Bioresource Technology 245 (2017) 1616–1626

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Gene silencing of stearoyl-ACP desaturase enhances the stearic acid content in *Chlamydomonas reinhardtii*

L. de Jaeger [a,b], J. Springer [b], E.J.H. Wolbert [b], D.E. Martens [a], G. Eggink [a,b], R.H. Wijffels [a,c,*]

[a] Bioprocess Engineering and AlgaePARC, Wageningen University and Research Centre, PO Box 16, 6700 AA Wageningen, The Netherlands
[b] Food and Biobased Research and AlgaePARC, Wageningen University and Research Centre, PO Box 17, 6700 AA Wageningen, The Netherlands
[c] Faculty of Biosciences and Aquaculture, University of Nordland, N-8049 Bodø, Norway

HIGHLIGHTS

- Stearoyl-ACP desaturase is silenced by artificial microRNA in the green microalga *Chlamydomonas reinhardtii*.
- Increased stearic acid content in triacylglycerol molecules in mutants of *Chlamydomonas reinhardtii*.
- Temperature impacts the accumulation rate of stearic acid.

ARTICLE INFO

Article history:
Received 30 March 2017
Received in revised form 21 June 2017
Accepted 22 June 2017
Available online 30 June 2017

Keywords:
Chlamydomonas reinhardtii
RNA silencing
Enhanced stearic acid

ABSTRACT

In this study, stearoyl-ACP desaturase (SAD), the enzyme that converts stearic acid into oleic acid, is silenced by artificial microRNA in the green microalga *Chlamydomonas reinhardtii*. Two different constructs, which target different positions on the mRNA of stearoyl-ACP desaturase, were tested. The mRNA levels for SAD were reduced after the silencing construct was induced. In one of the strains, the reduction in SAD mRNA resulted in a doubling of the stearic acid content in triacylglycerol molecules, which shows that stearic acid production in microalgae is possible.

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1. Introduction

The current increase in oil consumption, combined with the predicted population growth for the coming decades, requires an alternative, sustainable and renewable source of oils. Most renewable oil resources are based on extraction of oil from plant species such as palm oil, rapeseed, corn and others (Carlsson, 2009). Increased use of these resources will have a great impact on the available arable land, fresh water, and delicate nature. An alternative oil production crop are microalgae, which have a very high areal productivity compared to land plants (Chisti, 2007).

Currently, the most used feedstock for vegetable oil is derived from palm, in which palmitic acid accounts for 45% of the total oil in the seeds (Table 1). Although palmitic acid rich oils are suitable for high temperature applications (Guinda et al., 2003), it is known that saturated fatty acids, can raise the health risk by increasing low-density lipoprotein cholesterol (Cox et al., 1995). Furthermore saturated fatty acids are regarded as unhealthy as high intake is associated to cardio-vascular diseases (Mensink et al., 2003). Structural properties of oils are a result of the higher melting point of these saturated fatty acids compared to the lower melting point of mono- and poly-unsaturated fatty acids present in triacylglycerol. Oils rich in saturated fatty acids are semi-solid at room temperature and can therefore be directly used in products that require structural properties (Fernandez-Moya et al., 2002).

Oils that are low in palmitic acid and rich in stearic acid and oleic acid can provide the structural properties and have less negative health effects compared to oils rich in palmitic acid. Therefore the presence of stearic acid rich oils in the diet is preferred over palmitic acid rich oils (Mensink et al., 2003).

An effective chemical method to produce saturated fatty acids is hydrogenation of mono- and poly-unsaturated fatty acids by removing the double bonds. Disadvantage of this method is, that partial hydrogenation will lead to trans fatty acids, which are known to have potentially negative health effects and can raise cholesterol levels and therefore this process is not desirable (Ascherio and Willett, 1997).

* Corresponding author at: Bioprocess Engineering and AlgaePARC, Wageningen University and Research Centre, PO Box 16, 6700 AA Wageningen, The Netherlands.
E-mail address: rene.wijffels@wur.nl (R.H. Wijffels).

http://dx.doi.org/10.1016/j.biortech.2017.06.128
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Table 1
Fatty acid composition of several oil rich plants and microalgae.

Species	Common name	Fatty acid (CDW%)							Reference
		C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	Other	
Higher plants[a]									
Elaeis guineensis	Palm	45	5	39	11	0	0	1	Nzikou et al. (2010)
Butyrospermum parkii	Shea	4	42	46	7	0	1	0	Maranz et al. (2004)
Theobroma cacao	Cocoa	25	35	35	3	0	0	2	Ramadan et al. (2006)
Allanblackia floribunda	Allanblackia	1	56	43	0	0	0	0	Foma and Abdala (1985)
Gossypium hirsutum	Cotton	26	2	13	59	0	0	0	Liu et al. (2002)
Glycine max	Soybean	16	3	13	58	10	0	0	Singh et al. (2010)
Microalgae[b]									
Chlamydomonas reinhardtii		29	4	12	12	28	2	15	James et al. (2011)
C. reinhardtii BAFJ5		30	3	24	16	16	1	10	James et al. (2011)
Chlorella vulgaris		17	2	47	10	14	0	10	Breuer et al. (2012)
Chlorella zofingiensis		15	3	47	17	8	0	10	Breuer et al. (2012)
Dunaliella tertiolecta		25	2	14	9	31	0	19	Breuer et al. (2012)
Neochloris oleoabundans		23	3	41	21	3	0	9	Breuer et al. (2012)
Porphyridium cruentum		28	4	5	30	0	0	33	Breuer et al. (2012)
Phaeodactylum tricornutum		27	1	3	1	0	0	68	Breuer et al. (2012)
Scenedesmus obliquus		14	5	50	7	8	0	16	Breuer et al. (2012)

[a] The values given for higher plants are fatty acid compositions of the oil rich seeds.
[b] These values are representing fatty acid profiles for nitrogen starved cells to induce lipid accumulation.

There are only few crops known that naturally produce large amounts of stearic acid, for example Shea and Allanblackia (Table 1). But oils derived from those sources are expensive compared to the palmitic rich oils derived from palm oil and hydrogenated oils. There are various examples of organisms that have an enhanced stearic acid content derived via selective breeding, mutagenesis and targeted genetic engineering. Using hairpin mediated RNA silencing Liu et al. generated cottonseeds with elevated stearic acid contents up to almost 40% of total fatty acids (Liu et al., 2002). Other crops with enhanced stearic acid contents are soybean (chemical mutagenesis) (Graef et al., 1985), canola (antisense) (Knutzon et al., 1992), *Arabidopsis thaliana* (chemical mutagenesis) (Lightner et al., 1994), and also the yeast *Apiotrichum curvatum* (chemical mutagenesis) (Ykema et al., 1989).

Microalgae have an enormous potential for producing stearic acid, because the TAG molecules, which can be accumulated under unfavorable growth conditions, contain oleic acid as the predominant fatty acid (Table 1). The green microalgae *Chlamydomonas reinhardtii* has been used for many years as a model organism in research, which makes it the most extensively studied green microalgae available. *C. reinhardtii* has a very well-studied physiology, a sequenced and annotated genome, and there are many tools available that enable genetic and metabolic engineering (Merchant et al., 2007; Molnar et al., 2009; Day and Goldschmidt-Clermont, 2011).

