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ORIGINAL ARTICLE

Stearoyl-acyl carrier protein desaturase gene from the oleaginous microalga *Chlorella zofingiensis*: cloning, characterization and transcriptional analysis

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Abstract The green alga *Chlorella zofingiensis* can accumulate high level of oleic acid (OA, C18:1$\triangle^9$) rich oils in response to stress conditions. To understand the regulation of biosynthesis of fatty acid in particular OA at the molecular level, we cloned and characterized the stearoyl acyl carrier protein (ACP) desaturase (SAD) responsible for OA formation through desaturation of stearic acid (C18:0) from *C. zofingiensis*. Southern blot indicated that the *C. zofingiensis* genome contained a single copy of *SAD*, from which the deduced amino acid sequence shared high identity to the corresponding homologs from other microalgae and higher plants. The desaturation activity of SAD was demonstrated in vitro using C18:0-ACP as a substrate. Stress conditions such as high light (HL), nitrogen deficiency (N^-), or combination of HL and N^- (HL + N^-) drastically up-regulated the transcripts of biotin carboxylase (BC, a subunit of ACCase) and SAD, and therefore induced considerably the cellular accumulation of total fatty acids including OA. Glucose (50 mM) gave rise to the similar up-regulation of the two genes and induction of fatty acid accumulation. The accumulation of intracellular reactive oxygen species was found to be associated with the up-regulation of genes. This is the first report of characterization of *Chlorella*-derived *SAD* and the results may contribute to understanding of the mechanisms involved in fatty acid/lipid biosynthesis in microalgae.

Keywords *Chlorella zofingiensis* · High light · Nitrogen deficiency · Oleic acid · Stearoyl-acyl carrier protein desaturase

Abbreviations

ACCase	Acetyl-CoA carboxylase
BC	Biotin carboxylase
HL	High light
LL	Low light
OA	Oleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-PCR
SAD	Stearoyl-acyl carrier protein desaturase
TFA	Total fatty acids

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Introduction

Fossil-based fuels are widely considered as unsustainable and will be depleted in a limited number of years (Chisti 2007). To cope with this coming energy crisis, there is an urgent need for renewable petroleum alternative fuels. Being sustainable and carbon neutral, the biodiesel derived from biologically sourced feedstocks is recognized to be a

promising alternative (Knothe 2008). To date, commercial biodiesel is mainly produced from plant oils, animal fats and waste cooking oil. However, plant-derived oils cannot realistically meet the existing needs for transportation fuels as it will dominate the arable lands currently used for food crops and potentially cause a fuel versus food conflict (Chisti 2008). In contrast, microalgae appear to be the most potential feedstocks for biodiesel production, because of their fast growth, easily obtained biomass, abundant oil and low land requirement (Chisti 2007, 2008; Hu et al. 2008).

Chlorella, a genus of green microalgae rich in protein, has long been used as human health food. Under certain stress conditions, *Chlorella* species are capable of accumulating as high as 66 % (w/w, on dry weigh basis) oil within cells (Hsieh and Wu 2009). Together with the characteristics of high growth rate and ease of culture and scale-up in open ponds or photobioreactors, *Chlorella* has attracted unprecedented interest as a feedstock for biofuels, in particular biodiesel (Xu et al. 2006; Li et al. 2007; Xiong et al. 2008; Hsieh and Wu 2009; Gao et al. 2010; Liu et al. 2010, 2012). Biodiesel derived from *Chlorella* lipids has been shown to comply with the specifications established by American Society for Testing and Materials (Xu et al. 2006). The key properties of a biodiesel, e.g., cetane number, viscosity, cold flow and oxidative stability, are regarded to be greatly related to the fatty acid composition of the biodiesel feedstock (Knothe 2009). As such, the understanding of alterations in fatty acid profile is of great importance using *Chlorella* as a biodiesel feedstock.

Among the enzymes involved in lipid biosynthesis, acetyl-CoA carboxylase (ACCase) catalyzes the first committed step of de novo fatty acid synthesis, which is considered as the rate-limiting flux control point for this pathway (Ohlrogge and Jaworski 1997). ACCase in chloroplasts is composed of four subunits; the expression of the genes coding the subunits is autoregulated to each other and the characterization of one subunit such as biotin carboxylase (BC) can be representative of ACCase (Bao et al. 1997; Ke et al. 2000). Stearoyl ACP desaturase (SAD) adds the first double bond to acyl chain and plays an important role in determining the degree of saturation of fatty acids (Ohlrogge and Jaworski 1997). The fatty acid and lipid profiles in microalgae are tightly regulated by the expression of genes involved in fatty acid and lipid biosynthesis, but the molecular mechanism is relatively poorly understood compared to higher plants (Hu et al. 2008). To elucidate the regulation of fatty acid biosynthesis in *Chlorella*, key genes such as *BC* and *SAD* need to be cloned and characterized. The characterization of the two genes, as far as we know, has not been reported for *Chlorella* species.

We previously investigated the ability of *Chlorella zofingiensis* to produce oils (Liu et al. 2010). *C. zofingiensis* could produce fatty acids up to 45 % of cell dry weight, with 36 % of the fatty acids being oleic acid (OA). In the present study, the gene encoding SAD, which desaturates stearic acid to OA, was cloned and characterized from *C. zofingiensis*. We examined the expression and regulation of the *SAD* as well as the *BC* gene (GenBank accession number GQ996717) during fatty acid biosynthesis in response to high light (HL), nitrogen deficiency (N^-), the combination of hight light and nitrogen dificiency (HL + N^-), or glucose induction. These results will expand our knowledge about the fatty acid biosynthesis and regulation in *C. zofingiensis* and may also benefit the genetic engineering of *Chlorella* to modify fatty acid composition for better biodiesel quality.

Materials and methods

Chlorella strain and culture conditions

Chlorella zofingiensis (ATCC 30412) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). This alga was maintained at 4 °C on an agar slant containing the Kuhl medium (Liu et al. 2010). Ten milliliter of liquid Kuhl medium was inoculated with cells from slants and the algal cultures were grown aerobically in flasks at 25 °C for 4 days with orbital shaking at 150 rpm and continuous illumination of 30 μmol photons m^{-2} s^{-1}. The cells were then inoculated at 10 % (v/v) into 100-mL columns provided with illumination of 60 μmol photons m^{-2} s^{-1} and aeration of 1.5 % CO_2-enriched air, grown to late exponential phase and used as seed cultures for the following experiments.

