homeostasis in *Chlamydomonas*.

Keywords (6): *Chlamydomonas reinhardtii*, lipid catabolism, SUGAR DEPENDENT1, triacylglycerol lipase, N recovery; TAG remobilization

Introduction

Microalgae are abundant primary producers of the food chain in nature serving as food source for both marine and fresh water animals (Guedes and Malcata 2012). This is due to their high photosynthetic efficiency in converting solar energy to chemical energy. Many algae synthesize and accumulate triacylglycerol (TAG), which has been the basis for many applications such as pharmaceutical products and foods (Hasan et al. 2006), and more recently for biofuels (Chisti 2007; Durrett et al. 2008; Li-Beisson et al. 2015; Zienkiewicz et al. 2016).

Rapid accumulation of TAG into lipid droplets (LDs) occurs in response to different environmental stresses such as high light intensity, elevated temperature and nutrient deprivation as summarized in (Zienkiewicz et al. 2016). Nitrogen (N) deprivation is best investigated and most widely used in a laboratory setting to induce TAG accumulation. Upon the resupply of N when the conditions are favorable, TAG is degraded to support growth and cell division. TAG degradation is carried out by a process called lipolysis. The process starts with sequential hydrolysis of acyl chains from the glycerol backbone of TAG by TAG-, diacylglycerol (DAG)- and monoacylglycerol lipases. The resulting free fatty acids (FFAs) and glycerol are either being recycled for synthesis of membrane lipids or further broken down by β-oxidation in peroxisomes for energy production as summarized in (Kong et al. 2018).

TAG lipase (EC 3.1.1.3) is the first enzyme during lipolysis, and one of the most targeted enzymes in industrial applications. It catalyzes the release of an acyl chain from TAG in an oil-water interface yielding DAG and FFA. The respective proteins often contain an α/β hydrolase fold commonly found in hydrolases (Ollis et al. 1992). In addition, the amino acid sequences of these enzymes contain patatin domains with a GXSXG (G: glycine, X: any amino acid, S: serine) motif representing the catalytic site (Kelly and Feussner 2016). The reaction is mediated by a catalytic dyad composed of the serine residue serving as nucleophile in the GXSXG motif and an aspartate residue further along the sequence. These features are common in TAG lipases of various biological systems from yeast to human.

Biochemical studies of TAG lipases have been proven very difficult, if not impossible. Identification and characterization of TAG lipases have only recently made a big leap-forward with the advent of molecular genetics tools, especially the study of gain- or loss- of-function mutants. This has led to the identification of major TAG lipases in *Saccharomyces cerevisae* (Tgl3p, Tgl4p and Tgl5p) (Athenstaedt and Daum 2003, 2005), in human adipose tissue (ATGL) (Jenkins et al. 2004; Villena et al. 2004; Zimmermann et al. 2004) and in *Arabidopsis* (the SUGAR DEPENDENT1 (SDP1) (Eastmond 2006) and SDP1-LIKE (SDP1L) (Kelly et al.

2011)). In *Arabidopsis*, SDP1 is the major TAG lipase in seeds during post-germination (Eastmond 2006). Loss of a functional gene leads to seedling growth retardation prior to establishment of photosynthetic metabolism. This phenotype can be rescued by sucrose supplementation. Together with its paralogue SDP1L, the two proteins are responsible for 95% of TAG hydrolysis in germinating *Arabidopsis* seeds (Kelly et al. 2011). Recently, two *Arabidopsis* SDP1 homologues have been characterized in an oleaginous microalga, *Lobospaera incisa*, and a diatom, *Phaeodactylum tricornutum*, have been named as LiSDP1 and Tgl1, respectively (Barka et al. 2016; Siegler et al. 2017). LiSDP1 was able to partially rescue the *Arabidopsis sdp1/sdp1l* mutant but was not found associated with the LD as SDP1. In the diatom, down regulation of *TGL1* with antisense RNA resulted in a strong increase in TAG content. In addition, recombinant Tgl1 showed esterase activity against a TAG substrate analogue.

