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Overexpression of acetyl-CoA synthetase (ACS) enhances the biosynthesis of neutral lipids and starch in the green microalga *Chlamydomonas reinhardtii*

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ABSTRACT

Genetic engineering can be the solution to achieve the economically feasible production of microalgal based biofuels and other bulk materials. A good number of microalgal species can grow mixotrophically using acetate as carbon source. Moreover, experimental evidence suggests that the biosynthesis of acetyl-CoA could be a limiting step in the complex multifactor-dependent biosynthesis of acylglycerides and point to acetyl-CoA synthetase (ACS) as a key enzyme in the process. In order to test this hypothesis we have engineered the model chlorophyte *Chlamydomonas reinhardtii* to overexpress the endogenous chloroplastic acetyl-CoA synthetase, ACS2. Expression of the *ACS2* encoding gene under the control of the strong constitutive *RbcS2* promoter in nitrogen-replete cultures resulted in a 2-fold increase in starch content and 60% higher acyl-CoA pool compared to the parental line. Under nitrogen deprivation, the *Cr-acs2* transformant shows 6-fold higher levels of *ACS2* transcript and a 2.4-fold higher accumulation of triacylglycerol (TAG) than the untransformed control. Analysis of lipid species and fatty acid profiles in the *Cr-acs2* transformant revealed a higher content than the parental strain in the major glycolipids and suggests that the enhanced synthesis of triacylglycerol in the transformant is not achieved at expense of membrane lipids, but is due to an increase in the carbon flux towards the synthesis of acetyl-CoA in the chloroplast. This data demonstrates the potential of engineering the chloroplastic ACS to increase the carbon flux towards the synthesis of fatty acids as an alternative strategy to enhance the biosynthesis of lipids in microalgae.

1. Introduction

In the last decades there has been an increasing interest in exploiting microalgae for the production of biofuel precursors, such as triacylglycerol (TAG) and starch, which can be transformed into biodiesel and bioethanol, respectively [1–5]. However, until now the commercially viable production of these compounds has been restricted by the high cost of producing algal biomass at large scale and by the fact that these compounds are usually accumulated under stress conditions, such as nitrogen starvation, which limits algal growth and therefore reduces their overall yield [6].

Genetic engineering of microalgae can provide a solution to increase strain productivity and facilitate the development of the economically feasible production of microalgal based biofuels and other bulk materials [7–11]. The first attempt to engineer TAG biosynthetic pathways in microalgae was the pioneering work of Dunahay and co-workers, carried out in the 1990's within the Aquatic species program (ASP) of the US Department of Energy, to increase the TAG productivity in the diatom *Cyclotella cryptica* by overexpressing the native acetyl-CoA carboxylase (ACCase) [12], which catalyzes the first committal step in fatty acid biosynthesis and is considered a limiting step in lipid biosynthesis; however, the 2-fold increase observed for ACCase activity in the transformants did not lead to an increase in lipid content [13]. Since then, extensive research has been done to increase the content of TAG in plants [14] and microalgae [8], or to modify their fatty acids profiles, enriching the presence of acyl groups that better conform the need of certain nutritional or industrial applications [15,16].

Attempts to engineer lipid production include overexpression of enzymes involved in the biosynthesis of fatty acids (Dunahay et al., 1996; [17]) and/or TAG assembly [18–21] as well as blocking competitive

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pathways, such starch biosynthesis [22] or catabolism of lipids [23]. Several approaches have also focused on using the transcription factors, which regulate lipid biosynthetic pathways [24,25], or enhancing availability of the reducing agent NADPH, by overexpression of genes encoding NADP-malic enzyme, which has been used to increase the lipid content in *Dunaliella salina* [26] and *Phaeodactylum tricornutum* [27]. The degree of success of these genetic approaches is largely variable, but in many cases the results obtained are not those expected [8].

Chlamydomonas reinhardtii has emerged as model organism to study lipid metabolism in green algae [28]. Much of the research effort in *C. reinhardtii* has focused on engineering the last steps of the lipid biosynthetic pathway, namely the diacylglycerol acyltransferase (DGAT), catalyzing the last acylation step, and considered a key limiting step with great impact on TAG accumulation. Two evolutionary unrelated DGAT isoenzyme families, DGAT1 and DGAT2, differing in acyl specifity and expression patterns have been described [29]. The diversification of DGAT2 acyltransferases has been observed in *C. reinhardtii*, where five related genes have been identified [30], complicating the selection of any target isoenzyme. Overexpression of three of the five type 2 acyl-CoA diaylglycerol acyl-transferase (DGAT) in *C. reinhardtii* had no effect on intracellular TAG levels or fatty acid profiles [31]. However, it was finally demonstrated that overexpression of the forth isoform, DGTT4, strongly induced TAG accumulation [32]. In this work we propose an alternative approach, focused on increasing the carbon flux towards the synthesis of fatty acids, by overexpression of the plastidic acetyl-CoA synthetase (ACS2), which catalyzes the conversion of acetate to acetyl-CoA in the model microalga *Chlamydomonas reinhardtii*. The study of the transformants obtained has contributed to increase our knowledge about the role of ACS in lipids and starch accumulation and has shown the validity of this approach to increase their content in microalgae.

C. reinhardtii is a chlorophyte that can grow mixotrophically using acetate as carbon source. However, despite the important role that acetate plays on fatty acid synthesis [33], many aspects of its assimilation and metabolism remain unclear. Acetate is converted into acetyl-CoA via two possible pathways: 1) through the action of acetyl-CoA synthetase (ACS); and (2) in two steps catalyzed by acetate kinase (ACK) and phosphate acetyltransferase (PAT), with acetate-phosphate as intermediate. The presence of three different ACS isoenzymes and two ACK-PAT with different predicted locations in *Chlamydomonas,* together with the fact that acetyl-CoA, a key molecule of the metabolic crossroads connecting lipid and carbohydrate metabolism in many cell compartments, cannot cross organelle membrane by simple diffusion, as does acetate, suggests that acetyl-CoA can be synthesized in several subcellular compartments [34].

Under nitrogen-replete conditions acetyl-CoA, derived from acetate is catabolised via the TCA cycle or anabolised via the glyoxylate cycle. In *C. reinhardtii*, all the enzymes of the glyoxylate cycle have been identified and most of them have been localized in the peroxisomal microbodies [35]. Acetyl-CoA could also be obtained, via a plastidic pyruvate dehydrogenase, from pyruvate, which can be synthesized in the plastid from the Calvin cycle intermediates (PGA) or through the glycolytic pathway; in *C. reinhardtii* many glycolytic enzymes have been found in both the plastid and the cytosol [36]. Additionally, under heterotrophic nitrogen-limiting conditions acetyl-CoA can also be obtained from the citrate available in the cytoplasm, which is cleaved into oxaloacetate and acetyl-CoA by the citrate lyase (ACL), as has been documented in oleaginous yeast [7] and in some microalgae [37].