The seed cultures were pelleted by centrifugation and resuspended with nitrogen-replete medium (unless otherwise indicated) at a concentration of 1 g L^{-1}. For the experiment of light intensity effect, the algal cultures were exposed to HL of 400 μmol photons m^{-2} s^{-1} with low light (LL) of 60 μmol photons m^{-2} s^{-1} as the control. For the experiment of nitrogen deficiency (N^-), the algal cultures were grown in the nitrogen-deficient medium under both LL and HL, with the nitrogen-replete medium under LL as the control. For the cerulenin experiment, the algal cultures were grown in the nitrogen-deficient medium under HL with addition of 10 μM cerulenin. For the induction by glucose supplementation, the algal cultures were dark adapted and then cultured with addition of 50 mM glucose in the absence of light; glucose-free cultures were used as the control.

Genomic DNA and RNA isolation

DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Stewart and Via 1993).

RNA was isolated from aliquots of about 1×10^8 cells using the TriPure isolation reagent (Roche, Mannheim, Germany) according to the manufacturer's manual. The concentration of DNA and total RNA was determined spectrophotometrically at 260 nm.

Cloning of stearoyl-ACP desaturase cDNA and its corresponding gene

The primer sets used in this study are listed in Table 1. To amplify the *SAD* cDNA from *C. zofingiensis*, degenerate primers Sad-dF and Sad-dR were designed according to the conserved motifs GDM/LITEEA and HGNTARQ/HA in SAD proteins from *Arabidopsis thaliana*, *Brassica napus*, *Helianthus annuus*, *Haematococcus pluvialis* and *Chlamydomonas reinhardtii*. First strand cDNA synthesis was carried out with 1 μg of total RNA extracted from 24-h HL-induced cells, using a SuperScript III First-Strand Synthesis System according to manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). PCR amplification was programmed with around 50 ng of cDNA as template (30 cycles of 94 °C for 30 s, 55 °C for 20 s, 72 °C for 1 min). The PCR product was gel purified and sequenced, based on which the specific primers (Sad-F1 and Sad-R2 for first round PCR, Sad-F2 and Sad-R2 for second round PCR) were designed for rapid amplification of 5′ and 3′ cDNA ends (RACE). RACE was performed using the method described by Huang and Chen (2006). The primer pair of Sad-F3 and Sad-R3 derived from the sequences of 5′ and 3′ RACE fragments was employed to amplify a full-length *SAD* cDNA and its corresponding gene. Genome walking of the *SAD* gene was performed according to the approach described by the instruction of the Universal Genome-Walker kit (Clontech, Palo Alto, CA, USA).

Table 1 Primers for gene cloning and expression

Aim	Oligonucleotide sequence 5′–3′
Partial *SAD* fragment	
Sad-dF	GGCGAYWTGATHACNGARGARGC
Sad-dR	GCNTGNCKNGCNGTRTTNCCRTG
5′ and 3′ *SAD* RACE	
Sad-F1	GGGTTTCATCTACACCTCCTTC
Sad-R1	TGTTCTCCTCAGCCGTCCACTCAC
Sad-F2	TCCAGGAACGTGCCACCAAG
Sad-R2	CACGCGTCCACCTGCCCCAAG
SAD gene	
Sad-F3	TTAGCCACCTTGTTCAGCCGC
Sad-R3	GCTTTCAATCTAGCTACTGTCG
SAD probe (425 bp)	
Sad-F4	ATATGATCACTGAGGAGGC
Sad-R4	GCTTGTCAACAATCCTGCTG
SAD expression	
Sad-F2 and Sad-R5	See Sad-F2, GCGCCCTGTCTTGC CCTCATG
BC expression	
Bc-F	GTGCGATTGGGTATGTGGGGGTG
Bc-R	CGACCAGGACCAGGGCGGAAAT

Southern blot

Eight microgram of genomic DNA was digested with *Bam*HI, *Eco*RV, *Sac*I, and *Nco*I + *Hin*dIII, which have no recognition sites in the probed regions of the *SAD* gene. The digested DNA was separated with an 0.8 % agarose gel, transferred to a positively charged nylon membrane (Roche), and hybridized with digoxigenin (DIG)-labeled DNA probes in the presence of 50 % (v/v) formamide at 47 °C for 16 h. DNA probe was prepared by amplifying a 425-bp fragment of *SAD* gene with a pair of specific primers (Table 1) and a plasmid containing the *SAD* gene template (28 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s). Probe labeling and hybridization were carried out according to the instructions of the DIG Non-radioactive Nucleic Acid Labeling and Detection System (Roche). After hybridization, the membrane was washed twice with 0.1× standard saline citrate (SSC) containing 0.1 % sodium dodecyl sulfate (SDS) at 68 °C for 15 min.

In vitro SAD desaturation assay

The *SAD* open reading frame was digested by *Eco*RI and *Hin*dIII and inserted into the corresponding sites of the expression vector pRSET-C (Invitrogen). The resulting plasmids, pR-czSad, was introduced into *Escherichia coli* strain BL21 (DE3) (Invitrogen) for SAD expession. Cells were grown in SOB medium containing 50 μg mL^{-1} ampicillin at 37 °C with vigorous shaking, and 1 mM IPTG was added when the optical density at 600 nm reached 0.5. Cells were harvested after a 6-h induction period, resuspended in 1/5 volume of 50 mM sodium phosphate buffer (pH 8.0, containing 0.5 M NaCl), and then passed through a French pressure cell (Spectronics Instruments, Rochester, NY, USA) at an internal pressure of 20 MPa. Ten μg mL^{-1} DNase was added to the broken cell extract and the mixture was incubated on ice for 15 min. Cell debris was removed from the suspension by centrifugation at 10,000*g* and the resultant supernatant was applied to the Probond™ column (Invitrogen) for the purification of recombinant SAD.

As described by Schultz et al. (2000), the desaturation assay was carried out in 1 mL of reaction mixture containing 2 mM ascorbate, 0.75 mM NADPH, 1 mM NADH (NADPH and NADH were freshly prepared in 100 mM

Tricine, pH 8.0), 3,000 U of catalase, 2 mM ATP, 30 mM Pipes (pH 6.0), 0.04 mM ferredoxin, 0.3 U of ferredoxin-$NADP^+$ reductase, 0.1 mg of purified SAD, and 1 nmol of C18:0-ACP. The C18:0-ACP was prepared according to Rock and Garwin (1979). The reactions were maintained at room temperature with shaking of 100 rpm for 30 min and then terminated by adding 50 μL of glacial acetic acid and 5 mL of acetone, followed by evaporation to complete dryness under nitrogen gas. The fatty acids were methyl esterified by heating in 2 % sulfuric acid in methanol (v/v) for 2 h. The resulting fatty acid methyl esters were subjected to GC–MS analysis for the determination of the double bond position.