Although lipid metabolism in the model microalga *Chlamydomonas reinhardtii* has recently been subjected to intensive studies (Kong et al. 2018; Li-Beisson et al. 2015; Liu and Benning 2013; Merchant et al. 2012), and despite the central importance of TAG lipases, no TAG lipase has yet been identified and characterized in this model green alga. Protein sequence comparison revealed the occurrence of one putative orthologue (CrLIP4) of the *Arabidopsis* SDP1. Here, we determined that CrLIP4 is a TAG lipase by studying its gene expression and characterizing artificial microRNA repression and insertional disruption mutants. Based on our results we conclude that CrLIP4 plays a key role in TAG degradation.

Results

CrLIP4 is an orthologue of *Arabidopsis* SDP1

A previous transcriptomic study in *Chlamydomonas* has identified CrLIP4 as a putative SDP1 orthologue (Miller et al. 2010). Here, we further investigated this putative TAG lipase. Based on amino acid sequence alignment, CrLIP4 shares 44% identity with the *Arabidopsis* SDP1 protein. To gain more insight into CrLIP4 function, we searched for protein domain in the amino acid sequence and other characterized TAG lipases searching a hidden Markov model (HMM) library (Pfam) through HMMSCAN. All of the tested amino acid sequences contain a patatin domain (PF01734.21) with a GXSXG motif and conserved serine and aspartate residues (**Figure 1A**). Therefore, we concluded that CrLIP4 belongs to the patatin-like (PTL) protein family. These proteins also contain a DUF3336 domain (PF11815.7). Its function remains to be resolved. As shown in **Figure 1A**, all TAG lipases included in the analysis except human

ATGL contain this protein domain. Therefore, the DUF3336 might not be required or essential for the catalytic reaction of a TAG lipase.

To investigate the evolutionary relationship between these characterized TAG lipases with CrLIP4, phylogenetic reconstruction was carried out. Because the DUF3336 domain is absent in the human ATGL protein, it was excluded from this analysis. As expected, CrLIP4 grouped into the same clade as LiSDP, and was more distantly related to SDP1 and SDP1L, the diatom Tgl1, and the *Saccharomyces* Tgl4p and Tgl5p, respectively (**Figure 1B**). *Saccharomyces* Tgl3p, however, seemed to be separated into another branch. This could be due to the fact that the aspartate residue of the catalytic dyad is not present in its patatin domain (Koch et al. 2014). Taken together, these results are consistent with CrLIP4 being an orthologue of *Arabidopsis* SDP1.

Expression of *CrLIP4* is decreased during N deprivation and increased upon N resupply

TAG content can be manipulated easily in *Chlamydomonas*; i.e., TAG content increases strongly upon N deprivation, and decreases immediately following N resupply (Moellering and Benning 2010; Siaut et al. 2011). In order to see the relationship between the *CrLIP4* transcript level and TAG accumulation, we monitored both at different growth conditions; N sufficiency (NS), N deprivation (ND) and N resupply (NR) (**Figure 2**). Cells were first cultivated in Tris-Acetate-Phosphate (TAP) medium until mid-log phase (NS), then N-deprived for a couple of days (ND), and followed by a 2-d N recovery period (NR) following N resupply. Cells were harvested at each stage for TAG quantification as well as for RNA extraction and quantitative reverse transcription PCR (qRT-PCR) analysis. We confirmed the fluctuation of TAG content in response to changes in the N status of the culture. The *CrLIP4* transcript was present at high levels in regular TAP medium (NS). Its abundance dropped strongly to 1.5% of that found in TAP medium (NS) after 20 h of N deprivation. When N was resupplied to the culture, the *CrLIP4* transcript level increased slowly to approximately 30% of the level in the original TAP medium after 96 h . Therefore, we concluded that *CrLIP4* transcript and TAG levels are inversely correlated.