Despite this complex panorama, experimental evidence microalgae [38]; ii) acetate availability has been shown to limit the synthesis of lipids in some microalgal species [39]; iii) and failure to get higher lipids content by overexpression of acetyl-CoA carboxylase (ACCase) in some species, could be due to a limitation of acetyl-CoA supply which is the substrate for ACCase. Moreover, several proteomic and transcriptomic comparative studies of algal cells grown in normal and in different oil-accumulating conditions demonstrated that *ACS2*, encoding a chloroplastic acetyl-CoA synthetase, is up-regulated under all conditions in *C. reinhardtii* [39,40] and other chlorophytes, such as *Dunaliella* [41]. Expression of a bacterial *ACS* gene in the heterotrophic microalga *Schizochytrium* sp. allowed consumption of the acetate produced during its fermentative growth and an important increase in lipid accumulation [42]. Furthermore, knockdown of citrate synthase (CIS) the first step of TCA cycle, which diverts acetyl-CoA flux to the synthesis of amino acids and carbohydrates via oxaloacetate, causes an increase of TAG level in *C. reinhardtii*, while its overexpression has shown to provoke a decrease of TAG level [43]. Similarly, a *C. reinhardtii* mutant lacking *ICL1*, encoding isocitrate lysase, a critical gene in the aforementioned glyoxylate cycle, was also documented to have enhanced TAG accumulation compared to wild type cells [44].

On the other hand, expression of plastid acetyl-CoA synthetase (ACS) in *Arabidopsis*, did not lead to significant changes in the lipid content of developing seeds or leaves [45,46], indicating that there are important differences in the regulatory mechanisms and the rate-limiting steps in different organisms.

In order to understand the role of ACS in lipid and starch accumulation in N-starved cells of *C. reinhardtii* and to find alternative strategies to enhance lipid content in microalgae, we have studied the accumulation of TAG and starch in *C. reinhardtii* transgenic cells overexpressing an endogenous chloroplastic acetyl-CoA synthetase, and characterized their lipid species and fatty acid profiles.

2. Materials and methods

2.1. Strains and culture conditions

C. reinhardtii cell-wall deficient strain 704 (*cw15,* arg*7*, mt+), denominated as control or parental strain within this manuscript, was kindly provided by Dr. Emilio Fernández [47] and cultured photomixotrophically in liquid or agar solidified Tris-Acetate-Phosphate (TAP) medium [34] at 20 °C and 70 rpm, under continuous white light irradiation of 100 μE m^{-2} s^{-1}. In order to induce TAG accumulation, *C. reinhardtii* cells at the middle of the exponential phase of growth (OD_{660} = 0.8–1) were harvested by centrifugation, washed and resuspended in N-free TAP medium. When indicated additional acetate was supplied to the TAP medium to achieve a final acetate concentration of 50 mM. Microalgal growth was followed by spectrophotometrical measurement of the optical density at 660 nm or by determination of the dry weight. For dry weight determination, 2 mL of algal culture was harvested by centrifugation, washed with ammonium formate 50 mM, dried at 90 °C or freeze-dried for 48 h and the mass determined using an analytical balance. For cell density, Cellometer Auto T4 Bright Field Cell Counter (Nexcelom Bioscience, LLC.) was used loading 20 μl of algal culture into the cell chamber, and data were processed by the software.

The DH5α *Escherichia coli* strain, used for in vivo amplification of DNA, was cultured in LB medium as previously described [48].

2.2. Construction of the transformation plasmid Phyco105-CrACS2

suggests that the biosynthesis of acetyl-CoA could be a limiting step in the complex multi-factor dependent biosynthesis of acylglycerides and point acetyl-CoA synthetase (ACS) as a key enzyme in the process: i) A correlation has been observed

The binary plasmid, Phyco105, derived from the plasmid pSI103 built by Sizova et al. [49], was used for expression of the

contains two cassettes, one with the *APHVIII* gene from *Streptomyces rimosus*, coding for an aminoglycoside 3'phosphotransferase that confers resistance to the antibiotic paromomycin, under the control of the strong constitutive promoters *RBCcS2* and *HSP70A* and terminated by the 3′ untranslated region of *RBCS2* and other with a polylinker region flanked by the same regulatory regions.

In order to isolate the ACS2 (EDO98345) encoding gene, total RNA was obtained by the RNAeasy plant minikit from Qiagen according to manufacturer's instructions and single strand cDNA was synthesized from total RNA according to the Superscript II RNaseH-reverse transcriptase manual (Invitrogen). *ACS2* was amplified by PCR, using 1 μL of the cDNA as template, and the Phusion High-Fidelity DNA Polymerase (Thermofisher). Specific forward and reverse primers were modified to contain the *XhoI* and *EcoRI* restriction sites. The resulting PCR product was ligated into the pSpark vector (Canvax, Spain) and further subcloned into the *XhoI-EcoRI* sites of polylinker region of the microalgal expression vector Phyco105 to generate the Phyco105-CrACS2 plasmid (Fig. 1 suppl mat).

2.3. Nuclear transformation of C. reinhardtii

Transformation was carried out using the glass-bead method of Kindle [50] with minor modifications. *C. reinhardtii* cells were grown to a cell density of about $10^{\square 7}$ cells per mL, harvested by centrifugation and resuspended in fresh TAP medium to obtain a 100-fold concentrated cell suspension. The concentrated cell suspension (0.6 mL) was added to a conical tube containing 0.3 g of sterile glass beads (0.4–0.6 mm Φ), 0.2 mL of 20% polyethylene glycol 8000 and about 1 μg of the desired plasmid. Cells were vortexed for 8 s, resuspended in 50 mL of fresh sterile TAP medium and incubated in the dark overnight. After this incubation in the absence of antibiotic, the cells were pelleted and spread onto agar plates containing TAP medium with paromomycin (30 μg $mL^{\square -1}$). Transformed colonies were visible after 5 days.

2.4. Lipids extraction and fractionation

For total lipid extraction, 10 mL aliquots of *C. reinhardtii* cultures grown in TAP nitrogen-free medium were pelleted by centrifugation and freeze dried. Total lipids were extracted from freeze dried cells, following the protocol described by Abida et al., [51] and separated into neutral lipids, glycolipids and phospholipids fractions by solid-phase extraction (SPE) on silica PreSep columns (LiChrolut Si 500 mg 2 mL) from Merck Millipore. The three fractions were evaporated under nitrogen and dissolved in 200 μL of chloroform (neutral lipids) or 200 μl of chloroform/methanol 2:1 (polar lipids). Each extract was split into two 100 μL fractions; one fraction was used for lipids species separation and quantification by ESI-MS/MS, and the other was subjected to methylation, for GC-FID analysis.

2.5. GC-FID analysis of fatty acid methyl esters

Fatty acid methyl ester (FAMEs) derivates, obtained as previously described [52], were analyzed by gas chromatography flame ionization detection (GC-FID) using an Agilent 7890A gas chromatograph fitted with an Agilent DB-23 (30 m × 0.25 mm × 0.25 μm) column as described previously [53], with minor modifications. A 1 μL aliquot of each sample was analyzed with a 1:5 split injection and constant flow rate of 1.5 mL $min^{\square -1}$. The oven temperature cycle was initially held at 150 °C for 2 min and then increased to 250 °C at a rate of 10 °C $min^{\square -1}$. The temperature was then held for 5 min, for a total run time of 17 min per sample. Analysis was carried out using Agilent Menhaden oil (Larodan) and quantified using pentadecanoic acid (0.4 mg $mL^{\square -1}$) as internal standard.