In this study *C. reinhardtii* was used to show the possibility of increasing the stearic acid content in algae by means of genetic engineering. Wild type *C. reinhardtii* does not accumulate large amounts of TAG under unfavorable growth conditions and therefore the starchless mutant BAFJ5 was selected. The BAFJ5 starchless mutant is derived from *C. reinhardtii* and has a disrupted small subunit of ADP-glucose pyrophosphorylase (Zabawinski et al., 2001), and is unable to synthesize starch and therefore accumulates more TAG than the wild type strain. Oleic acid rich TAG molecules are accumulated under nitrogen depleted in the BAFJ5 starchless mutant (James et al., 2011) (Table 1). The soluble stearoyl-ACP desaturase (SAD) enzyme introduces a cis-double bond in stearic acid, resulting in the mono-unsaturated fatty acid oleic acid. Reducing the activity of this enzyme could potentially result in an enrichment of stearic acid in the TAG molecules. The effect of reducing the expression of the (SAD) gene using artificial microRNA (amiRNA) was studied. RNA interference (RNAi) is a tool to knockdown gene expression at the post-transcriptional level and in that way enables the study of gene function and the reprograming of specific metabolic processes.

2. Material and methods

2.1. Strains, growth conditions

The starchless mutant BAFJ5 (cw15 sta6), derived from *Chlamydomonas reinhardtii* 330 (mt1 arg7-7 cw15 nit1 nit2), which has a disrupted small subunit of ADP-glucose pyrophosphorylase (Zabawinski et al., 2001) was obtained from the Chlamydomonas Resource Center (University of Minnesota). All strains were grown on Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965) with 15 g/L agar for plates.

2.2. Construction of transformation vector

For the knockdown of the plastid stearoyl-ACP desaturase (also known as the FAB2 gene, XM_001691545) gene, artificial microRNA (amiRNA) sequences were designed using the web tool Web - Micro RNA designer 3 (http://wmd3.weigelworld.org). Two different targets on the SAD gene were chosen and specific amiRNA constructs were developed. One sequence was designed to target the end of the eighth exon of the SAD mRNA, and the other target was the second exon of the SAD mRNA. The 90 base pair long oligonucleotides that were used to make the silencing construct can be found in Table 2. Both double stranded annealed oligo's were cloned into the pChlamiRNAi3int vector (Chlamydomonas Resource Center at University of Minnesota) according to Molnar et al. (2009) to generate pΔ9DSamiRNA_I and pΔ9DSamiRNA_II respectively. An empty pChlamiRNAi3int plasmid was used as a negative control during the whole experiment.

2.3. Transformation

The BAFJ5 starchless mutant was grown to mid log phase (around 5×10^6 cells ml^{-1}), concentrated to 3×10^8 cells ml^{-1} by centrifugation and resuspended in TAP medium enriched with 40mM sucrose. From this cell suspension, 250 μl was mixed with 1 μg linearized plasmid, digested with *Xmn*I, and transferred to a 4 mm electroporation cuvette. Cuvettes were incubated at room temperature for 10 min, before electroporation using the Gene-

Table 2
Primers used in this study. amiRNA; artificial microRNA construct I and II. SAD; stearoyl-ACP desaturase. CBLP; chlamydomonas beta subunit-like polypeptide, reference gene. β–tub; β-tubulin, reference gene.

Fwd amiRNA I	ctagtCTGCATTATGTAGCCAATTAAtctcgctgatcggcaccatgggggtggtggtgatcagcgctaTTAAATGGCTACATAATGCAGg			
Rvs amiRNA I	ctagcCTGCATTATGTAGCCATTTAAtagcgctgatcaccaccacccccatggtgccgatcagcgagaTTAATTGGCTACATAATGCAGa			
Fwd amiRNA II	ctagtTCGGAGTATGTTCCTTGCGTAtctcgctgatcggcaccatgggggtggtggtgatcagcgctaTACGTGAGGAACATACTCCGAg			
Rvs amiRNA II	ctagcTCGGAGTATGTTCCTCACGTAtagcgctgatcaccaccacccccatggtgccgatcagcgagaTACGCAAGGAACATACTCCGAa			
Name	Fwd 5′-3′	Rvs 5′-3′	Accession	Source
SAD	CCTCGTGTGCTGAATGTTGT	CTGCACCCTGTGACTTCTGA	XM_001691545.1	This study
CBLP	CTTCTCGCCCATGACCAC	CCCACCAGGTTGTTCTTCAG	ACB05909.1	Moseley et al. (2006)
β-tub	GCCTCTTCCTCTTCGTTTCAGT	GAGACGACCTCCCAGAACTTG	XM_001693945.1	This study
$Paro^R$	CCCTCAGAAGAACTCGTCCAACAGC	GAGGATCTGGACGAGGAGCGGAA		This study
Insert	GGTGTTGGGTCGGTGTTTTTG	TAGCGCTGATCACCACCACCC		This study

Pulser (Bio-Rad) with the following settings: 600 V cm^{-1}, 25 μF and infinite resistance. Cells were allowed to recover for 15 min at room temperature, diluted to a total volume of 10 ml in TAP-sucrose (40mM) and allowed to recover in low light for 24 h at 25 °C. After recovery the cells were concentrated and plated on TAP selection plates, containing 10 μg ml^{-1} paromomycin (Duchefa, Haarlem, The Netherlands) and 100 μM oleic acid. Plates were incubated at 25 °C for 9 days in continuous light (50 μmol m^{-2} s^{-1}). Transformants were assessed for their oleic acid dependence by replica-plating the colonies on TAP agar plates with and without 100 μM oleic acid. All plates contained 1 g l^{-1} Tween 80 to disperse the oleic acid in the medium.

2.4. Experimental set up

Strains were cultivated in 100 ml TAP medium in 300 ml Erlenmeyer flasks in continuous light (100 μmol s^{-1} m^{-2}) at either 25 °C or 35 °C and shaken at 125 rpm. Each strain was grown in duplicate and inoculated 48 h before medium replacement at an OD_{750} of approximately 0.5. After 48 h the cells were harvested by centrifugation and washed twice with nitrogen depleted TAP medium and resuspended in 100 ml of nitrogen free TAP medium to induce the TAG accumulation. Immediately after the medium replacement (T = 0 h), the cells were heat shocked for 60 min at 40 °C to induce transcription of the amiRNA construct. Samples were taken at −24 h, 8 h, 24 h, 48, and 72 h to measure the dry weight content, total fatty acid and triacylglycerol content, and to extract RNA for real-time quantitative PCR.

2.5. Determination of dry weight concentration

Dry weight concentrations were determined at −24 h, 8 h, 24 h, 48 h, and 72 h on biological replicates. Around 1.5 mg of biomass was filtered through Whatman glass fiber filter paper (GF/F; Whatman International Ltd, Maidstone, UK) (de Jaeger et al., 2014).