Repression of the *CrLIP4* transcript level delayed TAG mobilization during N recovery

To investigate the *in vivo* function of CrLIP4, artificial microRNA was employed to repress the transcription of *CrLIP4*. Two artificial microRNA constructs were generated to target either the 5’ or 3’ portions of the gene. The linearized plasmid containing the paromomycin resistance gene *AphVIII* was introduced into the *Chlamydomonas* dw15.1 genome. Fifteen transformants

for each construct were tested for their *CrLIP4* expression levels with qRT-PCR. From a total of 30 transformants, three knockdown lines targeting the 3' region exhibited 60% *CrLIP4* levels compared to the dw15.1 parental line (PL) (**Figure 3A**). These three lines named amiRNA1 to -3 were selected for further analysis. The algae were grown in TAP medium until mid-log phase. To induce TAG accumulation the cultures were then transferred to TAP-N medium and incubated for 48 h. Finally, TAG mobilization was initiated by transferring the N-deprived cultures to TAP medium followed by incubation for another 24 h. During this 24 h period of recovery from N deprivation, the ratio of fatty acids in TAG over total fatty acids in the cell was determined at 0, 12, 16, 20 and 24 h (**Figure 3B**) to observe relative changes in TAG content. During the first 12 h of N recovery, TAG over total fatty acid ratio of the amiRNA lines were not significantly different from that of the dw15.1 parental line. However, at later time points, 20 and 24 h, the amiRNA lines had a higher TAG over total fatty acid ratio than dw15.1 parental line did. Based on this result we concluded that reduced levels of CrLIP4 led to a delay in TAG degradation consistent with the predicted TAG hydrolytic activity of CrLIP4.

Loss of function *Crlip4* insertional mutant alleles

To obtain a clearer picture of CrLIP4 function, we extended our *in vivo* analysis to insertional mutants that recently became available from the Chlamydomonas mutant library project (Li et al. 2016). We identified seven putative *Crlip4* mutant alleles which could harbor possible insertions either within an intron or 3' UTR of the *CrLIP4*. Lines with insertions in exons were not available. Among the mutants with an insertion within the second intron, three mutants named *Crlip4-1* to -3 were selected for further analysis. According to the information given by the mutant library project, *Crlip4-1* and *Crlip4-3* only have one insertion junction identified and their flanking sequences are identical (**Table S1**). However, their internal barcodes are different. In addition, *Crlip4-3* seemed to have larger cell diameter (data not shown). This could be due to an additional insertion in its background. For *Crlip4-2*, both insertion junctions are just a few bases apart. One of the junctions is overlapped with that of *Crlip4-1* and *-3* (**Table S1**). Based on this information, we postulated that all three mutants are independent and were chosen for characterization of their phenotype.

We first verified the insertion site. Two sets of PCR reactions were performed to corroborate the insertion sites determined by the developers of the mutant library project (**Figure 4A**). In the first reaction, primers CLiP-F3 and CLiP-R5 were designed to amplify the genomic region across the insertion site. Only the CC5325 parental line gave rise to a detectable

product (**Figure 4B**). In the second set of analysis, primers oMJ944 and CLiP-R4 were used to amplify DNA from inside the insertion to the flanking genomic region. In this PCR reaction, only the mutants gave rise to detectable PCR products (**Figure 4B**). The results confirmed the insertion sites for the three mutants.

Because the insertions were in the second intron, we further tested for the presence of *CrLIP4* transcripts by RT-PCR. Two positions along the gene were tested (**Figure 4A**) in parallel to the transcript of the receptor for activated protein kinase C 1 (*RACK1*) as a control. The RT-PCRs were carried out for 25, 30 and 35 cycles. Both RT-PCR reactions showed that all three mutants were not be able to amplify any product even after 35 cycles, while the CC5325 parental line started to show a band after 30 cycles (**Figure 4C**). The *RACK1* reactions all produced products ensuring the quality of the RNA/cDNA preparations. Based on these results we concluded that these three *Crlip4* mutants are true loss of function mutants.

