Growth dependent silencing and resetting of *DGA1* transgene in *Nannochloropsis salina*

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Abstract

Here we report recombinant expression and activity of the *Saccharomyces cerevisiae* type 2 diacylglycerol acyltransferase *DGA1* functioning in parallel with the native *Nannochloropsis salina* genes. Expression of *DGA1* shifted the chain length distribution of fatty acids produced and reflected an oleoyl- CoA substrate preference. Effect on the total FAME content was moderate and elevated by a maximum of 38%. Expression of the *DGA1* transgene varied throughout the culture life cycle and evidence of growth dependent environmental silencing of the transgene was observed. This is to our knowledge the first example of silencing and subsequent resetting in a transgenic microalga. Results from this study add valuable insights into the efficacy of algal genetic engineering and use of these microorganisms as bio-platforms for chemical manufacture.

Key words

Nannochloropsis; microalgae; lipid; biofuels; silencing; chromatin.

1. Introduction

While significant progress has been made in recent years in seeking alternative renewable forms of energy, at present most of these advances provide energy in the form of electricity. Oil remains the world's leading fuel amounting to 32.6% of the global energy consumption in 2014 (BP, 2015) and highlights the need for alternative sources of liquid transportation fuels to replace the over reliance on this diminishing commodity.

Liquid biofuels offer a promising alternative to petroleum based transportation fuels (Demirbas, 2011; Mata et al., 2010). Production from seed oil and cellulosic ethanol however cannot satisfy the current demand and impact negatively on both food and water security (Chisti, 2007; Norsker et al., 2011). In addition, whilst substituting gasoline with biofuels should reduce global CO_2 emissions which grew by an estimated 0.5% in 2014 (BP, 2015), changes in land use to provide sufficient feed stock is predicted to actually increase the greenhouse gas emissions by as much as 50% (Searchinger et al., 2008).

The use of microalgae has generated considerable interest as a high impact source of bioenergy and chemical feedstock (Hannon et al., 2010); growth facilities could be located adjacent to or within aquatic environments or on marginal land which, if coupled with the use of marine algal species, would reduce the impact on decreasing fresh water supplies (Chen and Smith, 2012). These photosynthetic microorganisms have low input nutrition requirements when compared to non photosynthetic microbes for light and whilst producing large amounts of biomass over short periods of time (Brennan and Owende, 2010) Additionally light delivery can be optimised, nutrients recycled and more importantly using closed systems the rate of photosynthesis can be improved through the maintenance of high CO_2 concentrations and optimal production conditions such as temperature and pH. All algae have the capacity to produce energy rich oils

and indeed a number of algal species have been found to accumulate oils up to 70% of their dry biomass under optimal conditions (Hannon et al., 2010; Scott et al., 2010).

Despite technical advances algal derived biofuels remain uncompetitive with present day fossil fuels (Norsker et al., 2011). The use of hydrothermal liquefaction (HTL) may improve the economics since there is no need to dry the material first and the process can recover up to 80% of the carbon and up to 90% of the chemical energy originally present in the microalga as either bio-oil or gas products (Brown et al., 2010; Elliott et al., 2015).

Improving the overall lipid accumulation within algae normally requires an increase in the lipids stored in the form of triacylglycerol (TAGs) and it is well-known that many algae accumulate TAGs in large quantities during the stationary phase of culture growth (Spolaore et al., 2006). To be commercially viable however, production requires a non–stop, semi-continuous culturing regime where the cells are maintained in the exponential phase. This will consequently require an increase in the natural level of lipid accumulated during the early phases of culture growth (Chisti, 2007). Whilst lipid accumulation increases can be achieved via strain selection under selective pressure or by means of random mutagenesis (Beacham et al., 2015), genetic engineering of optimal strains is likely to be faster and more efficient in terms of TAG production (Chen and Smith, 2012). It should be noted however that engineering an efficient pathway for the production of a specific product could then inhibit through feedback inhibition.