2.6. Total fatty acid and triacylglycerol analysis

Total fatty acid (TFA) and triacylglycerol (TAG) extraction and quantification were performed as described by Breuer et al. (2013) with the following adjustments. Around 10 mg of biomass was transferred to bead beating tubes (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) and lyophilized and disrupted by bead beating in the presence of a chloroform:methanol mixture (1:1.25) to extract the total lipids from the biomass. Two internal standards were added to the chloroform:methanol mixture to enable quantification of both the polar lipids and TAG content. 1,2-Dilauroyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt (Avanti polar lipids) and tripentadecanoin (Sigma-Aldrich, St Louis, MO, USA) were used as internal standards for polar lipid and TAG quantification respectively. The chloroform-methanol mixture was evaporated under N_2 gas and the total lipids pellet was dissolved in hexane and separated based on polarity using a Sep-Pak Vac silica cartridge (6 cc, 1000 mg; Waters, Milford, MA, USA). The neutral TAG fraction was eluted with 10 ml of hexane:diethyl ether (7:1 v/v) and the polar lipid fraction containing the glycolipids and phospholipids (PL) was eluded with methanol: acetone:hexane (2:2:1 v/v/v). Methylation of the TAG and polar lipid fraction to fatty acid methyl esters (FAMEs) and the quantification of the FAMEs were performed as described by Breuer et al. (2013) TFA concentration was calculated as the sum of the polar and neutral lipid fraction.

2.7. DNA extraction

Around 2 g (wet pellet) was ground for 5 min in liquid nitrogen using a mortar and pestle to disrupt the cells. DNA was isolated using hot phenol-chloroform extraction followed by isopropanol sodium acetate precipitation. The unwanted RNA that was extracted simultaneously was removed using 10 mg μl^{-1} RNase-A treatment for 60 min at 37 °C. The quality of the extracted DNA was checked by agarose gel electrophoresis and quantified using a NanoDrop 1000 spectrophotometer (NanoDrop).

2.8. Southern blot

Genomic DNA (2.5 μg) was digested with *Bam*HI, separated on gel and transferred to positively charged nylon membrane by Southern blotting. Digoxigenin (DIG) labeled probe was generated by PCR using the PCR DIG labeling mix (Roche Diagnostics, Almere) (Table 2). Membranes were sealed in hybridization tubes (Techne) with DIG easy hyb solution (Roche). The pre-hybridization (2 h) and hybridization (o/n) with DIG labeled probe (20 ng/ml) was performed at 42 °C. After the overnight incubation, the membrane was washed twice (15 min) in low stringency buffer (2× SSC/0.1% SDS) and high stringency buffer (15 min) (0.2× SSC/0.1% SDS). The probe-target hybrids were detected with an enzyme-linked immunoassay according to the DIG application manual from Roche.

2.9. RNA extraction

Samples for RNA extraction (2 ml) were immediately processed after sampling by 3 min centrifugation at 12.000*g* at 0 °C and snap frozen in liquid nitrogen before storage at −80 °C until further processing. The RNA was extracted using the RNeasy Plant mini kit (Qiagen) according to the manufacturer's recommendations, with the exception, that the initial cell lyses was by bead beating the cells for 10 s at 5500 rpm (Lysing Matrix E; MP Biomedicals, Santa Ana, CA, USA) with a Precellys 24 homogenizer (Bertin Technologies, Orléans, France). After the first wash step, on column DNA digestion was performed using RNase-Free DNase kit (Qiagen). RNA was eluted in 40 μl RNAse free water and quantified and qual-

ity controlled using the NanoDrop 1000 spectrophotometer (NanoDrop) and gel electrophoresis.

2.10. Complementary DNA synthesis and real-time quantitative PCR

Single-stranded complementary DNA (cDNA) was obtained from 1 μg of total RNA using the reverse transcriptase system (Promega) following the suppliers protocol. The knockdown of the SAD gene was studied by real-time quantitative PCR (qPCR) using SAD specific primers (Table 2). cDNA was PCR amplified with Platinum Taq DNA polymerase (Invitrogen). The cDNA was analyzed by qPCR using SYBR green dye and a MyIQ thermal cycler (Bio-Rad) using the following program: 8 min at 94 °C, followed by 45 cycles of 94 °C for 15 s and 60 °C for 1 min. Each qPCR assay was executed in duplicate using RNA isolated from independent cultures and values were normalized using *C. reinhardtii* housekeeping genes *Chlamydomonas* beta subunit-like polypeptide (CBLP) and β-tubulin (βtub). For all samples, the starting quantity of the target gene expression was determined and compared to the geometric average of CBLP and βtub gene expression.

3. Results and discussion

In this study we generated stearoyl-ACP desaturase (SAD) knockdown strains from the TAG accumulating *C. reinhardtii* starchless mutant BAFJ5 using artificial microRNA post translational gene silencing (Molnar et al., 2009). SAD, also known as delta-9 fatty acid desaturase (EC:1.14.19.2), functions by catalyzing the insertion of a double bond at the delta 9 position of stearoyl-ACP (C18:0) resulting in the monounsaturated fatty acid oleoyl-ACP (C18:1). Two different constructs were used to reduce the SAD activity and study the effect on fatty acid synthesis in *C. reinhardtii*. The first construct (named: DSI) targeted the last exon of the SAD mRNA and a second construct (named: DSII) targeted the mRNA more upstream in the region that is encoded from the second exon. A third construct was used which did not contain an amiRNA target and was used as a control vector (named: Empty). Three transformants were selected for each amiRNA construct, containing either the DSI or DSII silencing cassettes, from 40 and 35 screened transformants respectively. Two transformants were selected containing the empty vector out of 35 transformants screened. Transformants were selected based on the demonstration of the presence of the construct by PCR. All eight transformants (empty 10, empty 24, DSI7, DSI8, DSI11, DSII1, DSII2, and DSII8) plus the wild type strain were checked for the presence of the construct. Successful integration of the construct was confirmed by Southern blotting for all the strains with the exception of strains empty 10 and DSI7. All transformants tested showed a positive result for the paromomycin resistance gene in the PCR check. For the presence of the amiRNA construct all transformants were PCR positive as well, with the exception of DSI7. An explanation could be that the silencing construct was only partially integrated in the *C. reinhardtii* genome of DSI7. The Southern blot shows that most transgenic strains contain multiple copies of the silencing construct, depicted by multiple fragments on the Southern blot.

The transgenic lines plus the wild type strain were cultivated in shake flasks to assess the differences in growth rate and to study the effect of silencing the SAD gene on the fatty acid composition of the different strains. In Fig. 1 the growth curves of all strains are shown, cultivated at 25 °C and 35 °C. All transgenic strains show a similar growth rate under nitrogen replete and depleted conditions compared to the wild type strain.

The temperature of the culture does seem to influence the growth rate of all *C. reinhardtii* strains. The slope of the growth curves was steeper for the strains cultivated at 35 °C under both nitrogen replete and depleted conditions (Fig. 1). The growth of cultures kept at 35 °C levelled off towards the end of the experiment and after 5 days the cultures died (data not shown), which has been observed before (James et al., 2013). The growth of cultures maintained at 25 °C leveled off after five days and no decline was observed (data not shown).