***Crlip4* mutants are impaired in TAG breakdown following N resupply and accumulated TAG during normal growth** Artificial microRNA repression of *CrLIP4* led to a delay in TAG degradation and we expected that the insertional mutant had a more pronounced effect. Therefore, we determined the TAG fatty acid over total fatty acid ratio in cells cultivated under four different growth conditions as shown in **Figure 5A:** NS, ND, NR using TAP medium (NRT) and NR using minimal medium (NRM). Moreover, N resupply was done in the dark to force the utilization of TAG for the production of energy and cell building blocks. During the NS phase of the experiment, three cultures for each line were grown in TAP medium under normal growth conditions in the light until mid-log phase was achieved. The cells were then transferred to TAP-N medium and incubated for 48 h in the light to achieve N deprivation (ND). Finally, to induce maximum TAG mobilization, ND cultures were divided and half of the cells were resuspended following centrifugation in regular TAP medium (NRT) or minimal medium (MM, NRM) and incubated for 24 h in the dark. During the normal growth in N-replete medium in the light (NS), the *Crlip4* mutants showed significantly higher ratio of TAG over total fatty acids than the CC5325 parental line (**Figure 5B**). During the TAG accumulation phase (i.e. following N deprivation), the *Crlip4* mutants also had an increased TAG over total fatty acid ratio compared to the CC5325 parental line (**Figure 5B**). However, this difference was only statistically different for one of the mutant lines. When the cultures were subsequently incubated in N-replete media, the *Crlip4* mutants were strongly delayed in TAG degradation especially in MM as indicated by their significantly higher TAG over total fatty acid ratio after 24 h compared to the respective parental line (**Figure 5B**). Taken together this result for the

Crlip4 insertional lines along with that for the lines with artificial microRNA repression of *CrLIP4*, strongly indicated that CrLIP4 is involved in degradation of TAG during N resupply. Moreover, we showed that removal of CrLIP4 increased oil content in general and especially following N starvation providing a promising engineering strategy for increasing the TAG content in the algal biomass.

Fatty acid compositional changes in the *Crlip4* mutants To test the effect of CrLIP4 ablation on the fatty acid profile, TAG acyl groups and total fatty acids were analyzed. During normal N-replete growth conditions (NS), TAG acyl groups and total fatty acid profiles of the mutants were similar to those of the CC5325 parental line (**Figure S1**). In cultures during later stages of the experiment, saturated (16:0 and 18:0; number of carbons : number of double bonds, with position of double bonds indicated counting from the carboxyl end) were lower and unsaturated fatty acids (especially $18:1^{\Delta 9}$) were more abundant in TAG of the mutants compared to those of the CC5325 parental line. (**Figure S1** and **Figure 5C**). The strongest differences were observed in cells following N resupply in MM medium (NRM) (**Figure 5C**). The reverse effect was observed for the total fatty acid profile (**Figure S1** and **Figure 5D**). One interpretation of this result might be that CrLIP4 has a preference for a subfraction of TAG molecular species. However, other changes in lipid metabolism due to the loss of CrLIP4 could cause this phenotype.

Discussion

TAG lipases play important functions in maintaining lipid homeostasis, energy balance, and physiological activities in living organisms. Here we identified and characterized a TAG lipase in the model green alga *Chlamydomonas*. The phenotype of both amiRNA repression and insertional disruption mutants suggested that CrLIP4 plays a role in TAG degradation particularly during recovery from N deprivation when TAG is abundant and needs to be rapidly degraded. *CrLIP4* transcription is most abundant during normal growth conditions when the N supply is sufficient, but decreases following N deprivation when TAG is synthesized and TAG degradation should slow down. While the abundance of the *CrLIP4* transcript does not necessarily reflect the abundance or activity of the CrLIP4 protein, its presence under normal growth conditions highlights the importance of TAG homeostasis during active growth. Aside from providing insights into TAG homeostasis under different conditions, this study of CrLIP4

and its role in lipid degradation in microalgae is providing an avenue towards the engineering of TAG content of interest to various industries, not the least biodiesel production.

CrLIP4 transcript level is conversely related to TAG accumulation

In this study we observed an inverse correlation between *CrLIP4* transcript and TAG level similar to what was initially observed by Miller et al. (2010) and corroborated in other transcriptomic studies (Boyle et al. 2012; Lv et al. 2013; Tsai et al. 2018; Tsai et al. 2014).