2.6. Quantitative lipid analysis

Quantitative analyses of triacylglycerols (TAG), phospholipids (PL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), the galactolipids digalactosyl diacylglycerol (DGDG) and monogalactosyl diacylglycerol (MGDG), and the betaine lipid diacylgliceryltrimethylhomoserine (DGTS) were carried out using electrospray ionization tandem triple-quadrupole mass spectrometry (4000 QTRAP; SCIX; ESI-MS/MS). Phosphatidylcholine (PC) which seems to be absent in *C. reinhardtii* [28,54] and phosphatidylinositol (PI), present at very low level, were not targeted for analysis. The lipid extracts were infused at 15 μL $min^{\square -1}$ with an auto-sampler (HTS-xt PAL, CTC-PAL Analytics AG, Switzerland). Data acquisition and acyl group identification of the polar lipids was as described in Ruiz-Lopez et al., [55] with modifications. The internal standards for polar lipids were supplied by Avanti (Alabaster, AL, USA), incorporated as 0.080 nmol of di14:0-PE, 0.800 nmol of di16:0 DGTS, and 0.080 nmol of di14:0-PG. The standards dissolved in chloroform and 25 μL of the samples in chloroform were combined with chloroform/methanol 300 mM ammonium acetate (300:665:3.5 v/v/v) to make a final volume of 1 mL.

The ESI-MS/MS method described by Li et al. [56] was modified to quantify TAG contents. For quantifying TAG, 15 μL of lipid extract and 0.857 nmol of tri15:0-TAG (Nu-Chek Prep, Elysian, MN, USA) were combined with chloroform/methanol/300 mM ammonium acetate (24:24:1.75: v/v/v), to final volumes of 1 mL for direct infusion into the mass spectrometer. TAG molecular species were detected as $[M + NH_{\square 4}]^{\square +}$ ions by a series of different neutral loss scans, targeting losses of fatty acids. The as processed using the program Lipid View Software (SCIEX, Framingham, MA, USA) where isotope corrections are applied. The peak area of each lipid was normalized to the internal standard and further normalized to the weight of the initial sample. There is variation in ionization efficiency among acyl glycerol species with different fatty acyl groups, and no response factors for individual species were determined in this study; therefore, the values are not directly proportional to the TAG contents of each species. However, the approach does allow a realistic comparison of TAG species across samples in this study.

2.7. Fluorescence assay of neutral lipids labeled by Nile red staining

Non polar lipids were measured by Nile Red (Sigma-Aldrich) fluorescence staining (excitation 485–512 nm and emission at 590–610 nm) using a fluorescent microplate reader (Fluostar Omega, BMG Labtech). A 0.2 mL aliquot of *C. reinhardtii* culture was incubated with 2 mL of PBS buffer and 10 μL of the fluorescent dye Nile Red (0.1 mg $mL^{\square -1}$ in acetone) at 40 °C for 10 min. Blank measurements (cells incubated without Nile Red stain) were subtracted from test samples and total fluorescence was expressed as arbitrary fluorescent units or normalized to the fluorescence of control cells. All measurements were done in triplicate.

2.8. Determination of Acyl-CoA pool composition

To analyze the intracellular acyl-CoA pool, 2 mL of freshly harvested cells were frozen in liquid nitrogen and acyl-CoAs were extracted as described by Larson and Graham [57] and analyzed using LC-MS/MS + MRM in positive ion mode. The LC-MS/MS + MRM analysis (using an ABSciex 4000 QTRAP) was performed as described by Haynes et al. [58] (Agilent 1200 LC

Chemstation software. Retention time and identify of each peak was calibrated using FAME 37 Component FAME mix (Supelco) and methylated Qualmix system; Gemini C18 column (Phenomenex), 2 mm inner diameter, 150 mm length, particle size

5 μm). For the purpose of identification and calibration, standard acyl-CoA esters with acyl chain lengths from C14 to C20 were purchased from Sigma as free acids or lithium salts.

2.9. Starch analysis

Intracelullar starch content was determined in freeze dried algal pellets from 2 mL of *C. reinhardtii* culture, using a commercial amyloglucosidase/α-amylase enzymatic kit (K-TSTA, Megazyme) according to the manufacturer's instructions.

2.10. Neutral lipid staining and visualisation by confocal microscopy

Non polar lipids and oil droplets were visualized using BODIPY staining and confocal microscopy essentially according to Govender et al. [59]. Cells were grown in nitrogen replete or deplete conditions and non-polar lipids and neutral lipids were stained after 48 h with BODIPY 490/503 (Sigma). An aliquot of 0.5 mL cells were pelleted at 12000 rpm and re-suspended in TAP medium containing 1 μg mL^{-1} BODIPY 490/503 (diluted from a stock of 1 mg mL^{-1} in DMSO). Cells were incubated for 10 min, pelleted by centrifugation and washed with TAP medium. Cells were then re-suspended in 15 μL TAP medium prior to confocal microscopy (Zeiss LSM 780, 63× objective lens). Images were processed using the Zen 2010 software (Carl Ziess Microimaging).

2.11. Genomic DNA preparation and PCR screening of transformants

Integration of the *RBCS2-ACS2* cassette in the genome of *C. reinhardtii* transformants was checked by PCR using 1 μL of genomic DNA in a total volume of 25 μL containing 10 pmol of each primer, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 2.5 μl of specific 10× buffer (containing 2.5 mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). The PCR program was: 0.5 min at 96 °C, 0.5 min at annealing temperature, and 1.5 min at 72 °C for 30 cycles. Algal genomic DNA was isolated by the GeneJET genomic DNA purification kit from Life Technologies.

2.12. qRT-PCR analysis

qPCR experiments were performed on a Mx3000P Multiplex Quantitative PCR System (Stratagene) using 1 μL of the cDNA, synthesized from total RNA according to the SuperScript II RNaseH-reverse transcriptase manual (Invitrogen), as template and Brilliant SYBR® Green QPCR Master Mix (Agilent Technologies, La Jolla, CA, USA). Cycling conditions were: 10 min at 95 °C for activation of the Hot start Taq polymerase and 40 cycles for the melting (30 s at 95 °C), annealing (30 s at 62 °C) and extension (30 s at 72 °C). Each qPCR measurement was carried out in triplicate using primers for either the chloroplastic acetyl-CoA synthetase (*ACS2*), or the alpha-carboxyltransferase subunit (*ACX1*) of chloroplastic acetyl-CoA carboxylase encoding genes (Table 1, suppl material). The *UBC8* gene, encoding an ubiquitin ligase polypeptide (XM_001697453), which expression was previously shown to be constitutive under the different conditions used [60,61] was used as housekeeping gene to normalize mRNA abundance. $2^{-\Delta\Delta CT}$ approach was used to calculate fold change [62].

3. Results and discussion

3.1. Generation and screening of CrACS overexpression lines

C. reinhardtii was transformed with the Phyco105-CrACS2 plasmid, obtained as described in Section 2.2, in which the chloroplastic acetyl-CoA synthetase encoding cDNA is under the control of the chimeric constitutive tandem *HSP70A-RbcS2* promoter. The resulting transformants were grown under antibiotic selective pressure, and the correct integration of the *RbcS2-CrACS2* cassette in the selected transformants was checked with specific primers that anneal with the *RbcS2* promoter and the *ACS* cDNA (Fig. 1a). In all the transformants studied, a 1600 bp fragment corresponding to the *RbcS2-ACS2* construction was found, confirming its correct insertion in the genome of the microalga (Fig. 1b).