Samples were taken during the cultivation experiment to analyze the fatty acid composition of the triacylglycerol (TAG) and polar lipid (PL, phospholipids and other membrane lipids) for the different transgenic lines. In Fig. 2 the lipid content of all the tested strains is shown. Under nitrogen replete conditions TAGs could not be detected. The polar lipid fraction was always around 10% of the cell dry weight (CDW) (TFA-TAG fraction in Fig. 2). Under nitrogen depleted conditions, there seems to be a quicker accumulation of TAG molecules at higher temperatures in the initial phase (8 h) of nitrogen starvation, but the final TAG content after 72 h of nitrogen starvation was similar for the strains cultured at both temperatures. The total amount of TAG accumulated in the transgenic lines compared to the wild type was similar. All strains accumulated around 36% of their CDW as TAG molecules and around 10% of their CDW was consisting of polar lipids throughout the experiment. The transgenic lines containing the DSII silencing construct have a slightly reduced TAG content, when cultured at 25 °C, during the first 2 days of the nitrogen depletion phase compared to the wild type.

Examination of the fatty acid profiles of the wild type and all transgenic strains, cultivated under the different temperatures conditions, demonstrates an effect of silencing the SAD gene. In Table 3 the fatty acid profiles are shown for the first time point after nitrogen depletion showing the contribution of each measured fatty acid to the total fatty acid content. The variation in the PL between the transgenic lines and the wild type is very small. The major differences can be found in the TAG fatty acid profiles. The differences between both temperatures are most pronounced in the total saturated fatty acid content. At 25 °C the TAG molecules in the wild type and empty vector transgenic lines contain slightly more (C14:0), and less palmitic acid (C16:0) compared to 35 °C cultivated wild type strains. The stearic acid (C18:0) content is higher at elevated temperatures for wild type and empty vector containing transgenic strains (Fig. 3 and Table 3), as described for *C. reinhardtii* previously (James et al., 2013). The oleic acid content is reduced under the higher temperatures.

The transgenic lines DSI show similar fatty acid profiles to the wild type and there is no effect on the stearic acid content, in contrast to the stearic acid content in the DSII transgenic strains, all three of which showed elevated stearic acid content relative to the wild type. This was particularly evident for DSII8 in which the stearic acid content in the TAG molecules at 25 °C was 7.1% compared to 3.6% in the wild type. At 35 °C an overall increase in stearic acid content within all strains was found. The wild type accumulated 4.6% stearic acid in the TAG molecules, and the DSII1 and DSII2 strains showed an increase of stearic acid content in TAG molecules up to 5.9% and 6.5% respectively (Fig. 3). Surprisingly, the DSII8 strain had a lower stearic acid content when grown at 35 °C. An explanation for this phenomenon could be that as the silencing construct is randomly integrated into the *C. reinhardtii* genome, this could lead to differences in expression of the construct. Possibly the construct in DSII8 strain was inserted in a region that is more highly expressed at lower temperatures, since DSII8 probably only has one integration site which would make it more susceptible to genomic position effects.

The DSI silencing construct does not seem to be effective in post transcriptional gene silencing of the SAD gene, since the stearic acid content in the lipids did not change compared to the wild type strain. This could be explained by the fact that the silencing con-

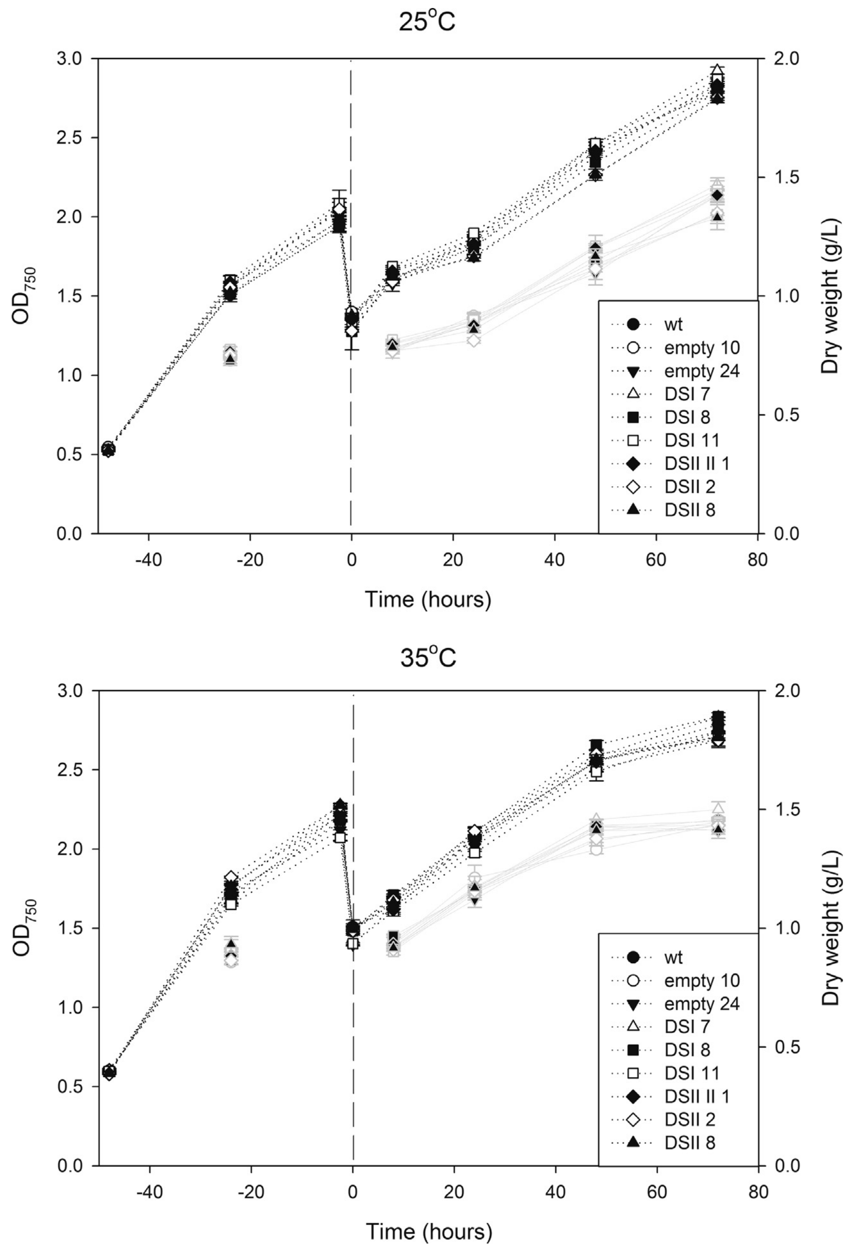

Fig. 1. Comparison of growth rate between transgenic lines and wild type. The effect of temperature and nitrogen depletion is shown. The dotted line indicates the moment of medium replacement with nitrogen depleted TAP medium followed by a heat shock. Black dotted lines represent the OD_{750} and the grey dotted line represent the cell dry weight. Error bars indicate the distance to the mean between two biological replicates.

struct DSI is targeting the final exon of the SAD gene and that the successful reduction of this part of the gene translation is not influencing the overall efficiency of the SAD gene.

The stearic acid content is higher in all transgenic strains containing the DSII construct (Fig. 4). The biggest relative increase in stearic acid is found in strain DSII8 at 25 °C, which doubled the stearic acid content in TAG compared to the wild type. Also at 35 °C all DSII containing constructs show an increased stearic acid content compared to the wild type, but strains DSII1 and DSII2 show the biggest fold change 1.2 and 1.4 times respectively. The fold change of stearic acid at 35 °C is not as high as at 25 °C and this can be explained by the fact that the stearic acid content in the wild type strain is already elevated by the higher temperature and that the absolute amount of stearic acid does not exceed the values that are found for the DSII8 strain at 25 °C. It is known that at lower temperatures plants and microalgae upregulate the expression of the SAD gene to produce more unsaturated fatty acids to maintain membrane fluidity at lower temperatures (Falcone et al., 2004; An et al., 2013).