Because the *CrLIP4* transcript level sharply fell during N deprivation, it was expected that following N resupply, the *CrLIP4* transcript level would recover rapidly to the same level as during normal growth conditions. However, following N resupply, the level of *CrLIP4* increased slowly and had not reached the original levels by the end of the experiment. This result was similar as observed in transcriptomics data from (Tsai et al. 2018). It seems possible that transcription of *CrLIP4* requires more time than we allowed in this experiment to fully recover its original level. Another possibility is that expression of *CrLIP4* is dependent on the stage of the culture as shown by (Lv et al. 2013), which could mean that the level of *CrLIP4* transcript will not recover to the same level until the culture is diluted and allowed to grow to mid-log phase again.

CrLIP4 is involved in TAG degradation during N recovery

The *in vivo* functions of CrLIP4 was addressed in two independent experiments using artificial microRNA repression of the gene and loss-of-function insertional mutants. Data from both sets of experiments were consistent with a role of CrLIP4 in degrading TAG during N recovery. In the artificial microRNA lines, delayed TAG degradation was not observed until later time points (**Figure 3B**). This could be due to the fact that the level of *CrLIP4* transcript was still more than 50% of the parental line giving rise to substantial levels of the CrLIP4 protein during N recovery. Furthermore, during this experiment TAG mobilization might have coincided with *de novo* TAG synthesis due to the incubation in the light as discussed below. Ultimately, the actual level of a metabolic end product or in this case intermediate such as TAG is determined by its concurrent synthesis and degradation.

During the analysis of the loss-of-function mutants, the highest difference in the ratio of TAG over total fatty acids between *Crlip4* mutants and the parental line was observed when the cultures were kept in the dark after N was resupplied (NRM) (**Figure 5B**). We assumed that this condition allowed us to examine TAG degradation only without interference from concurrent TAG synthesis. Under this condition, because no photosynthesis occurred to

provide reductant and ATP for TAG biosynthesis nor acetate was provided to serve as precursor for TAG biosynthesis in the dark, only minimal *de novo* fatty acid synthesis and therefore TAG assembly should occur at a limited level. Nevertheless, we observed the TAG mobilization defect in the mutants under all conditions tested, albeit to different extent, suggesting that CrLIP4 plays a key role in TAG degradation during recovery from N degradation.

Substrate specificity of CrLIP4 can be inferred from fatty acid compositional changes

Lipases have a diverse range of substrate specificities. *Saccharomyces* Tgl3p hydrolyzes a wide range of TAG substrates (Athenstaedt and Daum 2003), while Tgl4p and Tgl5p are more specific for TAGs with 14:0 and 16:0, and 26:0 fatty acids, respectively (Athenstaedt and Daum 2005). The recombinant *Arabidopsis* SDP1 protein was able to use a broad range of TAG substrates (Eastmond 2006). Fatty acid in the TAG fraction of *Crlip4* mutants following N resupply showed reduced 16:0 and increased $18:1^{\Delta 9}$ fatty acids compared the PL. Based on this result one might infer that CrLIP4 prefers $18:1^{\Delta 9}$. However, the real substrate specificity of an enzyme may not be able to determine only from the composition of fatty acids in the mutants. This is because the phenotype could be a result of pleiotropy. In addition, substrate specificity should be inferred from an in vitro assay by using recombinant protein as the TAG lipases mentioned above. Nonetheless, lipase assay alone may not indicate the true substrate in the biological system as in the case of Arabidopsis PLIP1, 2 and 3 (Wang et al. 2017; Wang et al. 2018). Therefore, for CrLIP4, more study needs to be carried out to determine the substrate preference.