In order to perform a preliminary screening, untransformed control and *Cr-acs2* transformants were transferred to nitrogen-depleted TAP media at the same initial cell density to induce the accumulation of

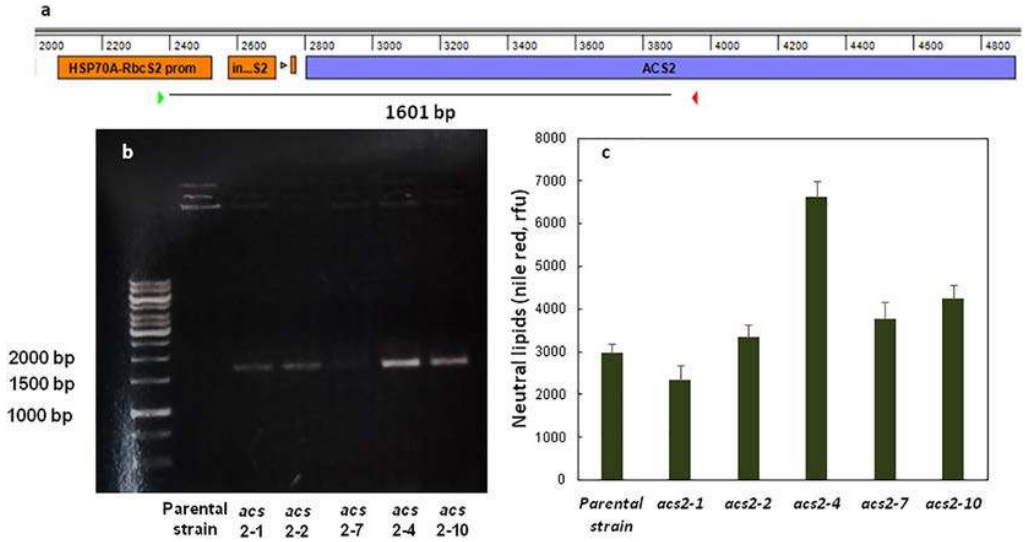

Fig. 1. Screening of *Cr-acs2* overexpression transformants. (a) Schematic map of the *RbcS2-HSP70A-ACS2-RbcS2 UTR* cassette, indicating the annealing sites for the PCR primers and the amplicon length. (b) Agarose gel electrophoresis showing the integration of the *RbcS2-ACS2* cassette in the microalgal genome of five transformants. The parental strain has been included as a negative control; the PCR was carried out as described in Section 2.11. (c) Comparison of neutral lipids content in the parental strain and the transformants, determined by staining with Nile Red after 72 h of nitrogen starvation. Data generated from three independent experiments. Error bars indicate standard deviation.

neutral lipids, which content was determined by staining with Nile Red dye and measuring fluorescence emission as detailed in Methods section 2.7. The cell density did not increased significantly over the three days in which the cultures were maintained without nitrogen (Fig. 2-suppl mat). Transformant *Cr-acs2-4* consistently accumulated more than twice the neutral lipid content of the parental strain after 72 h of nitrogen starvation (Fig. 1c). This clone was therefore selected for further studies.

3.2. TAG accumulation under nitrogen starvation in the Cr-ACS2-4 transformant

To further characterize the response to nitrogen starvation in the ACS2-overexpressing transformant (*Cr-acs2-4*), the TAG content was studied by electrospray ionization tandem triple-quadrupole mass spectrometry (ESI-MS/MS) before and 48 h after transfer to N-deprived medium and expressed as molecular species within the synthesized TAG (Fig. 2a) and as total TAG (Fig. 2b). In addition, the accumulation of lipid bodies was examined by fluorescence microscopy with the staining fluorophore BODIPY, in both the parental and the transformant strains (Fig. 2c–h).

Cells from the *Cr-acs2* transformant and the untransformed parental line were grown in standard conditions; at the middle of the exponential phase of growth both cultures were harvested by centrifugation, washed and transferred to TAP medium without a nitrogen source. The *Cr-acs2* transformant shows more and larger lipid droplets than the control untransformed strain cultured without N for the same time (Fig. 2c–h). The TAG content in nitrogen replete medium is negligible, however after transference to nitrogen free medium it increases to 15 and 30 fold relative to the corresponding N replete cultures, for the parental and the transformant strain respectively. Furthermore, the total TAG content in the transformant was 2.4-fold higher than in the parental strain after 48 h of N deprivation (Fig. 2a–b). All the TAG molecular species are higher in the transformant, being the increase of the same order for all the species. This data supports the hypothesis that overexpression of the chloroplastic ACS enhances accumulation of TAG, and confirms this enzyme as an effective target to increase neutral lipids accumulation in *C. reinhardtii* by genetic engineering.

3.3. The acyl-CoA intracellular pool in the ACS transformant is larger than in the wild type

Monitoring of the intracelullar levels of acyl-CoA offers interesting information about the potential bottlenecks for TAG biosynthesis. Therefore, cells from both the *Cr-acs2* transformant and the corresponding parental line were grown, harvested, transferred to nitrogen deprived TAP medium, as described in Section 3.2., and used to follow the intracellular acyl-CoA evolution over time of N deprivation, which was analyzed by LC-MS/MS as detailed in Section 2.8. In N-replete cultures, *Cr-acs2* transformant exhibited 60% higher intracellular content of acyl-CoAs relative to the parental strain (Fig. 3). The acyl-CoA pool is rapidly consumed when *C. reinhardtii* cells are transferred to N deprived medium due to the demand of substrates for the synthesis of TAG, which, as we will see later, is higher and more prolonged in the time in the *Cr-acs2* transformant as compared with the parental strain. In the absence of nitrogen source, acyl-CoA reserves are severely reduced in both the ACS transformant and the parental control strains, but remain higher in the transformant.

The fact that the intracellular pool of acyl-CoA is consumed during N starvation provides strong evidence to suggest that fatty acid synthesis, rather than TAG assembly, is the main bottleneck for the synthesis of neutral lipid in this microalga, in agreement with previous reports, such as those from La Russa et al. [31], who did not find significant alteration in the intracellular accumulation of TAG by overexpression of several DGATs enzymes, or Fan et al. [63], who demonstrated that the addition of oleic acid to the growth medium of *C. reinhardtii* increased oil production. Furthermore, our data suggests that carbon supply, rather than fatty acid synthesis itself, is the limiting factor in the production of oils in microalgae. The higher acyl-CoA intracellular level in *Cr-acs2* transformant corroborates that overexpression of *ACS2* provides higher availability of substrates for the synthesis of acyl-CoAs, which contribute to the synthesis of TAG during nitrogen deprivation. However the main source of acyl groups for the synthesis of TAG in these conditions is degradation of membrane polar lipids and their de novo biosynthesis, as will be discussed below.