Real time RT-PCR assay

Total RNA (1 μg) extracted from different samples was reverse transcribed to cDNA using a SuperScript III First-Strand Synthesis System (Invitrogen). Real time RT-PCR analysis was performed according to Liu et al. (2012), from 1 μL of the RT reaction mixture in a total volume of 20 μL with specific primers and the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The primers for *BC* and *SAD* are listed in Table 1. PCR was run in a BIO-RAD iCycler IQ Multi-Color RealTime PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative levels of the amplified mRNA were evaluated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using actin gene for normalization.

Fatty acid analysis

Lipids were extracted from lyophilized algal samples with a solvent mixture of chloroform, methanol and water (2:1:0.75, by vol.) according to the modified Folch procedure (Christie 2003). Fatty acid methyl esters (FAMEs) were prepared by direct transmethylation with sulfuric acid in methanol (Christie 2003).

The FAMEs were analyzed by gas chromatograph as described previously (Liu et al. 2012). The double bond position of the product from in vitro SAD desaturation assay was determined by GC–MS using the Agilent 7890 capillary gas chromatograph equipped with a 5975 C mass spectrometry detector and a HP-88 capillary column (60 m × 0.25 mm) (Agilent Technologies, Wilmington, DE, USA). The temperature program consisted of an initial hold at 100 °C for 5 min, ramping at 3.5 °C min^{-1} to 240 °C, and a final hold at 240 °C for 5 min. The injector was kept at 250 °C with an injection volume of 2 μL under splitless mode. The flow rate of the carrier gas (Helium) was 1.5 mL min^{-1}, and the ionization energy was 70 eV (EI, full scan mode).

Measurement of reactive oxygen species levels

Reactive oxygen species (ROS) were measured using the cell-permeant fluorescent dye, 5-(-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA), according to a method described by Baroli et al. (2004). The 5-mL culture samples were harvested by centrifugation at 3,000*g* for 3 min and resuspended in 750 μL of fresh culture medium (adjusted to pH 5.7 with acetic acid) to bring the total volume to 1 mL. Five microliters of CM-H_2 DCFDA (freshly dissolved in Me_2SO; final concentration 10 μM) or an Me_2SO-only control was added to the cell samples, which were incubated at room temperature for 20 min in the dark. The cells were then centrifuged and resuspended as mentioned above, and fluorescence of 10^4 cells was measured on an EPICS XL-MCL flow cytometer (BeckmanCoulter, Miami, FL, USA).

Statistical analyses

All experiments were determined in biological triplicate to ensure the reproducibility. Experimental results were obtained as mean value ± SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Paired-samples *T* test was applied. The statistical significances were achieved when $P < 0.05$.

Results

Cloning and characterization of *SAD* gene from *C. zofingiensis*

A 330-bp fragment of *SAD* cDNA was amplified by RT-PCR with the degenerate primers Sad-dF and Sad-dR. On the basis of this sequence information, two pairs of specific primers (Sad-F1 and Sad-R1, Sad-F2 and Sad-R2) were designed for 5′ and 3′ RACE, which generated a 1.6-kb fragment. The sequence of the fragment was determined as a fusion of the 5′ and 3′ ends of a putative *SAD* cDNA. The coding region of the cDNA amplified using the primers Sad-F3 and Sad-R3 was revealed to contain a 1,251-bp ORF (GenBank accession no. GQ996719). Upstream of the translation start codon is a 76-bp 5′ untranslated region and between the stop codon and poly (A) tail is a 3′ untranslated region of 413-bp nucleotides. TGTAA, the sequence considered as a potential polyadenylation signal in green algae, is also found in the 3′ untranslated region of this *SAD* gene. The GC content of the *SAD* coding region is 53.2 %, which is slightly higher than that of *SAD* from *A. thaliana* (47.9 %) but lower than those from *C. reinhardtii* (64.0 %) and *H. pluvialis* (60.4 %). The coding

region of *SAD* gene is interrupted by six introns of 77, 224, 283, 264, 233, and 319 bp, respectively (Fig. 1a; GenBank accession no. GQ996720). Intron/exon splice sites of the *SAD* gene are highly conserved, and all introns start with GT and end with AG.

To detect the number of *SAD* gene in the genome of *C. zofingiensis*, genomic DNA was digested with different restriction enzymes and subjected to Southern-blot analysis. Using a 0.43-kb fragment of *SAD* as a probe, the homologous fragments showed strong hybridization signals (Fig. 1b). Each of the four separate digests showed only one band, suggesting the presence of only one *SAD* gene copy in the genome of *C. zofingiensis*.

The deduced SAD protein is composed of 416 amino acids with a calculated molecular mass of 47.04 kDa, a theoretical isoelectric point of 6.42 and an instability index of 30.98 (data obtained with ProtParam program). The primary sequence of the SAD contains two EXXH motifs (residues 188–191 and 274–277), which are iron binding sites important for enzyme activity. The two motifs are spanned by 82 amino acid residues proceeded by either an E or D residue. SAD is a soluble enzyme localized in the chloroplast (Haralampidis et al. 1998). The Predotar program predicted the presence of a possible plastid targeting sequence with a processing site after the F at the residue 26. Protein sequence alignments showed the SAD of *C. zofingiensis* shared high identity to the published sequences of other algae and higher plants: *A. thaliana*, 65 %; *B. napus*, 64 %; *H. annuus*, 59 %; *H. pluvialis*, 75 %; *C. reinhardtii*, 75 %. The phylogenetic analysis of the SADs from microalgae, higher plants, and bacteria is illustrated in Fig. 2. Analysis was conducted by the MEGA4 programme (Tamura et al. 2007) using the neighbor-joining method. The predicted *C. zofingeinsis* SAD forms a cluster with those of other microalgae analyzed, which is much closer to higher plants than to bacteria.