To assess the level of silencing, the SAD mRNA was quantified using real-time qPCR and compared to that of selected housekeeping genes. It can be seen that the expression of the SAD gene in all strains is similar before the heat shock and the SAD mRNA levels are reduced in DSII containing strains after applying a heat shock, especially at 25 °C (Fig. 5). This shows that the silencing construct indeed is inducible by a heat shock. The SAD expression seems to be transiently enhanced when the culture experience nitrogen depleted conditions. The increase in SAD expression upon nitrogen depletion, results in the formation of the oleic acid, which is one of the major constituents of TAG in many green microalgae. The reduction in activity of this important enzyme for oleic acid production and TAG accumulation could explain the decrease in the

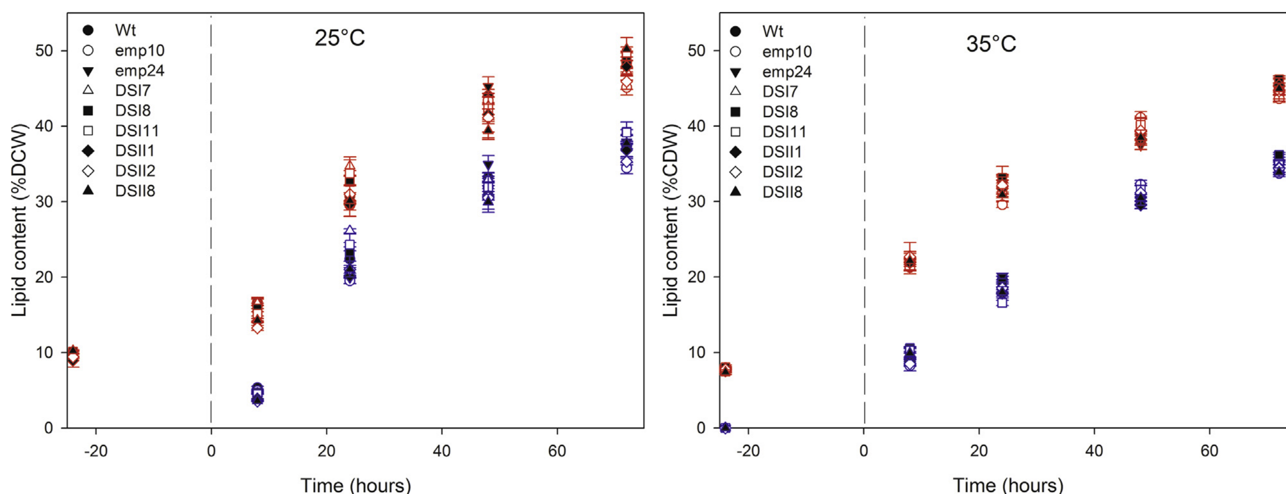

Fig. 2. Fatty acid content, red symbols represent the total fatty acid content and the blue symbols represent the triacylglycerol content for all tested strains. Dotted line is the time point at which the nitrogen depletion started and the heat shock applied. Error bars indicate the distance to the mean between two biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Fatty acid profiles for T1, 8 h after nitrogen depletion as a percentage of the respective lipid types.

	TAG 25 °C									PL 25 °C								
	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8
C14:0	0.4*	0.3	0.6	0.4	0.5*	0.3**	0.4**	0.3**	0.5**	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
C16:0	30.7	30.5	30.4	30.6	30.0	30.6	28.0	28.6	30.3	24.7	24.6	24.5	24.5	24.6	24.3	24.4	23.8	24.2
C16:1	6.2	6.4	6.0	6.2	5.8	5.8	6.0	5.7	5.7	6.7	6.9	6.7	6.8	6.5	6.6	6.6	5.9	6.5
C16:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.9	0.9	0.9	1.0	1.0	1.3	1.5	1.1
C16:3	1.1	1.0**	1.3	1.6**	2.5*	1.4**	1.3**	1.8**	1.6*	3.0	3.0	3.0	3.0	3.1	3.2	3.2	3.5	3.3
C16:4	2.5	2.7	2.7	2.5	2.7	2.6	3.3**	3.0	3.0	13.9	13.6	13.9	13.7	13.7	13.6	13.0	13.9	14.0
C18:0	3.6	3.6	3.6	4.0	3.6	4.2	4.7	4.7	7.1	1.8	1.8	1.8	1.8	1.8	1.9*	2.2	2.0	2.7
C18:1	39.9	39.1	39.0	38.6	38.0	38.8	36.7	36.3	34.8	22.3	22.3	21.9	22.1	21.8	21.7	21.3	19.7	20.2
C18:2	8.1	8.9	8.3	8.3	8.5	8.9	10.7	10.7	9.1	7.3	7.9	7.8	8.0	8.1	8.4	9.6	9.6	8.2
C18:3	5.3	5.5	5.8	5.2	5.5	5.4	6.5*	6.2	5.7	16.6	16.3	16.7	16.5	16.6	16.5	15.8	17.0	16.9
C18:4	2.2**	2.1	2.3**	2.5**	3.0*	2.0**	2.5*	2.8	2.1**	1.7	1.6	1.7	1.7	1.8	1.7	1.7	2.1**	1.9
SFA	34.7	34.4	34.6	35.1	34.1	35.1	33.2	33.6	37.9	27.5	27.5	27.3	27.3	27.4	27.3	27.7	26.8	27.9
MUSFA	46.1	45.5	45.1	44.7	43.8	44.7	42.7	42.0	40.6	29.1	29.2	28.6	28.8	28.3	28.3	27.8	25.7	26.7
PUFA	19.2	20.2	20.3	20.1	22.1	20.2	24.2	24.4	21.6	43.4	43.3	44.1	43.8	44.3	44.4	44.5	47.5	45.4
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
SFA/UFA	53.0	52.4	52.9	54.1	51.7	54.1	49.6	50.5	60.9	37.9	37.9	37.6	37.6	37.7	37.5	38.2	36.6	38.8
	TAG 35 °C									PL 35 °C								
	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8	wt	emp 10	emp 24	I7	I8	I11	II1	II2	II8
C14:0	0.5	0.6	0.5	0.6	0.5	0.6	0.6	0.6	0.6	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
C16:0	26.8	28.0	27.9	27.5	28.0	28.4	26.0	25.3	27.9	24.3	24.1	23.6	23.1	23.1	24.0	24.1	24.4	23.7
C16:1	8.9	8.5	9.0	9.4	9.1	9.0	8.5	8.4	8.9	10.0	9.8	9.9	10.7	10.1	9.7	9.4	9.5	9.9
C16:2	0.6	0.6	0.6	0.4	0.6	0.6	0.8	0.7	0.5	1.3	1.4	1.4	1.1	1.4	1.5	1.7	1.5*	1.3
C16:3	2.2	2.0	2.1	1.9	2.1	2.1	2.2	2.2	2.1	6.0	5.9	6.3	5.9	6.6	6.5	6.1	5.7*	6.5
C16:4	1.5	1.5	1.4	1.4	1.3	1.4	1.5	1.7*	1.3	8.3	8.5	8.7	9.0	9.1	9.2	8.2	8.1*	8.7
C18:0	4.6	4.5	4.6	4.8	4.6	4.8	5.9	6.5	5.3	2.1	1.9	1.9	1.9	1.9	2.0	2.4	2.6*	2.1
C18:1	33.9	33.8	33.8	34.2	33.9	33.6	32.2	31.4	33.3	20.5	19.9	19.6	19.8	19.1	18.1	19.7	20.1*	19.2
C18:2	14.8	14.5	14.5	13.8	14.1	13.5	16.0	17.1	14.0	12.1	12.3	12.3	12.0	12.1	12.3	13.0	12.6	12.0
C18:3	4.8	4.7	4.8	4.8	5.0	5.1	4.8	4.9	4.8	13.8	14.1	14.4	14.4	14.6	14.6	13.5	13.5	14.2
C18:4	1.4**	1.3*	0.9*	1.1*	0.8*	1.0*	1.4**	1.3**	1.2*	0.9*	1.3	1.1*	1.2**	1.2*	1.4**	1.0*	1.3*	1.4
SFA	32.0	33.1	33.0	32.9	33.1	33.8	32.6	32.3	33.7	27.1	26.8	26.3	25.9	25.8	26.7	27.2	27.7	26.6
MUSFA	42.7	42.3	42.7	43.7	42.9	42.6	40.7	39.8	42.2	30.5	29.7	29.5	30.5	29.2	27.8	29.1	29.6	29.1
PUFA	25.3	24.6	24.3	23.4	23.9	23.7	26.7	27.9	24.0	42.5	43.5	44.2	43.6	45.0	45.5	43.7	42.7	44.3
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
SFA/UFA	47.1	49.5	49.3	49.0	49.5	51.0	48.3	47.7	50.9	37.1	36.6	35.6	34.9	34.8	36.4	37.4	38.3	36.2