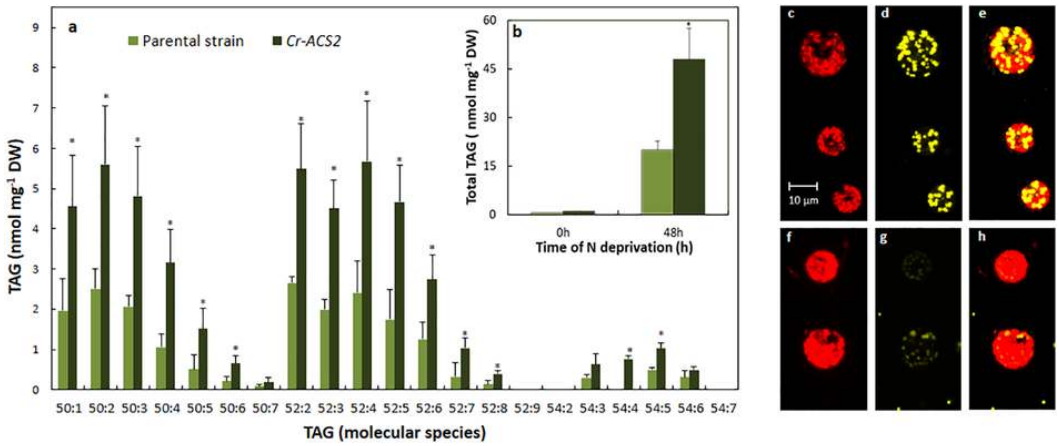

Fig. 2. Accumulation of neutral lipids and TAG in the control and the *acs2 Chlamydomonas* transformant. (a) The abundance of different molecular species within the TAG synthesized after 48 h of N starvation in the control strain (■) and in the *Cr-acs2* transformant (■) was determined by ESI-MS/MS. (b) Total TAG content obtained adding the content of all the molecular species was determined in the control and the transformant before and 48 h after transference to N deprived medium. Asterisks denote statistically significant differences in the transformant regards to the parental strain (*t*-test, $p < 0.05$). (c–h) Fluorescence microscopy detection of neutral lipid accumulation by BODIPY staining in *ACS2* overexpressing transformant (c-e) and control (f-h) strains, after 48 h of nitrogen starvation: (c,f) chlorophyll autofluorescence, (d,g) BODIPY stained lipids (e,h) combination of both. Bar: 10 µm.

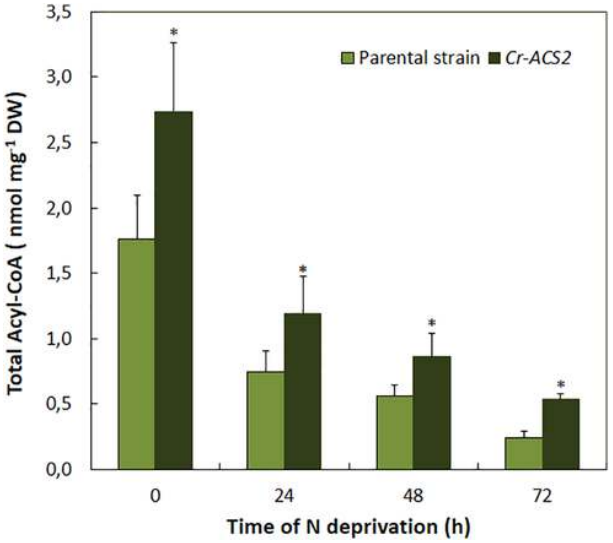

Fig. 3. Effect of ACS2 overexpression on the acyl-CoA pool of nitrogen starved *C. reinhardtii* cells. Total Acyl-CoA pool of control untransformed (Parental strain,■) and ACS2 transformant (*Cr-acs2*, ■) lines of *C. reinhardtii* cultured in nitrogen depleted TAP medium was determined by LC-MS/MS over time of N deprivation. Values are the average of three biological replicates and bars indicate standard deviation. Asterisk shows significant differences in the Cr-*acs2* transformant with respect to the parental strain (*t*-test, $p < 0.05$).

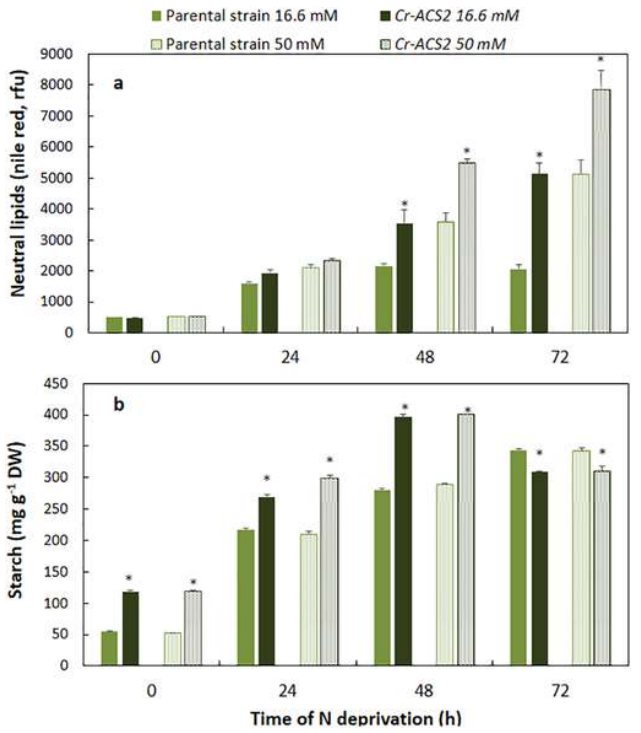

Fig. 4. Effect of acetate concentration on carbon storage products of ACS2 overexpressing *C. reinhardtii* transformant (*Cr-acs2*) and the corresponding untransformed parental line cultured under N deprivation. (a) Neutral lipids and (b) starch content in parental and *Cr-acs2* transformant strains were followed over time in N starved cultures with 16.6 mM and 50 mM as initial acetate concentration. Neutral lipids were measured by Nile Red fluorescence staining and expressed as relative fluorescence units. Values are the average of three biological replicates and bars indicate standard deviation. Asterisk indicates statistically significant differences in the transformant with regard to parental strain (*t*-test, $p < 0.05$).

3.4. Influence of differential initial acetate concentration on TAG and starch accumulation

To further investigate the influence of external acetate supply on TAG and starch accumulation, mid log cultures of the parental control and the *acs2*-transformant strains were transferred to nitrogen deprived TAP medium, with two different initial acetate concentrations of 16.6 and 50 mM; 16.6 mM is the standard acetate concentration in TAP medium [34], while 50 mM acetate was obtained by supplementing standard TAP medium with additional sodium acetate. Samples were collected every 24 h after nitrogen deprivation to determine the content of starch and neutral lipids, assayed using Nile red staining. We found that neutral lipids assayed by Nile Red method and TAG determined by ESI-MS/MS, show similar profiles over time in nitrogen deprivation, demonstrating that Nile red fluorescence assay accurately predicts the accumulation of neutral lipids in the *C. reinhardtii*. Other authors have previously validated the correlation between Nile Red fluorescence assay and the oil content in this microalga [64].

This experiment has allowed us to confirm that accumulation of neutral lipids under N deprivation is higher in the *Cr-acs2* transformant and is boosted by acetate in both the *Cr-acs2* and the corresponding parental line, and reveals that the increment is higher in the transformant (Fig. 4a). Increasing acetate concentration to 50 mM leads to recorded values of 8000 fluorescence units in the transformant after 72 h of nitrogen starvation, 50% higher than the neutral lipids accumulated in the parental strain in the same conditions (Fig. 4a) and 1.5 fold the value reached at 16.6 mM of acetate. Neutral lipid content increases in the parental strain during the first 48 h of nitrogen deprivation, but after this point further accumulation is only observed in the 50 mM acetate treatment. This limitation is not observed for the *Cr-acs2* transformant; in this case nitrogen starvation induces neutral lipid accumulation continuously over the whole experimental time under both acetate concentrations.