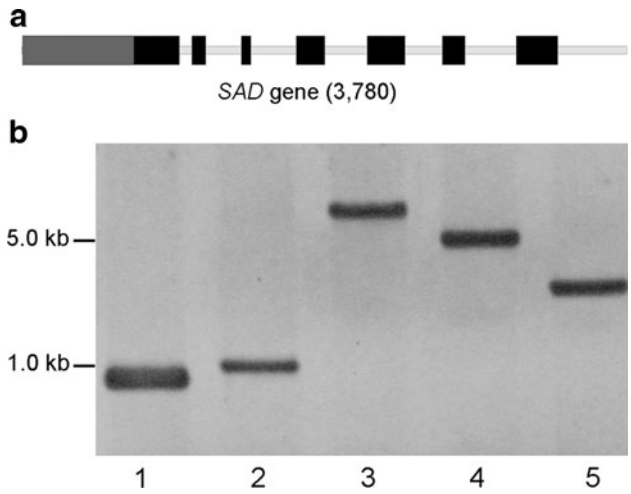

Fig. 1 Molecular characterization of *SAD* gene of *C. zofingiensis*. **a** Schematical illustration of the *SAD* gene. The promoter sequence is denoted by a *gray box*, the exons are showed by *black boxes* and the introns are indicated as *lines*. **b** Southern analysis of genomic DNA. Genomic DNA (10 μg) was digested with *Bam*HI (*lane 2*), *Eco*RV (*lane 3*), *Sac*I (*lane 4*), and *Nco*I + *Hin*dIII (*lane 5*), respectively, electrophoretically separated on a 0.8 % agarose gel, blotted onto a nylon membrane, and hybridized with a 425-bp *SAD* gene fragment amplified by PCR. Plasmids (300 pg) containing the *SAD* gene were cut by *Bam*HI and used as a positive control (*lane 1*)

In vitro analysis of desaturation activity of *C. zofingiensis* SAD

To confirm the biochemical activity of the *C. zofingiensis* SAD, the in vitro desaturation assay was performed, using the purified recombinant SAD protein expressed in *E. coli*. Incubation of the reaction mixture containing the SAD protein resulted in an additional peak, indicating the formation of a new fatty acid (Fig. 3a). Mass spectrum analysis revealed that the new fatty acid was oleic acid (OA, C18:1$\triangle^9$) from the addition of a double bond in $\triangle^9$ position of C18:0 catalyzed by SAD (Fig. 3b).

High light and nitrogen deficiency up-regulate the transcripts of *BC* and *SAD* and enhance the biosynthesis of fatty acids

High light is considered an effective stimulus to induce accumulation of fatty acids in microalgae (Zhekisheva et al. 2005; Chen 2007). As revealed by real time RT-PCR results, upon exposure of *C. zofingiensis* cells to HL (400 μmol photons m^{-2} s^{-1}), a significant increase in the steady-state mRNA level of *BC* was observed and the mRNA level reached its maximum at 24 h (Fig. 4a). HL also up-regulated *SAD* transcripts to much stronger extent as compared to *BC* transcripts (Fig. 4b). The steady-state *SAD* mRNA level began to decline after 24 h of HL induction (Fig. 4b). The fatty acid profiles were examined over the period of induction to correlate the transcript levels of *BC* and *SAD* genes with the biosynthesis of fatty acids. The *Chlorella* cells without HL stress accumulated only a relatively small amount of total fatty acids (TFA); whereas a great increase in the TFA content was observed with HL (Fig. 4c). Similarly, HL stimulated the accumulation of OA (Fig. 4d). After 72 h of induction, the contents of TFA and OA reached 21.2 and 5.26 % of dry weight, 41.3 and 164.3 % higher than those of control, respectively (Fig. 4c, d).

Nitrogen deficiency (N^-) also markedly up-regulated the expression of *BC* and *SAD* and accordingly induced the accumulation of fatty acids (Fig. 4). HL together with N^- (HL + N^-) stimulated the up-regulation of *BC* and *SAD* more significantly than HL or N^- alone. Consequently,