All samples, unless otherwise indicated have less than 5% variation between the duplicates.
* Samples with 5–10% variation between duplicates.
** Samples with >10% variation between duplicates.

TAG content that is found in *C. reinhardtii* when SAD is silenced and the increase in TAG content when SAD is overexpressed (Hwangbo et al., 2014).

After 24 h, the expression starts to decline and after 72 h the abundance of the SAD mRNA was lower than under nitrogen replete conditions for each strain. At the first two time points after

Fig. 3. Stearic acid content per lipid type for each strain over time. Error bars indicate the distance to the mean of duplicate samples. T-1 is not included in the TAG measurement, since under nitrogen replete conditions TAG contents did not exceed threshold limits. Error bars indicate the distance to the mean between two biological replicates.

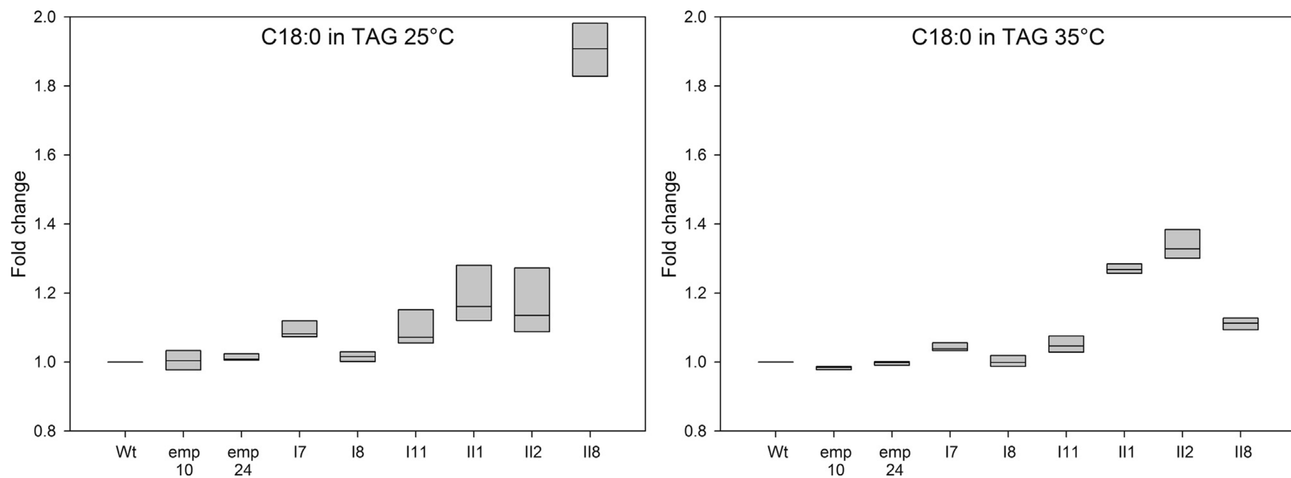

Fig. 4. Stearic acid content and the fold change in TAG relative to wild type at 25 °C and 35 °C. The wild type is shown as 1. The grey boxes indicate the spread of the data points throughout the time series and the black line in the box indicates the mean value for each strain.

the heat shock, the mRNA levels of the SAD gene in the strains that contain the DSII silencing construct and are grown at 25 °C were reduced. This shows that the silencing construct was indeed able to reduce the mRNA levels of the SAD gene, causing an enrichment of stearic acid in the TAG of *C. reinhardtii*. The silencing at 35 °C is less efficient and this can be caused by the temperature effect, being that SAD expression is higher at lower temperatures (James et al., 2013). The DSII containing strains show a relatively higher SAD expression at the end of the experiment and the silencing effect has faded. It could be that the DSII containing transfor-