In contrast to lipids, starch accumulation is not enhanced by increasing acetate, neither in the control, as expected from previous reports [65], nor in the transformant line. The starch levels observed in N-replete cultures of the control and the *Cr-acs2* transformant are 55 and 115 mg g^{-1} DW, respectively. Upon transfer to nitrogen lacking medium, starch content is significantly increased. Interestingly, the differences between the *Cr-acs2* transformant and the untransformed control get smaller over time, becoming practically insignificant after 72 h of nitrogen starvation. The maximum intracelullar concentration of starch reached is practically the same for the two acetate concentrations assayed, around 40% of the dry weight for both the *Cr-acs2* transformant and the untransformed control, although this level is reached earlier in the transformant (Fig. 4b).

The differences found in the accumulation of starch and TAG in the transformant and the control cells suggest that the origin of the carbon source for the synthesis of each storage compound under nitrogen starvation is different. In stress conditions the metabolism of acetate via acetyl-CoA synthetase pathway seems to be much more important for the production of TAG than for the accumulation of starch, which must be mainly synthetized from other carbon intermediates.

Overexpression of the chloroplastic acetyl-CoA synthetase, besides providing higher levels of basal starch and acyl-CoA to the transformant than to the parental strain in N-replete conditions, can, under nitrogen stress, provide higher flux of acetyl-CoA for fatty acids. The data obtained in the present research is in agreement with previous evidence that pointed acetate availability as a key factor for the accumulation on TAG in *C. reinhardtii* [65,66] and other chlorophytes [67] and with recent studies that correlate the intracellular level of acetyl-CoA in different chlorophyte microalgae with its ability to accumulate triglycerides [38].

3.5. Characterization of lipids species and fatty acid profiles in transgenic lines overexpressing acetyl-CoA synthetase

To study the influence of *ACS2* overexpression on the composition of membrane lipids, the fatty acid profile of total lipids (Fig. 5) and the distribution of the main lipid species (Fig. 6) were followed over time of nitrogen deprivation in both the *Cr-acs2* and the parental strain, cultured in standard TAP medium (16.6 mM acetate). The total lipids accumulated under N-starvation in the *Cr-acs2* transformant and in the control untransformed cells were extracted, separated into the different lipid fractions (neutral, phospholipids and glycolipids) by SPE and analyzed by ESI-MS/MS. Total lipid were methylated and the (FAMEs) analyzed by GC-FID.

Globally the fatty acid profiling corresponds to that observed in other studies of nitrogen stressed *C. reinhardtii* cells [68,69], however, there are small differences between the parental and *Cr-acs2* transformant strains. Our results show that in both strains, nitrogen starvation causes a bias in the fatty acid profile towards saturated and low unsaturated fatty acids, specifically palmitic (C16:0) and oleic (C18:1) acids, which are the more abundant fatty acids in TAG, while the polyunsaturated fatty acid, such as 16:4n6 and 18:3n3, more abundant in membrane lipids, suffer a strong decrease under nitrogen deprivation. A more detailed look reveals that in the transformant line the increase in the monosaturated oleic acid is around 48% after 72 h of nitrogen deprivation, much higher than in the parental strain, for which oleic acid increase barely reach 29%. Interestingly, this observation supports the hypothesis that oleic acid accumulation in TAG, may be sensitive to acetyl-CoA availability (Smith R., personal communication). It can also be observed that the polyunsaturated acids, 16:4n6 and 18:3n3 suffer a > 50% decrease in the parental strain, while are only reduced in around 30% in the transformant. This indicates that degradation of membrane lipids, which are rich in these PUFAs, contributes to the synthesis of neutral lipids; however the higher content of neutral lipids observed in the transformant is not due to a higher degradation of the membrane lipids.

Additionally, separation and quantification of the major polar lipids reveals that, in the control strain, the big increase in TAG accumulation upon transference to nitrogen starvation is accompanied by a reduction in the main polar lipid species, in agreement with previous reports [68,69]. In the transformed strain, nitrogen depletion causes, as happens in the control untransformed strain, a decrease in some of main lipid components of the thylakoid membrane, such as monogalactosyl diacylglycerol (MGDG), sulfoquinovosyl diacylglycerol (SQDG) or phosphatidylglycerol (PG), although this decrease is lower than in the parental strain. The decrease of MGDG is particularly noteworthy, which after 72 h without nitrogen is reduced in >70% compared to the parental strain, while in the *acs2* transformant MGDG declines only 37%. Furthermore, digalactosyl diacylglycerol (DGDG), the second most abundant lipid in the plastid membranes, which slightly decreases in the control, increases around 30% in the transformant (Fig. 6). The betaine lipid diacyglyceryltrimethylhomoserine (DGTS), the major extraplastidic lipid in *C. reinhardtii,* which is slightly reduced in the control (5%), does not show a significant increase in the *Cr-acs2* transformant, after 72 h of nitrogen deprivation.

It is interesting to note that we have not found phosphatidylcholine (PC) neither in the parental, nor in the transformed strain. Although this ubiquitous phospholipid is essential for most eukaryotes and has been found in several species of *Chlamydomonas* [54], it seems to be absent in *Chlamydomonas reinhardtii*, where the role of PC could have been replaced by the non-phosphorous betaine lipid DGTS [28].

Thus, overexpression of *ACS2* results in higher TAG production and a certain bias to saturated or low unsaturated fatty acids upon transference to nitrogen deprived medium. Additionally, after some days in the absence of nitrogen a considerable degradation of membrane lipids, which contributes to the synthesis of neutral lipids can be observed; however this degradation is less acute in the *acs2* transformant, indicating that the higher TAG increment in the modified strain is due to an increase in the carbon flux towards the synthesis of acetyl-CoA in the chloroplast rather than to a higher decrease of polar lipid species.

It is generally accepted that fatty acid synthesis is largely reliant on exogenous acetate in *C. reinhardtii*, but there is certain controversy about the contribution of preexisting membrane lipids as source of fatty acids for the synthesis of TAG. Fan et al. [63,65] maintain that oil accumulation in response to N starvation is mainly dependent on de novo fatty acid synthesis in the chloroplast, while Juergens et al. [33] have observed, by 13C labelling time course studies, that there is a considerable turnover of membrane lipids during nutrient deprivation in mixotrophic cultures of *C. reinhardtii*. Similar results were observed by Siaut et al. [69] who concluded that there is a considerable degradation of plastidial membrane lipids at the beginning of the oil accumulation phase in N deprived *C. reinhardtii* cultures. Our analysis of the

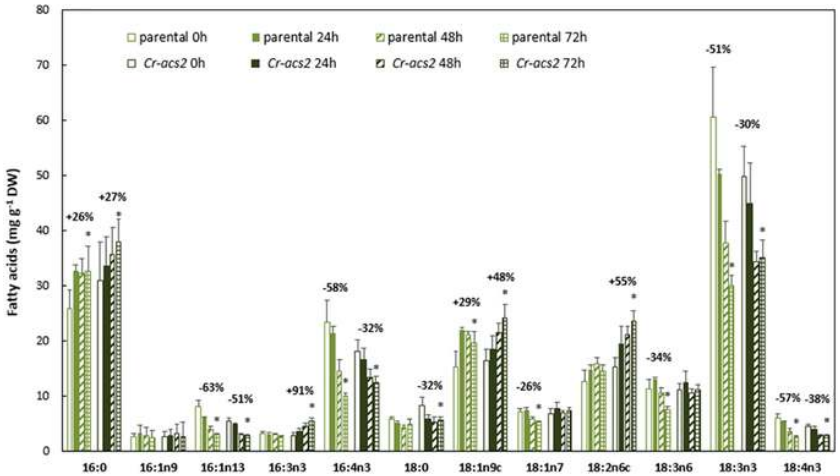

Fig. 5. Time course evolution of fatty acid composition of total lipids isolated from N-deprived control (PARENTAL STRAIN) and transformant (*Cr-acs2*) *C. reinhardtii* strains cultured in standard TAP medium (16.6 mM acetate). Samples from both strains were collected before and after 24, 48 and 72 h of N starvation and fatty acid methyl esters (FAME) prepared from total lipids were separated and analyzed by FID-GC. Values are the average of four measurements from two biological replicates and bars indicate standard deviation. Asterisks denote significant variations in lipid content at 72 h with respect to 0 h (*t*-test, $p < 0.05$).