It should be noted that 16:0 and $18:1^{\Delta 9}$ in the total fatty acid pool seem to increase, while $16:4^{\Delta 4,7,10,13}$ and $18:3^{\Delta 9,12,15}$ seem to decrease in the mutants (**Fig. 5D**). This result was not unexpected because 16:0 and $18:1^{\Delta 9}$ are the major fatty acids in TAG (Zäuner et al. 2012) which contribute almost 40% of total fatty acids in the mutants. On the other hand, $16:4^{\Delta 4,7,10,13}$ and $18:3^{\Delta 9,12,15}$ are major fatty acids found in membrane lipids (Giroud et al. 1988), which were present in a lower ratio compared to the parental line simply refection the difference in relative TAG content. A similar result was observed for the *Tgl1* TAG lipase inactivation in a diatom mutant (Barka et al. 2016).

The role of CrLIP4 in actively growing cells

Chlamydomonas accumulates small amount of TAG during normal growth conditions. This could be due to the fact that flux of carbon in the form of acyl groups goes toward other pathways such as membrane lipids under these conditions. Deng et al. (2013) also reported that in *Chlamydomonas* citrate synthase (CrCIS) diverts flux of acetyl-CoA from TAG biosynthesis to the tricarboxylic acid cycle. In plants, during vegetative growth, fluxes of DAG and phosphatidylcholine go toward membrane lipids more than to TAG synthesis (Bates and Browse 2012). Based on analyses of transcript levels of genes involved in TAG metabolism during normal growth condition, *Chlamydomonas* synthesizes and degrades TAG concomitantly (Boyle et al. 2012; Lv et al. 2013; Miller et al. 2010; Tsai et al. 2018). This concurrent synthesis and break down results in a low TAG concentration during optimal growth. Once the breakdown of TAG is inhibited as is in the case of the *Crlip4* mutants, higher TAG was detected (**Fig. 5B**). Hence, CrLIP4 plays an important role in TAG and lipid homeostasis during normal growth as well as during the TAG mobilization phase, when energy and acyl-chains are needed to support growth and membrane synthesis. Therefore, TAG degradation in microalgae, i.e. CrLIP4, provides a potential engineering target to shift the balance towards accumulation of TAG in growing cells of microalgae.

Materials and methods

Chlamydomonas strains and growth conditions

The *Chlamydomonas* cell wall-deficient strain dw15.1 (cw15, nit1, mt^+) kindly provided by Arthur Grossman (Carnegie Institute for Science, Stanford University) was used for analysis of transcript levels and served as the parental strain for artificial microRNA studies. The *Crlip4* insertional mutant strains LMJ.RY0402.162064, LMJ.RY0402.162453 and LMJ.RY0402.216596 were obtained from the Chlamydomonas Resource Center (Li et al. 2016). The background strain CC-4533 (cw15, mt^-) was used as a parental strain in experiments of the *Crlip4* insertional mutants. Unless otherwise specified, the algal cultures were grown in Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine 1965) under continuous light at 80-100 μmol $m^{-2}sec^{-1}$and at 22°C. For N deprivation, the cells were collected by centrifugation at 3,000 *g*, washed twice and resuspended in TAP-N. For N resupply, the cell pellets were collected similarly but resuspend in either TAP or minimal medium (MM) (TAP medium adjusted pH with HCl instead of acetic acid) and incubated either under the light or in the dark, as indicated in the results part.

Phylogenetic tree reconstruction

The conserved domains, GXSXG motif and catalytic residues were predicted by HMMER (Finn et al. 2015). Amino acid sequences alignment was carried out with MUSCLE (Edgar 2004). The phylogenetic tree was reconstructed by the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992) using MEGA7 (Kumar et al. 2016). Test of phylogeny was carried out with the Bootstrap method using 1000 replicates (Felsenstein 1985).

RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

Cell pellets were harvested from 10-15 ml *Chlamydomonas* culture as described above. The pellets were flash frozen in liquid N_2 and stored at -80°C until use. Total RNA isolation from the frozen cell pellets was carried out with RNeasy Plant Mini Kit (QIAGEN). The total RNA was treated with RNase-Free DNase Set (QIAGEN). cDNA synthesis was performed with either RETROscript® Reverse Transcription Kit (Ambion) or QuantiTect Reverse Transcription Kit (QIAGEN). The PCR reactions were prepared with Applied Biosystems® SYBR® Green PCR Master Mix (ThermoFisher SCIENTIFIC). Primers Lip4-jw2-fwd and Lip4-jw2-rev were used to amplify the *CrLIP4* transcript. Sequences of these primers, and also all others used in this study, are given in **Table S2**. The reference gene used for *Chlamydomonas* samples was *RACK1*. The qRT-PCR reactions were carried out using the Applied Biosystem 7500 Fast real-time PCR system. The qRT-PCR data were calculated based on the $2^{-\Delta\Delta C_T}$ method as previously described (Livak and Schmittgen 2001).