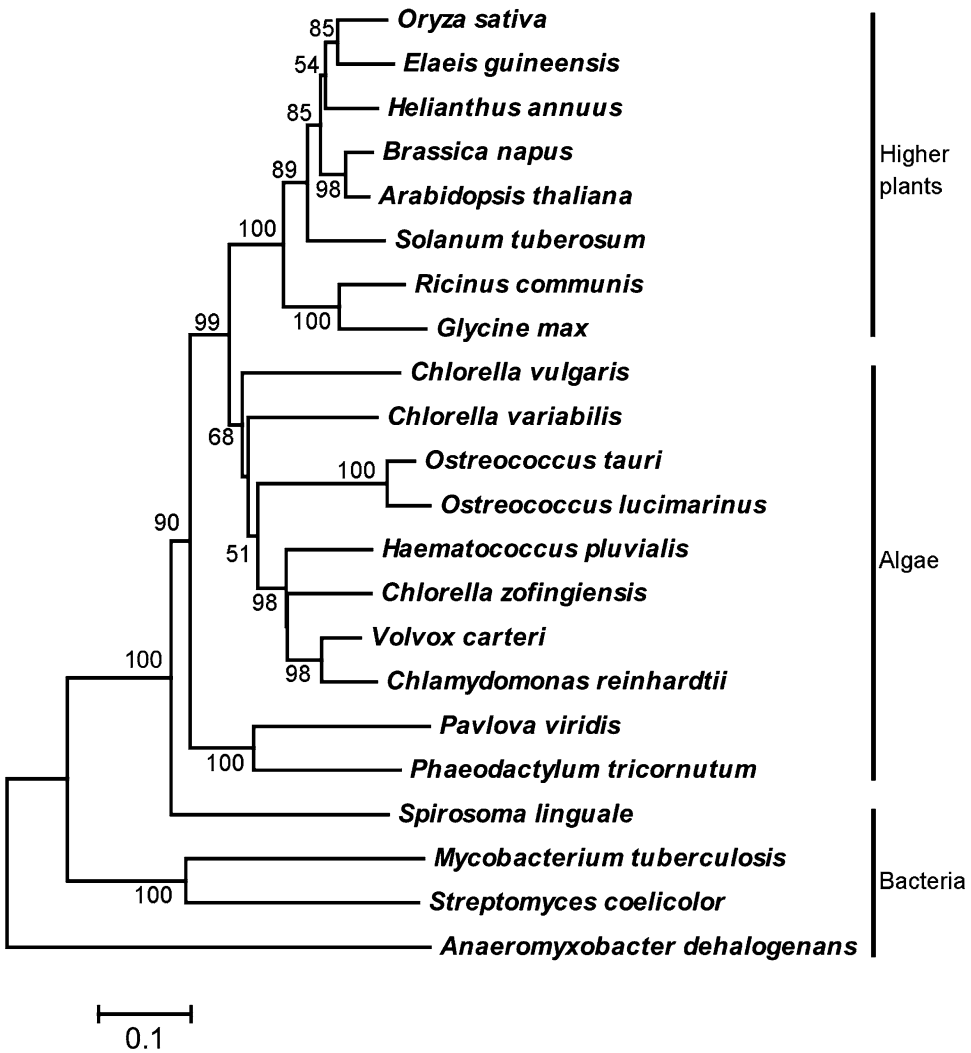

Fig. 2 Phylogenetic tree of SAD homologs from bacteria, algae and higher plants. The alignment was generated by the Clustal W program and the tree was constructed with neighbor-joining method using MEGA4 software (Tamura et al. 2007). The deduced SAD sequences of *Chlamydomonas reinhardtii* (estExt_gwp_1W.C_130244), *Chlorella variabilis* (estExt_Genewise1Plus.C_80339), *C. vulgaris* (estExt_Genewise1Plus.C_120391), *Ostreococcus lucimarinus* (fgenesh1_pg.C_Chr_4000263), *Ostreococcus tauri* (estExt_fgenesh1_pm.C_Chr_04.00010050), *Phaeodactylum tricornutum* (e_gw1.1.376.1), and *Volvox carteri* (estExt_fgenesh4_pm.C_150013) come from JGI database (http://www.jgi.doe.gov). Others are from GeneBank with following accession numbers: *Anaeromyxobacter dehalogenans* (ZP_02322836), *A. thaliana* (AAK85232), *B. napus* (AAT65205), *Elaeis guineensis* (AAB41041); *Glycine max* (ABM45912), *H. pluvialis* (ABP57425), *H. annuus* (AAB65144), *Mycobacterium tuberculosis* (CAE55326), *Oryza sativa* (BAA07631), *Pavlova viridis* (ABS20117), *Ricinus communis* (XP_002526163), *Solanum tuberosum* (AAA33839), *Spirosoma linguale* (ADB40562), and *Streptomyces coelicolor* (NP_630790)

C. zofingiensis produced significantly higher levels of fatty acids including OA in response to HL + N^-, such that at 72 h the TFA content was 31.6 and 22.9 % higher than that induced by HL and N^-, respectively, and the OA content was 173.8 and 153.1 % of that caused by HL and N^-, respectively (Fig. 4c, d). The addition of cerulenin, a specific inhibitor of β-ketoacyl-ACP synthase I (KAS I), caused a considerable decrease in the contents of both TFA and OA (Fig. 4c, d). The transcriptional expression of *BC* and *SAD*, however, was differentially regulated: the *BC* transcripts were greatly promoted while the *SAD* transcripts were only slightly affected.

Glucose triggers the up-regulation of *BC* and *SAD* genes and induces the enhanced biosynthesis of fatty acids

In addition to HL and N^-, glucose could stimulate the accumulation of fatty acids in *C. zofingiensis* (Liu et al. 2010). To survey the effect of glucose on fatty acid

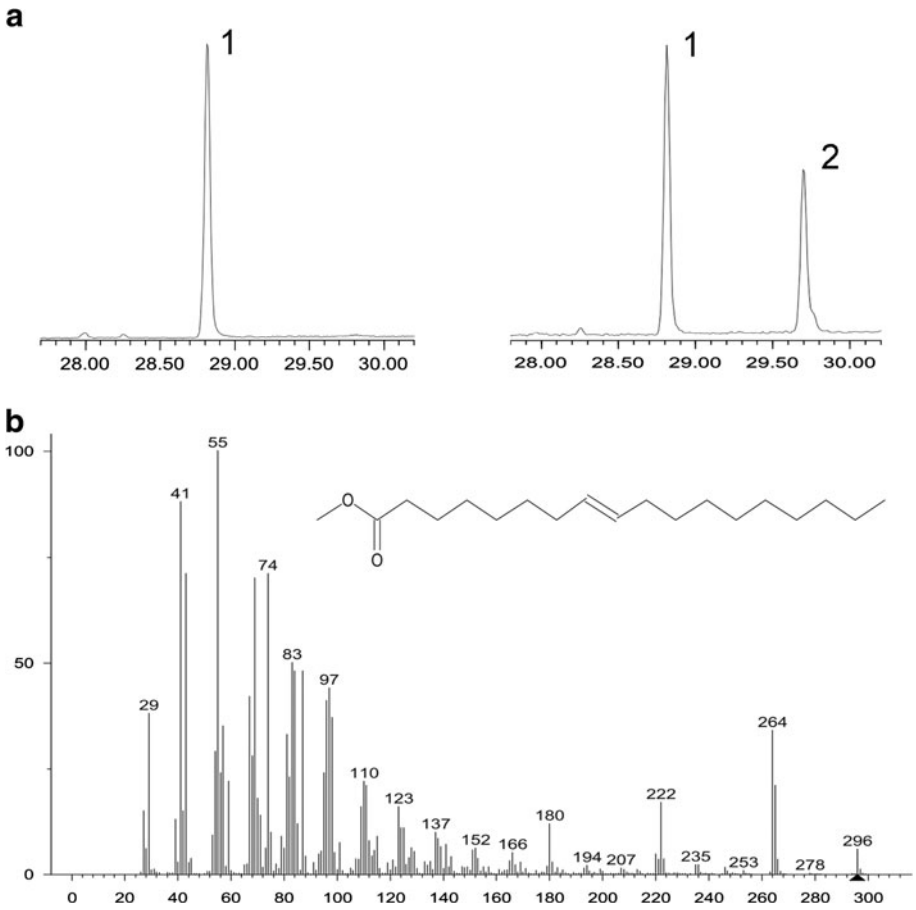

Fig. 3 In vitro conversion of stearic acid to oleic acid by the *C. zofingiensis* SAD. **a** GC separation of methyl esters from the reaction mixture without (*left panel*) and with (*right panel*) the purified *C. zofinginesis* SAD. Peak *1*, C18:0; peak *2*, C18:1$\triangle^9$. **b** Mass spectra of the 18:1$\triangle^9$ product of the *C. zofinginesis* SAD and its fragmentation ions

biosynthesis at transcriptional level, *C. zofingiensis* cells were induced by 50 mM glucose in the absence of light. The mRNA levels of both *BC* and *SAD* were drastically up-regulated, reaching their maximum at 48 h after glucose induction (Fig. 5a, b), 24-h delay as compared to under HL, N^- and HL + N^- conditions (Fig. 4a, b).