Conversion of fatty acids into TAGs serves the algae in two main ways, firstly allowing carbon storage in a very dense energy form and secondly it neutralises free fatty acids (FFAs) and other lipotoxic derivatives. There are two metabolic pathways for the production of TAG, an acyl-CoA dependent and acyl-CoA independent pathway. The major route of *de novo* TAG biosynthesis (KEGG pathway map ko00561) is thought to be via the acyl-CoA dependent pathway

commonly known as the Kennedy pathway. Acyl-CoAs are sequentially added to the sn-1, sn-2 positions of a glycerol-3 phosphate molecule followed by de-phosphorylation to form diacylglycerol (DAG). The last and only committed step in TAG biosynthesis is the acylation of DAG at the sn-3 position, and it is catalysed by the activity of diacylglycerol acyltransferase (DGAT) (Kennedy, 1961). This enzyme has significant potential for biotechnological purposes, offering the prospect of increasing the oil content of oleaginous species. Multiple DGATs are present in most eukaryotic organisms and there are at least two major classes of DGAT genes (type 1 and 2) that are frequently seen in algae, the enzyme products of both are membrane bound and catalyse the same reaction. DGAT1 and DGAT 2 belong to two different gene family's which likely evolved separately with functional convergence despite wide molecular and structural divergence (Kroon et al., 2006; Turchetto-Zolet et al., 2011). Studies have shown that both DGATs play a strong roll in TAG regulation with the specific enzyme activity often tissue and/or species specific (Chen and Smith, 2012). Additionally DGAT2 is often observed to have a critical role in the accumulation of unusual FAs (Oelkers et al., 2002; Xu et al., 2014). DGAT has also been proposed to be the rate limiting enzyme in storage lipid accumulation (Ichihara et al., 1988; Perry et al., 1999) and it has been shown that overexpression of this enzyme can lead to elevated lipid accumulation (Ahmad et al., 2015; Dey et al., 2014; Jako et al., 2001).

Previous work (Beacham et al., 2014) identified *Nannochloropsis salina* 849/3 as an ideal candidate for use as biofuel feed stock and target for genetic manipulation due to its capacity to accumulate high levels of lipid during stationary culture phase, coupled with a thin cell wall which should provide less resistance to DNA penetration than many other oleaginous algae species.

In this study, we sought to increase the availability of DGAT to determine if the level of TAG accumulation during the exponential phase of growth could be significantly enhanced. We used *Agrobacterium tumefaciens* mediated transformation to incorporate the *Saccharomyces cerevisiae* DGAT2 (*DGA1* gene) into *N. salina*. DGA1p is the sole member of the DGAT family of enzymes in *S. cerevisiae*. The substrate specificity of DGA1p is well defined and whilst it can utilise a range of acyl-CoA substrates the preferred substrates are oleoyl-CoA (C18:1) and palityl-CoA (C16:0) (Oelkers et al., 2002). This specificity makes this gene an ideal candidate for this study since *N. salina* naturally accumulates high levels of C16:0 and moderate levels of C18:1. Changes in the levels of these fatty acids as well as the impact of removal of C18:1 from the FA pool on the production of very long chain polyunsaturated fatty acids (PUFAs) was assessed alongside growth and productivity analysis over a period of 58 days. Transcript analysis indicated possible environmental conditioned silencing and the implications of this for future modifications are discussed. Results from this study add valuable insights into the efficacy of algal genetic engineering and use of these microorganisms as bio-platforms for chemical manufacture.

2. Methods

2.1 Strains.

Nannochloropsis salina (CCAP 849/3) was obtained from the Culture Collection of Algae and Protozoa (Scottish Association for Marine Science, Oban, Scotland, U.K.).

2.2 Culture conditions.

Stock cultures were maintained under batch culture conditions (0.5 L) in F/2 medium (Guillard, 1975) using fresh sterilised seawater at 90 % (30 g L^{-1}) salinity (F/2-90), pH 8.2, maintained under 100 µmols photons m^2 sec^{-1} irradiance on a 16 h: 8 h light: dark cycle at 25 ºC, agitated daily and sub-cultured on a bi-weekly basis. Experimental time course cultures in duplicate (1.5 L in 2 L growth vessels) were inoculated at a density of 1 x 10^5 cells ml^{-1} from stock cultures in mid logarithmic phase (to minimise lag phase between strains and favour close synchronisation during the growth phase). All cultures were maintained under the same lighting and temperature regime as for the stocks but additionally bubbled with 0.2µm filtered ambient air and maintained without sub-culturing into stationary growth phase.