Generation of artificial microRNA knockdown lines

Target sequences of artificial microRNA were determined through WMD3- Web MicroRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd). The Designer page of WMD3 was used for primer design. Target sequence and primers can be found in **Table S2**. Oligonucleotides were then inserted into the pChlamiRNA3int vector according to (Molnar et al. 2009). The plasmid was linearized with *Kpn*I restriction enzyme and transformed into *Chlamydomonas* dw15.1 using the glass bead method (Kindle 1990) with minor modifications; Cells were grown in liquid TAP medium and the use of polyethyleneglycol (PEG) was omitted. Transformants were selected on agar-solidified TAP medium containing 10 μg/ml paromomycin. Single colonies were picked and tested for the *CrLIP4* transcript level by qRT-PCR as described above. Lipid analysis was performed as described below.

Genotyping of *Crlip4* insertional mutants

PCR was used to test the genotype of the *Crlip4* mutants. Three single colonies were tested for each mutant. Genomic DNA was extracted with Chelex-100 (Sigma-Aldrich) as previously described (Cao et al. 2009). PCR across the predicted insertion site was performed with CLiP-F3 and CLiP-R5 primers. While PCR from the insert to the flanking sequence was performed with oMJ944 and CLiP-R4 primers. The PCR was carried out with KOD DNA polymerase (MERCK) for 35 cycles.

Reverse transcription PCR for *Crlip4* insertional mutants

RNA extraction was performed as previously described (Nguyen et al. 2013). DNase treatment was carried out with TURBO™ DNase (Thermo Fisher SCIENTIFIC). The treated RNA was then purified with NucleoSpin® RNA Clean-up (MACHEREY-NAGEL). cDNA was synthesized with SuperScript™III First-Strand Synthesis System (Thermo Fisher SCIENTIFIC). PCR was performed at two locations with CrLIP4-RT-F1 and CrLIP4-RT-R1, and CrLIP4-RT-F4 and CrLIP4-R4 primers. The PCRs were conducted at 25, 30 and 35 cycles. In addition, *RACK1* was amplified with LP-F and LP-R primers for 25 cycles as an internal standard.

Lipid analysis

Four to 15 ml of *Chlamydomonas* cultures were harvested and stored at -80°C as described above. Total lipid was extracted from the frozen cell pellets using the method from (Bligh and Dyer 1959). The extracted lipids were dried under an N_2 stream and stored at -20°C. TAG was separated from total lipids (30 µl from 200 µl resuspended in chloroform) with thin layer chromatography (TLC) using a solvent system for neutral lipids as described in (Warakanont et al. 2015). TAG bands were isolated from the TLC plate and being converted to fatty acid methyl esters (FAMEs) as described in (Benning and Somerville 1992) with some modifications. Generally, after incubation with 1 ml of 1 N methanolic HCl (Sigma-Aldrich) at 80°C for 30 min, 1 ml of 0.9% NaCl (w/v) was added for phase separation. FAMEs were extracted into 1 ml hexane which was then dried and resuspend in 50-100 µl hexane for quantification with gas chromatography using DB-23 column (Agilent Technology) as described in (Zäuner et al. 2012). For all experiments the instrument was HP6890 coupled with

a flame ionization detector (Agilent Technology), except GCMS-QP2020 (Shimadzu) was used for the lipid analysis of *Crlip4* mutants.

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Disclosures:

The authors declare no conflicts of interest.

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Supplemental information:

Supplemental Table S1. Information of *Crlip4* insertional mutant alleles

Supplemental Table S2. Sequences of primers and target sequences used for different purposes as indicated.