Consistent with the up-regulation of *BC* and *SAD* transcripts, *C. zofingiensis* accumulated TFA and OA within cells (Fig. 5c, d). After 72 h of induction, the TFA content reached 29.9 % of dry weight, which was 89 % higher than that of control; the OA content was 8.97 % of dry weight, 3.6-fold higher than that of control.

Discussion

The production of unsaturated fatty acids relies on the desaturases, which contribute to the introduction of double bonds into fatty acids via an oxygen dependent mechanism (Ohlrogge and Jaworski 1997). Fatty acid desaturases are usually membrane bound and utilize complex lipid substrates such as phosphatidylcholine or monogalactosyldiacylglycerol (Harwood 1996). An exception is the stearoyl-ACP desaturase (SAD) that is soluble and localized in the chloroplast stroma and catalyzes the insertion of a *cis* double bond between carbons 9 and 10 of C18:0-ACP to form C18:1-ACP (Sobrado et al. 2006). Because of the availability of increasing full genomic sequences of microalgae, the genes encoding the putative SAD have been annotated for several algal species (Chi et al. 2008). The cloning and characterization of SAD from microalgae, however, was rarely reported. The capacity of *C. zofingiensis* to produce large quantities of OA-rich TAG (Liu et al. 2010, 2011, 2012) suggested that it would be of great interest to study the genes and enzymes involved in the accumulation of fatty acids particularly OA. In this study, we isolated and characterized from *C. zofingiensis* a key *SAD* gene involved in desaturation of saturated fatty acids. *C. zofingiensis* contained only one copy of *SAD* in its genome as revealed by Southern blot (Fig. 1b), which is consistent with *Haematococcus pluvialis* (Chen 2007). Similar to SADs from other algae and higher plants, the deduced amino acid sequence of *C. zofingiensis* SAD contains two EXXH motifs that are located within a predicated α-helix where the E and H residues are on the same surface. The presence of EXXH motifs that are conserved in the diiron-oxo proteins suggests the desaturase belongs to a general class of diiron-oxo proteins (Haralampidis et al. 1998).

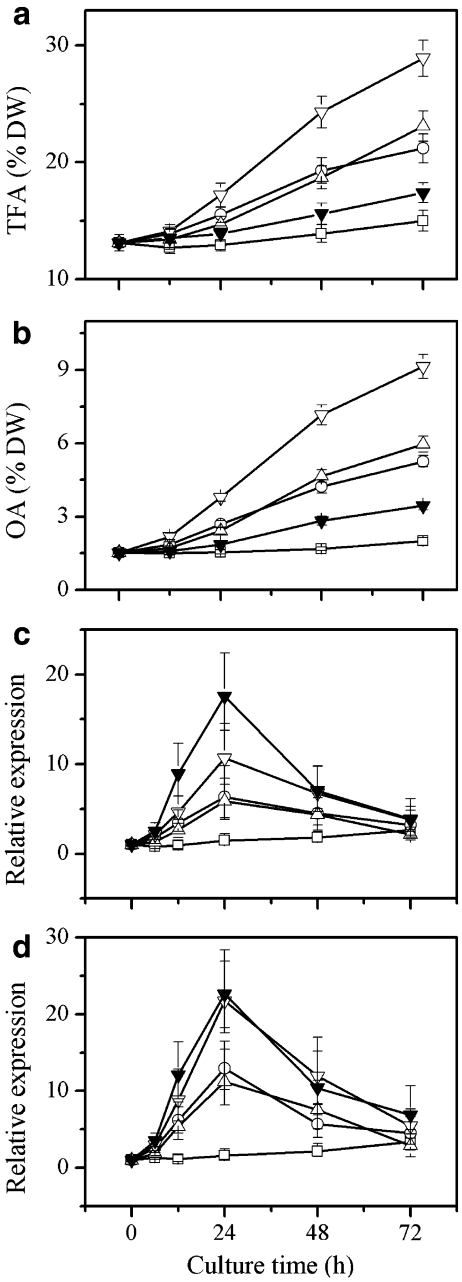

Fig. 4 Total fatty acids (TFA, **a**), oleic acid (OA, **b**), and relative expression of *BC* (**c**) and *SAD* (**d**) of *C. zofingiensis* under different culture conditions. *Square*, control; *circle*, high light (HL); *triangle*, nitrogen deficiency (N^-); *inverted triangle*, HL together with N^- ($HL + N^-$); *filled inverted triangle*, $HL + N^-$ with addition of 10 μM cerulenin. The levels of gene expression were normalized relative to the control cultures at day 0, which was set to 1

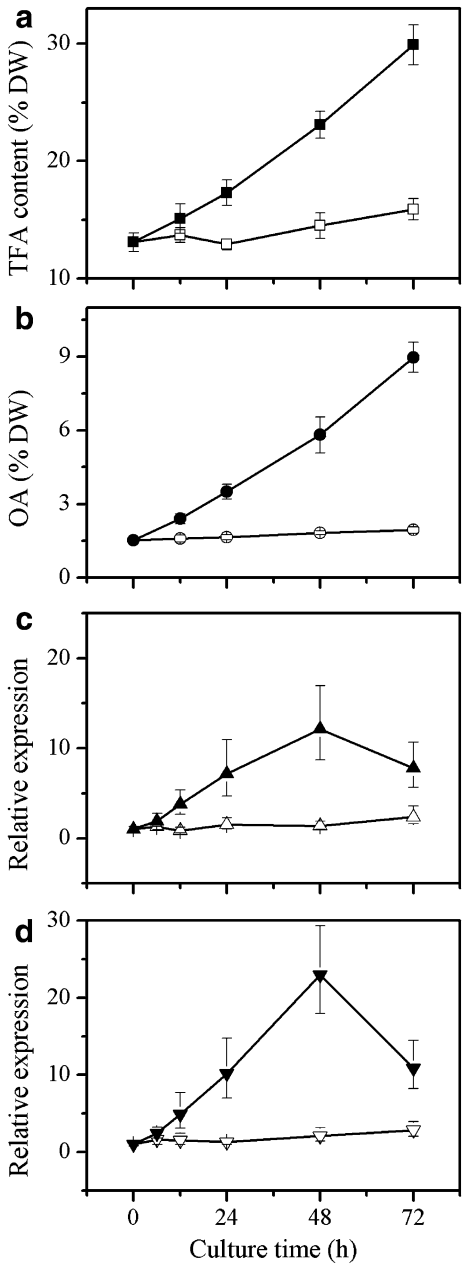

Fig. 5 Glucose-induced TFA (**a**), OA (**b**), and relative expression of *BC* (**c**) and *SAD* (**d**) of *C. zofingiensis* cultured in the dark (*filled symbols*). Glucose was added at a concentration of 50 mM. Glucose-free cultures were used as the control (*open symbols*). The levels of gene expression were normalized relative to the control cultures at day 0, which was set to 1