2.3 Cloning *DGA1* (T-DNA vector construction)

Full length *DGA1* gene (NCBI Reference Sequence: NM_001183664.1) (Goffeau et al., 1996) was amplified from the gDNA of *Saccharomyces cerevisiae* strain BY4742 using primers GW_Y_DGAT_F (5'GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GTC AGG AAC ATT CAA TGA TAT AAG '3) which targets the first 26bp of the gene and includes a flanking wing region which includes an upstream kozak sequence and *att*B1 sequence and GW_Y_DGAT_R2 (5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA CCC AAC TAT CTT CAA TTC TGC 3) targeting the last 24bp of the gene and includes a downstream wing region containing the *att*B2 sequence. Purified gene fragment was

transferred via the GATEWAY™ *att*B1 and *att*B2 sequences into pDONOR-zeo and then recombined in to the T-DNA destination vector pEG101B (an adaptation of the pEARLYGATE101 vector (Earley et al., 2006) modified to include the Hygromycin B resistance gene (*hyg*) under the control of the tef promoter and terminator) such that the DGA1 gene falls under the control of the CaMV35S promoter and the resulting plasmid was designated pEG101B:DGAT. ElectroMAX™ *A. tumefaciens* LBA4404 (Invitrogen) were transformed with 100 ng DNA (pEG101B:DGA1) plated on selective medium (YM supplemented with 50μg ml^{-1} kanamycin and 100μg ml^{-1} Streptomycin) and incubated for 48 hours at 30 °C. An individual colony (ABF10) containing the peg101b:DGA1 was isolated and the fidelity of the construct was rechecked by DNA sequencing. Vector map is provided in supplementary materials Figure 1.

2.4 Agrobacterium mediated transformation of *N. salina*

N. salina 849/3 cells at mid log phase (5 $x10^7$ cells) were washed and then re-suspended in 600 μl fresh sterilised sea water at 10% (3.3 g L^{-1}) salinity, (F/2 -10) at pH 5.6. Freshly cultured *A. tumefaciens* ABF10 was washed and then re-suspended in the F/2-10, pH 5.6, at A_{600} 0.5. The microalga was then mixed with 1200 μl ABF10 and vanillin added to give a final concentration of 400 μM. Cells were co-incubated as a thin liquid layer in a 25 ml vented culture bottle in the dark at 25 ^{0}C for 3 days. Samples were exposed to light for 30 min., 20 ml fresh F/2-90 medium containing cefotaxime to a final concentration 500 μg ml^{-1} added and then placed back in the dark for a further 3 days. Samples were allowed to recover under standard 16:8 light:dark conditions for a period of approximately 4 generations (7 days) then pelleted and plated for single colonies on solid media (F/2-90 with 0.8 % agarose containing hygromycin B at 300 μg mL^{-1}). Plates were initially incubated for 3 days in the dark for antibiotic selection then transferred to the light until colony appearance. All incubation steps were performed at 25 ^{0}C.

160 **2.5 Growth rate determination.**

161 Culture density was determined via light microscope cell enumeration in a haemocytometer
162 following staining with Lugols iodine solution (2 %). Specific growth rates (K) were calculated
163 according to the following equation:

164 $K = \ln [(n2/n1) / (t2-t1)]$

165 where n2 and n1 are the total cells mL^{-1} at time point (t2) and time point (t1) respectively, and
166 where t2 > t1.