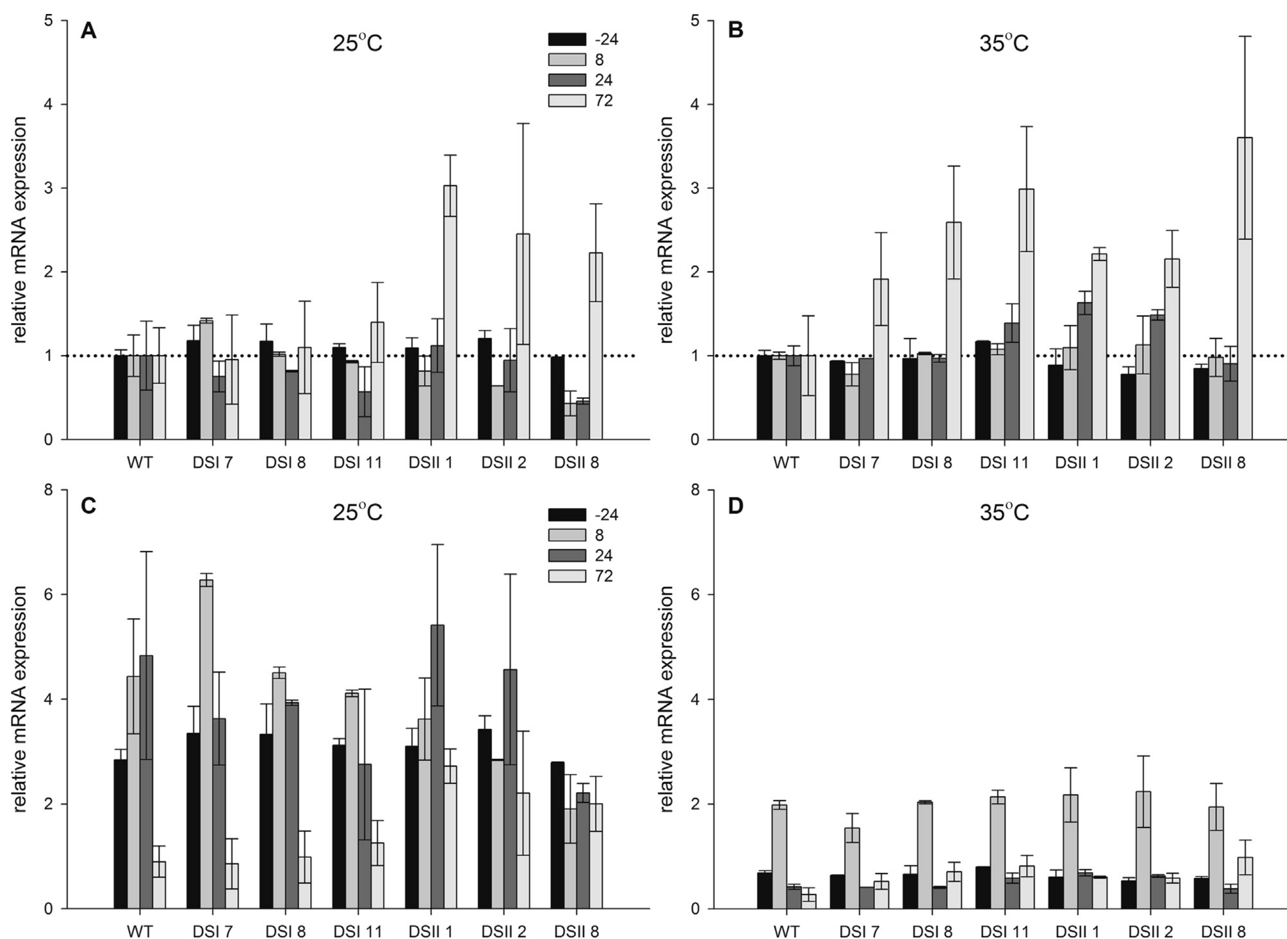

Fig. 5. Relative mRNA expression of SAD gene in the tested strains and temperature conditions. The mRNA expression values are relative to the housekeeping genes β-tubulin and CBLP. The geographic mean was taken from both housekeeping genes and normalized (set as 1) to the wild type (A and B). The relative mRNA expression values (to housekeeping genes) not normalized to the wild type (C and D). Error bars represent the standard deviation between the biological replicates.

mants are overexpressing SAD to compensate for the higher stearic acid content in the cells. The DSI containing constructs do not show a different SAD expression compared to the wild type and this corresponds with the unchanged stearic acid content in the DSI containing strains.

In the beginning of the nitrogen depletion phase and the expression phase of the silencing construct, the stearic acid content is quickly increased in the DSII containing transgenic lines. The percentage of stearic acid in TAG lipids decreases after the first 24 h, indicating that the silencing of the SAD gene is most likely most active during the first hours, which is supported by the SAD expression data (Fig. 5).

This could indicate that in the beginning, when silencing is strongest, C18:0 is integrated into TAG molecules and cannot be converted afterwards when SAD silencing is reduced. The C18:0 content does not increase much subsequently, because SAD is less silenced. In the study of Hwangbo et al. (2014) the SAD gene was overexpressed in wild type *C. reinhardtii* cells and they found an increase of oleic acid content compared to the wild type. They did not observe a decrease in stearic acid content however. This would suggest that the content of stearic acid is tightly regulated and that the increased demand for oleic acid does not result in an increased stearic acid pool. In addition, the stearic acid content was maintained at a constant level for all strains tested under different conditions (Hwangbo et al., 2014).

Under normal growth conditions, the stearoyl-ACP molecule is generally rapidly desaturated by SAD to form oleoyl-ACP inside the chloroplasts resulting in low presence of stearic acid in glycerolipids. The further desaturation or elongation of oleoyl-ACP into polyunsaturated fatty acids (PUFAs) can occur in either the chloroplast using the prokaryotic pathway (Roughan et al., 1980), or the acyl-ACP molecules are exported outside the chloroplast as acyl-CoA molecules and enter the eukaryotic pathway (Slack and Roughan, 1975; Dubacq et al., 1976; Slack et al., 1976; Simpson and Williams, 1979), taking place in the endoplasmic reticulum (Fig. 6). The steps towards PUFAs, are catalyzed by membrane bound enzymes in contrast to the soluble SAD, resulting in membrane glycerolipids or triacylglycerol of 16–20 carbon chain lengths long with various degrees of saturation. Over the past decades many SAD genes have been identified in a broad range of organisms and often these genes encode different isoforms (Byfield et al., 2006; Kachroo et al., 2007). These isoforms can differ from the original SAD in the location where double bonds are introduced or they have alternative substrate length specificity (Cahoon et al., 1997). The increase in the stearic acid content resulting from the transient silencing of the SAD gene is significant, but not as pronounced as has been observed in SAD mutants in plants and other organisms. A reason for this could be the presence of SAD isoforms in *C. reinhardtii*. However, there has been no evidence for other soluble SAD genes in the genome of *C. reinhardtii*. In *Arabidopsis*, expression of none of the six isozymes could recover