The soluble nature of SAD has allowed comprehensive studies of its structure and enzymatic activity (Harwood 1996; Schultz et al. 2000). The *C. zofingiensis* SAD was heterologously expressed in *E. coli* in its active form. The purified *E. coli* expressed SAD demonstrated to catalyze the desaturation of C18:0 to form C18:1$\triangle^9$ by an in vitro assay (Fig. 3). SAD accepts not only stearate but also palmitate as the substrate for producing monounsaturated fatty acids (Kachroo et al. 2007). The *C. zofingiensis* SAD was also capable of converting C16:0–C16:1, but with much lower conversion efficiency even in the large excess of the purified enzyme as compared to the conversion of C18:0–C18:1 (data not shown), suggesting the substrate preference on C18:0. This might also be explained by the fatty acid profile of *C. zofingiensis*, in which C16:0 is rich

Table 2 Fatty acid composition of *C. zofingiensis* under various growth conditions

Fatty acid	0 h	48 h of induction						
		LL	HL	N^-	HL + N^-	HL + N^- + C	DK	DK + Glu
C16:0	28.9 ± 1.52	27.7 ± 1.21	24.9 ± 1.15	25.4 ± 1.03	23.1 ± 0.97	25.4 ± 1.19	29.1 ± 1.48	24.1 ± 1.36
C16:1	2.4 ± 0.11	2.5 ± 0.13	2.2 ± 0.10	2.1 ± 0.13	2.6 ± 0.14	2.7 ± 0.15	2.7 ± 0.12	2.1 ± 0.15
C16:2	7.9 ± 0.44	8.2 ± 0.36	8.5 ± 0.48	8.1 ± 0.52	7.7 ± 0.28	8.1 ± 0.39	7.2 ± 0.25	8.3 ± 0.43
C16:3	6.9 ± 0.29	6.7 ± 0.35	3.7 ± 0.21	3.2 ± 0.18	2.6 ± 0.10	4.5 ± 0.23	7.4 ± 0.40	3.1 ± 0.25
C16:4	1.0 ± 0.07	0.8 ± 0.05	0.4 ± 0.03	0.3 ± 0.02	0.2 ± 0.02	0.5 ± 0.03	1.3 ± 0.07	0.4 ± 0.03
C18:0	3.7 ± 0.25	3.9 ± 0.19	2.4 ± 0.15	2.6 ± 0.11	2.8 ± 0.10	2.9 ± 0.13	3.1 ± 0.14	2.5 ± 0.18
C18:1	11.6 ± 0.74	12.8 ± 0.61	21.9 ± 0.98	24.9 ± 1.35	29.5 ± 1.72	18.1 ± 1.03	11.4 ± 0.71	24.8 ± 0.78
C18:2	20.8 ± 1.14	21.1 ± 0.91	22.4 ± 1.32	20.5 ± 1.25	19.7 ± 0.64	21.3 ± 1.19	20.1 ± 0.86	22.1 ± 1.23
C18:3 (n-6)	1.4 ± 0.11	1.6 ± 0.13	1.3 ± 0.09	1.4 ± 0.10	1.2 ± 0.12	1.6 ± 0.09	1.4 ± 0.12	1.6 ± 0.09
C18:3 (n-3)	13.8 ± 0.49	13.3 ± 0.78	11.1 ± 0.57	10.4 ± 0.37	9.3 ± 0.42	13.2 ± 0.54	14.4 ± 0.69	9.8 ± 0.38
C18:4	1.6 ± 0.10	1.4 ± 0.06	1.2 ± 0.08	1.1 ± 0.05	1.3 ± 0.07	1.7 ± 0.06	1.9 ± 0.11	1.2 ± 0.05
MUFA (%)	14.0 ± 0.84	15.3 ± 0.75	24.1 ± 1.28	27.0 ± 1.29	32.1 ± 1.82	20.8 ± 1.16	14.1 ± 0.81	26.9 ± 0.97
PUFA (%)	53.4 ± 2.87	53.1 ± 2.96	48.6 ± 2.12	45.0 ± 2.31	42.0 ± 2.11	50.9 ± 2.27	53.7 ± 2.56	46.5 ± 2.53
UFA (%)	67.4 ± 3.53	68.4 ± 3.65	72.7 ± 3.37	72.0 ± 3.58	74.1 ± 3.92	71.7 ± 3.11	67.8 ± 3.45	73.4 ± 3.58
DUS (∇/mol)[a]	1.48 ± 0.06	1.48 ± 0.08	1.41 ± 0.07	1.35 ± 0.05	1.32 ± 0.07	1.46 ± 0.06	1.51 ± 0.05	1.37 ± 0.08
TFA	13.1 ± 0.71	13.9 ± 0.76	19.3 ± 1.11	18.7 ± 0.99	23.3 ± 1.35	15.6 ± 0.91	14.5 ± 1.10	23.1 ± 1.15

Fatty acid content is expressed as percentage of total fatty acids

LL low light, *HL* high light, N^- nitrogen deficiency, *HL* + N^- HL together with N^-, *HL* + N^- + *C* HL + N^- with addition of 10 μM cerulenin, *DK* dark, *DK* + *Glu* dark together with glucose addition, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *UFA* unsaturated fatty acids, *TFA* total fatty acids (%, on dry weight basis)

[a] ∇/mol, the degree of fatty acid unsaturation = [1.0 (% monoenes) + 2.0 (% dienes) + 3.0 (% trienes) + 4.0 (% tetraenes)]/100

(over 23 % of TFA) but C18:0 percentage is relatively low (below 4 % of TFA) under all tested conditions (Table 2).