167 **2.6 Lipid analyses.**

168 Since HTL is impractical on a small scale, fatty acid concentrations and profiles in microalgal
169 cells were determined post conversion to fatty acid methyl esters (FAMEs) using GC-MS
170 (Agilent 7890A GC and 5975C inert MSD, Agilent Technologies Ltd., Edinburgh, UK). Culture
171 samples were centrifuged (10,000 × *g*), washed in distilled water and resulting pellets
172 lyophilised. Nonadecanoic acid (C19:0) was added as an internal standard and cellular fatty acids
173 were converted directly to FAMEs by adding 1 mL of transesterification mix (95:5 v/v 3 N
174 methanolic HCl; 2,2-dimethoxypropane) followed by incubation at 90 ºC for 1 h. After cooling,
175 FAMEs were recovered by addition of 1 % w/v NaCl solution (1 mL) and *n*-hexane (1 mL)
176 followed by vortexing. The upper hexane layer was injected directly onto the GC-MS system as
177 previously described (Beacham et al., 2015). FAMEs were identified using retention times and
178 qualifier ion response and quantified using respective target ion responses. All parameters were
179 derived from calibration curves generated from a FAME standard mix (Supelco, Sigma-Aldrich,
180 Gillingham, Dorset, UK).

181 **2.7 Transcript analysis.**

At each time point for each culture, 20 ml culture was pelleted and ground under liquid nitrogen and total RNA extracted using TRIzol® Reagent (Life Technologies). RNA samples were subsequently treated with RNase-Free DNase (Qiagen) and 1μg used as template for cDNA synthesis using SuperScript® III First-Strand Synthesis kit (Invitrogen). Analysis by qPCR was performed on an ABI Prism7000 system (Applied Biosystems) in triplicate for each sample. The cDNA samples were diluted 5-20 fold for amplification of PCR fragments using TaqMan® Gene Expression Master Mix (25 ul reactions) with forward primers at 3 μm, reverse primers at 9 μM and the probe at 10 μm final concentrations. Gene specific primers and probes used: *N. salina* ribosomal 18S using FAM –TAMRA labelled gene specific probe NS18S_Probe (5' TGG CCT ACC ATG GCT CTA ACG GG 3') and primers NS18S(TM)F (5' TTC TGC CCT ATC AGC TTT GG 3') and NS18S(TM)R (5' GTC TCT CAG GCT CCC TCT CC 3'); *S. cerevisiae DGA1* using FAM –TAMRA labelled gene specific probe YDGAT_Probe (5' CCA CTT CGC CAG TTG CAG GAG A 3') and primers YDGAT(TM)F (5'TGT GGG TTC TTG CTA TTC CA 3') and YDGAT(TM)R (5' AAT GGG CAA TGA ACG AAA TC 3'). The latter primer probe set was designed and checked such that no amplification of the native *N. salina* DGAT genes occurred. The amplicon size of *18S* and *DGA1* PCR reactions were 100bp and 107bp respectively. The cycling parameters were as follows; 2 minutes at 50 °C (UDG incubation), 15 minutes at 95 °C for AmpliTaq activation followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute.

No amplification was detected in the WT 849/3 controls when using the *DGA1* primer probe set indicating no cross amplification of the native genes was occurring. Controls lacking template were also used with each primer-probe pair to ensure that probe/primer dimer was not causing false signal detection. The cycle at which the florescence passed the threshold (Ct) was

determined automatically using the on-board software and used to calculate the transcript level by comparison to a standard curve generated from a standard dilution series of plasmids containing the specific PCR fragment (generated with the TM primers given above and cloned into pGEM®-T Vector (Promega). Samples were normalised to one another by using the relative expression of *DGA1* to *18S*. RNA quality can be reduced during the stationary phase and if this is the case this may affect gene expression estimates. The raw 18S QPCR data are there for presented in supplementary table to demonstrate that the RNA quality was maintained throughout the time course.