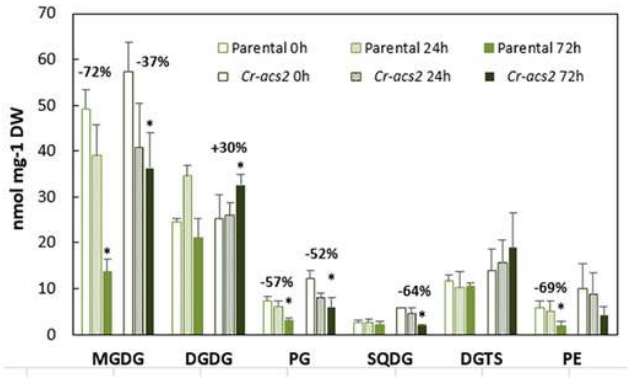

Fig. 6. Time course evolution of the major polar membrane lipids species in N deprived control (PARENTAL STRAIN) and transformant (*Cr-acs2*) *C. reinhardtii* strains cultured in standard TAP medium (16.6 mM acetate). The major lipid classes in glycolipids and phospholipids extracts from both strains were identified by ESI-MS/MS analysis before, and after 24 h and 72 h of N deprivation. Abbreviations: monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), phosphatidyl glycerol (PG); sulfolipid sulfoquinovosyl diacylglicerol (SQDG), diacyglyceryltrimethylhomoserine (DGTS), phosphatidylethanolamine (PE). Values are the average of four measurements from two biological replicates and bars indicate standard deviation. Asterisks denote significant variations in lipid content at 72 h with regard to 0 h ($p < 0.05$).

composition of membrane lipids in nitrogen deprived cultures shows a strong decrease of the main polar lipids over time of N deprivation, in agreement with the contribution of membrane lipids turnover to the accumulation of TAG, but comparative analysis between the transformant and the parental strain suggests that an increase in the carbon flux towards the synthesis of fatty acids in the chloroplasts, rather than higher membrane lipids turnover, is responsible for the higher accumulation of TAG in the *Cr-acs2* transformant.

3.6. Differential expression levels of chloroplastic acetyl-CoA synthetase and acetyl-CoA carboxylase

To complete this study we have analyzed the transcript levels of two enzymes: the chloroplastic acetyl-CoA synthetase (ACS2), the enzyme overexpressed in this work; and the α-carboxyltransferase subunit of the chloroplastic acetyl-CoA carboxylase (ACCase-ACX1), which is directly involved in the conversion of acetyl-CoA into malonyl-CoA, the first step in the biosynthesis of fatty acids. The expression profile of these two genes over time in N deprivation (Fig. 7a–b) and also the ratio between their expression levels in the transformant and the parental untransformed strain (Fig. 7c–d) have been analyzed by real-time quantitative PCR.

These experiments confirmed that *ACS2* is overexpressed in the transformant, which, resulted in a transcript level 50% higher than in the control parental strain (N-replete medium; Fig. 7c). Furthermore, profiling of *ACS2* expression over time during N deprivation showed that *ACS2* is up-regulated upon N deprivation both in the control and the transformant, reaching 6-fold the basal transcript level after 24 h of N deprivation (Fig. 7a). The *ACS2* transcript level is always higher in the transformant, e.g. 6-fold higher in the transformant than in the control after 48 h of nitrogen deprivation (Fig. 7c).

These results are in agreement with previous studies. Ramanan et al. [39] observed that the *ACS2* gene is highly up-regulated under mixotrophic and nitrogen-limited conditions founding a correlation between the up-regulation of the *ACS2* gene and the formation of lipid droplets in *C. reinhardtii*. Goodenough et al. [40] also concluded, after RNA-seq transcriptome analysis of several N-starved acetate-fed wild and mutant strains of *C. reinhardtii*, that *ACS2* is a sensitive gene for which expression was stimulated by acetate in N starved cultures. In other microalgae, such as *Dunaliella tertiolecta*, a similar increase of *ACS* transcript levels after nitrogen-deficient cultivation has been observed [41].

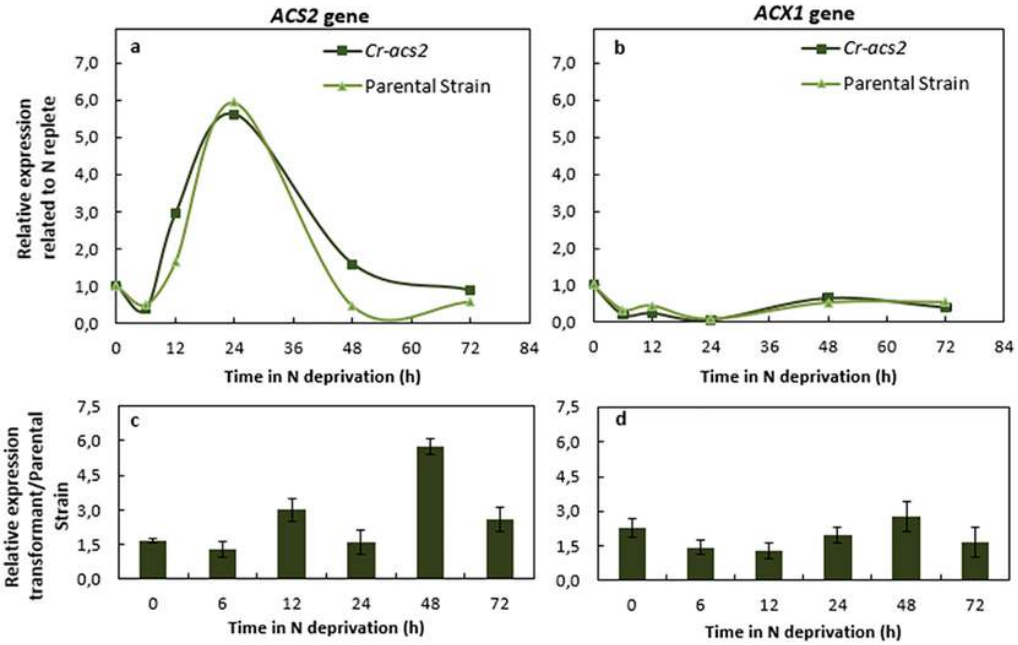

Fig. 7. Transcription levels of *ACS2* (a,c) and *ACX1* (b,d) genes in response to nitrogen deprivation. Samples of control and *acs2* transformant cultures, collected at the indicated time after transference to nitrogen free medium, were processed for mRNA isolation and the expression of the indicated genes analyzed by quantitative RT-PCR. Measurements were normalized to selected endogenous genes and presented as fold-change relative to transcript levels at 0 h (N replete cultures) (a,b) and as the ratio between the expression level in the transformant and in the parental untransformed strain (c,d). Error bars represent 95% confidence intervals from three replicates.