The biosynthesis of fatty acids and expression of related genes in microalgae are subjected to stress conditions, among which HL and nitrogen deficiency are of greatest importance (Zhekisheva et al. 2005; Choi et al. 2011). We examined the expression pattern of *BC* and *SAD* exposed to induction of HL, N^- and HL + N^- as well as glucose. All these inducers stimulated the rapid up-regulation of the two genes, though the extent of expression varied (Figs. 4, 5). In accord with the drastic up-regulation, a great increase in the cellular content of TFA including OA was observed (Figs. 4, 5), suggesting the possible transcriptional control on fatty acid accumulation in *C. zofingiensis* by *BC* and *SAD*. The up-regulation of the two genes, however, was not tightly coordinated with the accumulation of fatty acids (24–48 h delay), indicating possible involvement of translational or post-translational control in addition to the transcriptional control of fatty acid biosynthesis. Regardless of the inducers, *SAD* exhibited much stronger expression than *BC* (Figs. 4, 5). This may explain the larger fold increase of OA as compared to TFA (Figs. 4, 5), as well as the enhanced OA proportion on a TFA basis (Table 2). In the presence of 10 μM cerulenin, a specific inhibitor of β-ketoacyl-ACP synthase I (KAS I), the synthesis of TFA and OA was greatly decreased, but the transcripts of both *BC* and *SAD* were not attenuated (Fig. 4), indicating that cerulenin might have no effect on the expression of these two genes, but rather specifically targets KAS I to reduce the intracellular fatty acid synthesis. The observed increase in *BC* transcripts (Fig. 4a) was likely due to stimulation by the substrate acetyl-CoA that may accumulate when cerulenin is added. The fatty acid composition varied upon inducers such as HL + N^-: C18:1 drastically increased, C16:0, C16:3, C18:0 and C18:3 decreased, whereas other fatty acids remained relatively stable (Table 2). This may suggest that the desaturase genes and other involved genes were differentially regulated to control the fatty acid composition in *C. zofingiensis*. From the biodiesel's point of view, the neutral lipid triacylglycerol (TAG) is of interest. The stress conditions also caused a great increase in TAG level in *C. zofingiensis* (data not shown). Thus, we hypothesized the genes coding the enzymes for TAG assembly, e.g., acyl-CoA:diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT), were up-regulated in *C. zofingiensis* with these conditions. Recently, it has been reported that nitrogen deficiency induced the up-regulation of *DTAG* and *PDAT* genes in *C. reinhardtii* (Miller et al. 2010; Boyle et al. 2012; Msanne et al. 2012) and *Chlorella vulgaris* (Guarnieri et al. 2011), which might indirectly support the above mentioned hypothesis.

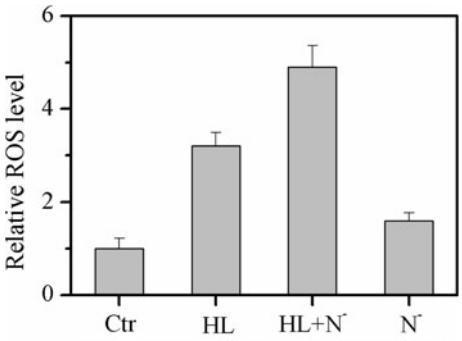

Fig. 6 Intracellular ROS abundance detected in *C. zofingiensis* following 24 h of different treatments. The ROS levels were normalized relative to the control cultures (low light in nitrogen replete medium), which was set to 1. For abbreviations, see Fig. 4

Oxygenic photosynthesis is usually accompanied by the generation of ROS, which can be accumulated during HL stress (Li et al. 2009). Nitrogen deficient conditions may also lead to an increase in intracelluar ROS through decomposing pigments and photosynthetic proteins (Sun et al. 2012). A significant buildup of ROS was observed in *C. zofingiensis* in response to stress conditions, in particular HL + N^- (Fig. 6), which may serve as a signal to trigger the up-regulation of fatty acid biosynthesis-related genes and accordingly the induced accumulation of fatty acids (Fig. 4). The enhanced de novo fatty acid synthesis, particularly the desaturation of C18:0–C18:1, consumes a large amount of NAD(P)H and oxygen, which in turn could contribute to the reduction of ROS and protect cells from damage (Chen 2007).

Glucose plays pivotal roles in regulating many genes involved in glycolysis, respiration, lipid biosynthesis and stress response (Rolland et al. 2006). It was suggested that glucose effects on the expression of genes might be mediated by glucose sensing, changes in flow of electrons during respiratory electron transport, or ROS (Moller 2001; Moore et al. 2003; Ryu et al. 2004; Li et al. 2008). In this study, glucose was found to be able to greatly up-regulate the expression of both *BC* and *SAD* genes and enhance the accumulation of TFA including OA (Fig. 5). We assume the mechanisms mentioned above might be involved in the regulation of the two genes, but this remains open and needs to be experimentally supported. In addition, glucose catabolism produces the pool of acetyl-CoA that can serve as precursors entering the fatty acid biosynthetic pathway. The rapid consumption of glucose by *C. zofingiensis* (data not shown) may result in a buildup of acetyl-CoA, which in turn stimulates the up-regulation of downstream genes involved in fatty acid synthesis including *BC* and *SAD*.

The well-studied pathway of fatty acid biosynthesis and characterization of genes involved in the fatty acid biosynthesis have greatly facilitated the genetic modification of higher plants for oil and fatty acid production, e.g., to increase the total oil content (Lardizabal et al. 2008), to alter the composition of fatty acids (Graef et al. 2009), or to produce new fatty acids for nutritional improvement (Cheng et al. 2010). Genetic manipulation of the fatty acid/lipid synthesis in microalgae has also been tried, e.g., the overexpression of ACCase gene in *Cyclotella cryptica* (Sheehan et al. 1998) and overexpression of *DGAT* genes in *C. reinhardtii* (La Russa et al. 2012). These attempts, however, failed to increase the intracellular oil level, suggesting the fatty acid/lipid synthesis in microalgae may be based on a complicated interplay of cellular pathways that are tightly controlled and overexpression of a single gene may not be sufficient to enhance the whole lipid biosynthetic pathway. Better understanding of regulatory processes of fatty acid biosynthesis and lipid metabolism and the availability of more sophisticated genetic manipulation tools might be necessary for the successful engineering of microalgae of a production strain such as *Chlorella* for enhanced oil production. The current study is the first report of the isolation and characterization of *Chlorella*-derived *SAD*. We investigated the expression of this desaturase gene as well as *BC* at the transcriptional level. These results might help better understand the regulation of fatty acid biosynthesis in *C. zofingiensis* at molecular level and benefit future genetic modification of this alga to enhance fatty acid production and/or alter fatty acid composition for improving its qualities as a biodiesel feedstock.

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