3. Results and Discussion

To determine if we could improve the overall lipid accumulation of *N. salina* during the active culture growth phase, we generated *DGA1+*, *hyg+* mutants, using *Agrobacterium tumefaciens* mediated transformation of strain CCAP 849/3. Genomic DNA was extracted from individual colonies of *N. salina* transformed with *Agrobacterium* ABF10, and was screened for the presence of the *hyg* and *DGA1* transgenes and the ribosomal RNA 18S control gene for gDNA quality. Of the colonies picked only 15% contained the transgenes and this was indicative of the poor selective nature of Hygromycin B that we have observed throughout this study which made clone selection challenging. This antibiotic appears to be very susceptible to light and to the changes in salinity and pH which occur in the micro environment around the plated algae cells. Transformation efficiency was approximately 1 in 0.132 x 10^{-5} cells (0.000132%). Two positive transformants NBF22-8 and NBF22-9 were taken forward for metabolic and *DGA1* expression analysis.

3.1 Growth.

Both NBF22-8 and NBF22-9 displayed a significant reduction in growth rate during the growth phase compared to the wild type control (Figure 1). The period of time spent in the growth phase was however extended by approximately 3 days for both *DGA1+* mutants and was thus accompanied by an increase in the maximal cell density of 13-15% though this increase was not statistically significant in either mutant.

Reduction in growth rate in the mutants was not unexpected and is similar to that observed in random mutagenized *N. salina* that over accumulate lipids (Beacham et al., 2015), and is likely a result of limited resource and energy requirements being funnelled to lipid storage thus slowing cell replication.

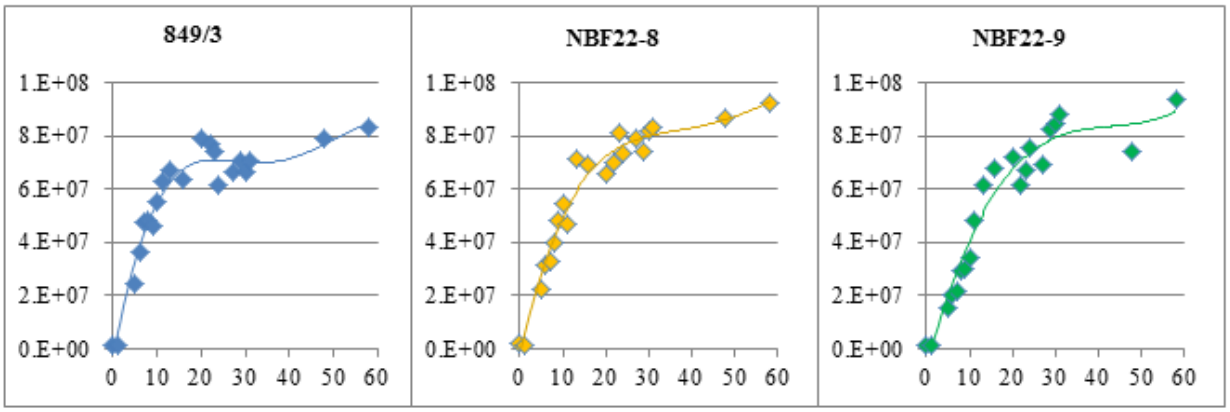

Figure 1 (double column fitting) Growth characteristics of wild type *N. salina* 849/3 and *DGA1+* mutants NBF22-8 and NBF22-9 over a 58 day period. Average data from 8 technical and 2 biological replicates for each strain. Doubling times during the exponential growth phase were: WT = 38.7hours, NBF22-8 = 64.3hours, NBF22-9 = 74.2 hours.

3.2 Expression.

Quantitative PCR was used for expression analysis of the *DGA1* transgene. Expression was plotted as a ratio of *DGA1 / 18S* and is given in Figure 2A. For both NBF22-8 and NBF22-9 *DGA1* was expressed only during the growth phase with little or no detectable transcript levels during the transition and stationary phases of growth. Besides the promoter and terminator sequences of the T-DNA construct no additional gene regulatory elements such as enhancer/silencers were co-transformed with the *DGA1* transgene which indicates a form of gene silencing has occurred via the native chromosomal environment. Silencing of a foreign gene shortly after integration is not uncommon (Dehio and Schell, 1994; Meins, 2000; Meyer, 2000) and depending on the type of silencing is often permanent. In this instance however our *DGA1+* strains had been propagated through many generations prior to the time course experiment presented here. If the genes had been permanently silenced shortly after insertion we would not have expected to detect any heterologous expression.