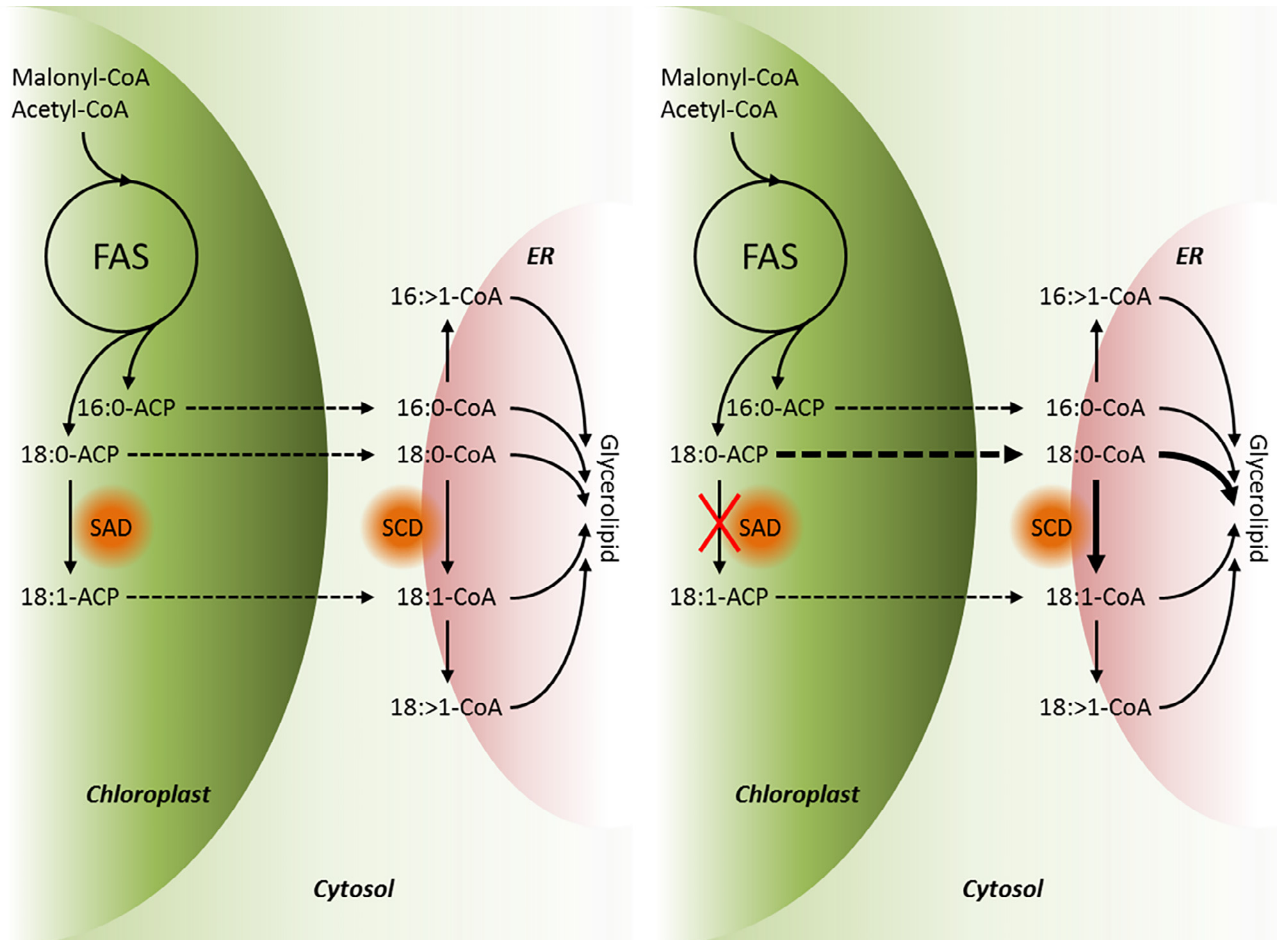

Fig. 6. Schematic representation of the fatty acid synthesis and desaturation in the chloroplast and endoplasmic reticulum. FAS: Fatty Acid Synthesis. SAD: Stearoyl-ACP Desaturase. SCD: Stearoyl-CoA Desaturase. ER: Endoplasmic Reticulum.

the SAD deficient mutant phenotype (Kachroo et al., 2007), indicating that these isoforms have a different function in this plant and supporting the hypothesis that there is just a single enzyme that has the SAD properties.

Plants are able to accumulate much more stearic acid than microalgae (Table 1). Note that these plants are all from areas with high temperatures, which enables the accumulation of higher saturated fatty acid contents. An explanation could be the different biological function of lipid storage in plant seeds compared to TAG in microalgae. The function of TAG in oleaginous microalgae is likely to be very different from the TAG that is accumulated and stored in plant seeds, and more research is needed to understand the role and mechanisms of TAG. A reason could be that the compartmentalization of microalgae is causing the difference in stearic acid over-accumulation. Potentially the stearoyl-ACP is transported out of the chloroplast and the fatty acid is desaturated in the ER by another enzyme that is not affected by the silencing (Fig. 6). Another bottleneck could be that the enzyme that liberates the acyl-ACP chain from the fatty acid synthesis cycle is poorly expressed or has a low affinity for stearoyl-ACP. Similarly the enzymes that incorporate the acyl chain in the glycerolipids might have low affinity for stearoyl chains compared to other fatty acids such as oleoyl. There are unicellular organisms found that can accumulate high amounts of intracellular stearic acid in (Ykema et al., 1989; Ratledge and Cohen, 2010), which is proof that stearic acid rich TAG molecules in microorganisms are feasible.

This study demonstrates a proof of concept to produce TAG enriched in stearic acid in green microalgae. However the yield of stearic acid present in TAG molecules in these transgenic lineages of *C. reinhardtii* is not yet high enough to make stearic acid production in microalgae economically feasible. How much the stearic acid content should be enriched to make production economically feasible is not exactly known today, but we roughly estimate that at least a further 2 to 3-fold increase of stearic acid in TAG is needed to make microalga interesting to be used as alternative to current oil producing plant species (Hinrichsen, 2016). To increase the stearic acid content of the TAG molecules in *C. reinhardtii* further, the cultures could be exposed to several heat shocks over time to maintain expression of the silencing construct. Although we expected that growing the transgenic lines at 35 °C would result in a continuous expression of the silencing construct because it is very close to the heat shock temperature (40 °C). This is not the case, probably because the heat shock protein needs a transient big shift in temperature to be active and not a constant high temperature. Furthermore, the higher temperature is another stress factor for the microalgae, explaining the cultures' loss of viability after five days of nitrogen depletion at 35 °C.

Another option would be to also reduce the mRNA level of the Stearoyl-CoA Desaturase (SCD) enzyme, which can desaturate the stearoyl-CoA acyl chain which is present outside the chloroplast (Fig. 6). It is very likely that the accumulated stearic acid is converted to oleic acid in the cytosol and endoplasmic reticulum. In *C. reinhardtii* the SCD is not well annotated. There is a predicted

protein sequence for this enzyme and therefore a more thorough study needs to be done on characterizing and annotating the desaturase genes involved in the desaturation of fatty acids outside the chloroplasts. Another possible way to reduce the enzyme function of the SCD would be by using inhibiting fatty acids that are known to reduce enzyme efficiency of specific desaturases. The cyclic inhibitor sterculic acid could be used to reduce the SCD efficiency (James et al., 1968; Moreton, 1985; Cao et al., 1993; Wältermann and Steinbüchel, 2000). This could be a potential way to reduce overall stearoyl desaturase effectivity in combination with amiRNA gene silencing, to study the effect of stearic acid accumulation in microalgae. An interesting next step would be silencing both SAD and SCD genes in oleaginous microalga, since the TAG accumulating mechanisms are more developed in these species and possibly the response to SAD silencing is different from *C. reinhardtii*. Alternatively, oleaginous microalgae that are isolated from high temperature ecosystems should be used, since these species normally contain a higher saturated fatty acid content. From a production point of view, thermophilic microalgae would be advantageous, since the energy needed to cool the photo bioreactors would be less compared to the cultivation of non-thermophilic microalgae (Ruiz et al., 2016).

4. Conclusion

Artificial microRNA was used to reduce the stearoyl-ACP desaturase activity in *C. reinhardtii*. The mRNA expression was transiently reduced upon the induction of the amiRNA construct by heat shock and nitrogen depleted growth conditions, which doubled the stearic acid content in *C. reinhardtii*. The overall stearic acid content could be further increased by blocking the conversion of stearoyl to oleoyl in other compartments. Optionally the enzyme activity can be reduced by using specific cyclopropene inhibiters, to circumvent silencing the poorly annotated SCD gene.

Acknowledgements

This research project is financially supported by the Food and Nutrition Delta program, of Agentschap NL (FND10007) and Unilever. The authors would like to thank Rene Draaisma and John Chapman for reviewing this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.06.128.

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