Chloroplastic ACCase is a tetrameric complex, consisting of biotin carboxylase, biotin carboxyl carrier protein, and α- and β- carboxyltransferases, responsible for the formation of malonyl-CoA in the chloroplast. Assembly of the four subunits of ACCase is very well coordinated, as is their regulation [70]. In this work we have chosen the α-carboxyltransferase subunit (*ACX1*) to evaluate the expression level of the Acetyl CoA carboxylase. We have found that the expression level of *ACX1* is not induced by N deprivation (Fig. 7b). Similar results were observed by Ramanan et al. [39] and Schmollinger et al. [71], who found that BCX1, the β-carboxylase subunit of ACCase, was down-regulated under N limitation. While Goodenough et al. [40] found a transient down-regulation followed by an induction after 24 h of N starvation for both the ACX1 and BCX1 subunits.

Curiously, although *ACX1* did not showed higher levels relative to N replete cells (time 0 h); *ACX1* transcript level is around 2-fold higher in the transformant than in the control strain (Fig. 7d). Higher substrate availability for the acetyl-CoA carboxylase in the transformant could be the cause for this induction. Although the influence of acetyl-CoA on ACCase at trancriptional level has not been studied, several previous studies have revealed that sufficient acetyl-CoA levels together with stromal appropriate redox pool are necessary to activate ACCase [36,38].

4. Conclusions

The ACS2 overexpressing *C. reinhardtii* transformant accumulated more starch reserves and had a larger acyl-CoA pool than the corresponding parental line. In nutrient replete non-stressed cultures, the mechanisms that trigger the accumulation of TAG are not active, and the excess acetyl-CoA is mainly stored as starch and accumulated as intracelullar acyl-CoA, both of which are higher in the ACS overexpressing transformant, reaching values 100% (in the case of starch), and 60% (in the case of acyl-CoAs) higher than in the parental untransformed strain.

Upon transfer to N-depleted medium, the biosynthesis of starch and TAG is rapidly induced, however their biosynthetic dynamics are very different. An example of this is that increasing acetate concentration enhances TAG production, but not starch accumulation, neither in the parental nor in the *acs2* transformant strain. TAG content of N-deprived *Cr-acs2* transformant cells is much higher than that of the parental untransformed line grown in the same conditions; after 3 days of nitrogen deprivation with an initial concentration of acetate of 50 mM, the content of neutral lipids in the ACS transformant is 52% higher than in the control grown in the same conditions and up to 16-fold higher than in the N replete control cells. Although the final oil content in microalgae is determined by many factors, we can conclude that the contribution of plastidic ACS is very important for essential the accumulation of storage lipids.

On the other hand, although the basal starch content is two-fold higher in the *Cr-acs2* than in the transformant, the starch accumulated after 72 h of N deprivation is practically the same in the transformant and the parental strains, demonstrating that the origin of these two C storages is clearly different and showing that in N deprived medium the acetate metabolized via acetyl-CoA synthetase is prioritized towards the biosynthesis of TAG. This is in agreement with recently published data, provided by carbon 13C isotope labeling, that showed that in mixotrophic cultures, starch is predominantly obtained from photosynthetically assimilated $CO_{□2}$, while fatty acid synthesis is mainly supplied by exogenous acetate [33] and with the observations of Fan et al. [65], that suggest that carbon availability is a key factor controlling carbon portioning between starch and oil in *C. reinhardtii*.

Overexpression of ACS2 results in higher *ACS2* transcript levels, enhanced TAG production and a major bias to urated fatty acids upon nitrogen starvation, but not in higher decrease of polar lipid species, indicating that the higher increment in the TAG content of the transformant is not achieved at expense of other polar plastidial lipids, but due to an increase in the carbon flux towards the synthesis of acetyl-CoA in the chloroplast.

Overexpression of *Cr-ACS2* by genetic engineering increases the availability of acetyl-CoA, such that under nitrogen limitation it is mainly used for the synthesis of TAG, thus this is a good strategy to increase the accumulation of lipids, particularly TAG, in *C. reinhardtii*, that could be successfully applied to other microalgal systems. Simultaneous overexpression of ACS with other enzymes catalyzing downstream steps of the pathway or the synthesis of specific high value fatty acids could provide higher yields of tailor-made lipids that better conform to the requirements of certain energetic or nutritional applications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.02.009.

Conflicts of interest and statement of informed consent, human/animal rights

Authors declare that they have no conflicts of interest. No conflicts, informed consent, human or animal rights and no financial or other interest that could influence the outcomes of this research applicable.

Author contributions

All authors have contributed to this research and agree to authorship and submit this manuscript for its revision and publication. R.L and O. S. designed research and wrote the paper. R. R. and M.V. obtained plasmids and algal transformants. R.R., R.S., and R.L. performed screening and analysis of transformants. R.H. performed mass spectrometry analysis. R.R did statistical treatment of the data. All authors contributed to analysis of results and corrected the written paper.

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In order to isolate the ACS2 (EDO98345) encoding gene, total RNA was obtained by the RNAeasy plant minikit from Qiagen according to manufacturer's instructions and single strand cDNA was synthesized from total RNA according to the Superscript II RNaseH-reverse transcriptase manual (Invitrogen). *ACS2* was amplified by PCR, using 1 μL of the cDNA as template, and the Phusion High-Fidelity DNA Polymerase (Thermofisher). Specific forward and reverse primers were modified to contain the *XhoI* and *EcoRI* restriction sites. The resulting PCR product was ligated into the pSpark vector (Canvax, Spain) and further subcloned into the *XhoI-EcoRI* sites of polylinker region of the microalgal expression vector Phyco105 to generate the Phyco105-CrACS2 plasmid (Fig. 1 suppl mat).

qPCR experiments were performed on a Mx3000P Multiplex Quantitative PCR System (Stratagene) using 1 μL of the cDNA, synthesized from total RNA according to the SuperScript II RNaseH-reverse transcriptase manual (Invitrogen), as template and Brilliant SYBR® Green QPCR Master Mix (Agilent Technologies, La Jolla, CA, USA). Cycling conditions were: 10 min at 95 °C for activation of the Hot start Taq polymerase and 40 cycles for the melting (30 s at 95 °C), annealing (30 s at 62 °C) and extension (30 s at 72 °C). Each qPCR measurement was carried out in triplicate using primers for either the chloroplastic acetyl-CoA synthetase (*ACS2*), or the alpha-carboxyltransferase subunit (*ACX1*) of chloroplastic acetyl-CoA carboxylase encoding genes (Table 1, suppl material). The *UBC8* gene, encoding an ubiquitin ligase polypeptide (XM_001697453), which expression was previously shown to be constitutive under the different conditions used [60,61] was used as housekeeping gene to normalize mRNA abundance. $2^{-\Delta\Delta CT}$ approach was used to calculate fold change [62].

In order to perform a preliminary screening, untransformed control and *Cr-acs2* transformants were transferred to nitrogen-depleted TAP media at the same initial cell density to induce the accumulation of neutral lipids, which content was determined by staining with Nile Red dye and measuring fluorescence emission as detailed in Methods section 2.7. The cell density did not increased significantly over the three days in which the cultures were maintained without nitrogen (Fig. 2-suppl mat). Transformant *Cr-acs2-4* consistently accumulated more than twice the neutral lipid content of the parental strain after 72 h of nitrogen starvation (Fig. 1c). This clone was therefore selected for further studies.