To confirm that the silencing of the *DGA1* gene was due to a transient effect, cultures of NBF22-8 and NBF22-9 that had been in stationary phase for 3 months were sub-cultured into fresh medium at a dilution of 1/20, and maintained under standard batch culture conditions (no additional aeration). Culture samples for transcript analysis were taken during the lag phase, the growth phase and stationary phases for both the refreshed cultures and the quiescent cultures from which they had been sub-cultured. Quantitative PCR analysis shows that the transcription of the transgene is indeed subject to growth phase specific silencing and can be reactivated upon returning the cells to an active growth state (Figure 2B).

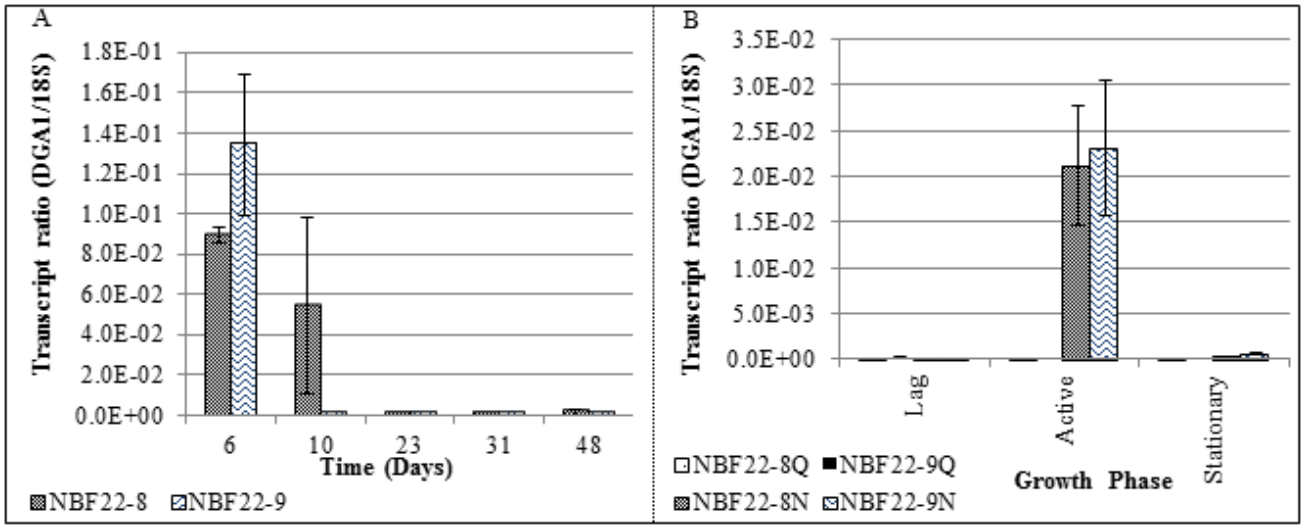

Figure 2. **(double column fitting)** QPCR analysis of DGAT transgene expression. Panel A - Expression over 48 day time course for clones NBF22-8 and NBF22-9. Panel B - Resetting of the *DGA1* gene following transfer to fresh medium. NBF22-8 and NBF22-9 refer to *DGA1+* clones. Q refers to a culture that has entered a quiescent stage. R refers to refreshed cultures (quiescent cells that have been sub cultured into fresh medium).

Based on investigations in plants, there are several possible explanations for the silencing and resetting observed. Individual genes are embedded in a highly complex chromatin structure and often groups of genes are packaged in a chromosomal matrix which is regulated by sophisticated chromatin remodelling mechanisms governing when a gene or set of genes are accessible for transcription (Meyer, 2000). An *N. salina* culture is not unlike a plant in the sense that the processes occurring within a given cell cycle vary widely between cells in early exponential growth and those in the quiescent state observed in nutrient deplete conditions. It is well know that *Agrobacter*ium-mediated gene transfer often favours T-DNA integration sites in transcriptionally active regions of the chromosome (Alonso et al., 2003; Gelvin, 2003; Tzfira et al., 2003), and it is likely therefore that the *DGA1* gene was integrated into a site that is active

during exponential growth (the state of the culture at time of transformation). Histone modification and DNA methylation act in accord in self-propagating epigenetic cycles that stabilise transcriptionally-active and -inactive states in response to environmental or developmental cues (Dehio and Schell, 1994). This transitioning from euchromatin to facultative heterochromatin causes gene silencing by way of inaccessibility of the transcriptional machinery and is fully reversible (Meyer, 2000). One possibility therefore is that the *DGA1* gene has integrated into a region of a chromosome that becomes inactivated in nutrient deplete conditions but is reactivated when environmental conditions become favourable once more.