Figure legends

Figure 1. Protein domain and phylogenetic analysis.

(**A**) A schematic diagram showing the positions of protein domains.

The DUF3336 (PF11815.7) and Patatin (PF01734.21) domains, GXSXG motif, serine and aspartate residues were predicted by HMMER (Finn et al 2015) of characterized TAG lipases and CrLIP4. The length is drawn to scale from N- to C-terminus.

(**B**) Phylogenetic analysis of CrLIP4 homologues.

The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 2. *CrLIP4* transcript level and TAG content during the three N status.

Cells were first cultivated under N sufficiency in TAP medium, then deprived for N for a number of days and followed by N recovery in TAP medium again. The transcript of *CrLIP4* was normalized to that of the receptor for activated kinase C 1 (*RACK1*). This experiment was carried out with *Chlamydomonas* dw15-1 as a parental line (PL). Noted that the culture in all conditions was incubated under the light. The error bars represent standard deviations calculated from three biological replicates.

Figure 3. Artificial microRNA knockdown of *CrLIP4*.

(**A**) Relative expression of *CrLIP4*.

(**B**) TAG concentration during N resupply of artificial microRNA knockdown lines 1 to 3 (amiRNA-1 to -3).

Transcript levels of *CrLIP4* from 3 amiRNAs were analyzed with *RACK1* as an internal control. The amiRNA lines were tested for their TAG content after 0, 12, 16, 20 and 24 h of N resupply under the light. The error bars represent standard deviations calculated from three biological replicates. Statistical analysis was carried out with two-tailed student *t*-test based on whether the variances are equal or not. * indicates statistically difference between amiRNA and dw15.1 PL with p-value ≤ 0.05. ns indicates no statistically different between amiRNA and dw15.1 PL at p-value ≤ 0.05.

Figure 4. *CrLIP4* gene model and genotyping of *Crlip4* knockout mutants.

(**A**) *CrLIP4* gene structure, antibiotic marker gene insertion position in *Crlip4-1* to *-3* mutants and primer binding sites are shown.

(**B**) PCR amplification of genetic insertion sites.

Genomic DNA amplifications from CC5325 PL against *Crlip4* mutants with primers covering the insertion region (CLiP-F3 and CLiP-R5) and from insertion cassette (oMJ944 and CLiP-R4). Three single colonies were tested for each mutant. White lines indicate removal of irrelevant lanes.

C) RT-PCR of *CrLIP4* expression.

RACK1 is used as a control.

Figure 5. TAG phenotype of *Crlip4* mutants.

(**A**) Schematic drawing shows growing conditions of *Chlamydomonas*.

The cultures were grown in TAP medium until mid-log phase was achieved. They were transferred to TAP-N for 48 h and to either TAP or MM for another 24 h under the dark. N sufficient (NS), N deprivation (ND), N resupply with TAP (NRT) and N resupply with MM (NRM) samples were taken as indicated.

(**B**) TAG/total fatty acids during NS, ND, NRT and NRM.

(**C**) Fatty acid composition of TAG.

(**D**) Total fatty acids of cultures from NRM condition. The error bars represent standard deviations calculated from three biological replicates. Statistical analysis was carried out with student *t*-test (one-tailed in B and two-tailed in C and D). The type of the *t*-test was chosen based on the equality of variances. * and ** indicate statistical difference with p-value ≤ 0.05 and 0.01, respectively. ns indicates no statistical difference at p-value ≤ 0.05.

Figure S1 Fatty acid composition of TAG and total fatty acids of four N status

Fatty acid compositions of TAG (left panel) and total fatty acid (right panel) fractions from NS (first row), ND (second row), NRT (third row) and NRM (fourth row) of WT and *Crlip4* mutants were shown. The error bars represent standard deviations calculated from three biological replicates. Statistical analysis between each *Crlip4* mutant and WT was carried out with two-tailed student *t*-test based on whether the variances are equal or not. * and ** indicate statistical difference with p-value ≤ 0.05 and 0.01, respectively. ns indicates no statistical difference at p-value ≤ 0.05.

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