A second possibility is homology dependent post transcriptional gene silencing. It has been frequently observed in plants that interactions between the trans and host genes of a similar sequence lead to transgene inactivation and targeted degradation at the mRNA level (Meyer and Saedler, 1996).

Evidence suggests that the mechanisms involved in post transcriptional gene silencing and resetting are closely linked to a variety of pathways involved in sensing stress and developmental cues and that sequence similarity of 60-70% between the trans and native genes is sufficient to activate this kind of RNA degradation leading to gene silencing (Meins, 2000). Whilst resetting of post transcriptional gene silencing has been detected in plants (Balandin and Castresana, 1997; Dehio and Schell, 1994) it is regularly observed to occur after meiosis, a process that is thought not to occur in *N. salina* though this does not necessarily preclude this method of silencing. Whilst we have no empirical evidence of the *DGA1* gene integration site for either NBF22-8 or NBF22-9 we suppose the former chromatin silencing model to be the more likely mode of action in this investigation.

306 **3.3 Lipid analysis.**

307 Batch cultures of wild type *N. salina* show a predictable shift in lipid storage, from lag where
308 stored lipid is reduced through exponential growth where lipid levels stabilise to between 5-15%.
309 During the transition from logarithmic to stationary growth, levels rise rapidly to as much as 30 -
310 50%, (Beacham et al., 2014; Beacham et al., 2015). We therefore assessed the contribution of the
311 *DGA1* transgene expression on lipid accumulation over a 58 day period from lag through to late
312 stationary phase.

	Total average FAME content (pg/cell)					Mid Exponential Productivity (µg ml-1 d-1)
	Day 10	Day 16	Day 23	Day 31	Day 48	
WT (849/3)	0.85 (±0.01)	1.25 (±0.02)	1.79 (±0.02)	4.59 (± 0.19)	6.32 (±0.29)	11.03(±0.2)
NBF22-8	0.97 (±0.02) **↑13.8 %**	1.40 (± 0.02) **↑11.3 %**	2.12 (± 0.03) **↑18.5 %**	4.33 (± 0.05)	5.88 (±0.15)	19.29 (±2.1) **↑74.9 %**
NBF22-9	0.94 (±0.02) **↑10.6%**	1.49 (±0.15) **↑18.8 %**	2.48 (±0.02) **↑38.3 %**	4.96 (±0.17)	6.60 (± 0.10)	11.43 (±0.3)

Table 1. Total FAME content over a 48 day period and productivity during mid exponential growth, with (SEM). Significant changes in the transgenic lines given as a % change from the WT control.

Total FAME content was elevated in both NBF22-8 and NBF22-9 during the first 23 days compared to the wild type control, but during subsequent time points the lipid content of these strains fell back to levels comparable with the control (Table 1). *N. salina*, like many marine algae, maintain a "reserve level" of TAG which has functions beyond energy storage; in maintaining a source of the long chain fatty acids required for plastid membrane maintenance, as a store for secondary carotenoids and preventing photo-oxidative injuries by consuming excess photoassimilates (Guschina and Harwood, 2006; Solovchenko, 2012). We speculate that the ability of NBF22-8 and NBF22-9 to extend the length of time spent undergoing cell proliferation prior to entering the semi-quiescent state of stationary phase may be due to the utilisation of the extra lipids accumulated during early exponential phase and would thus account for the normalising of lipid content to a level comparable to the wild type cultures.

Lipid production on an industrial scale will likely require a semi continuous culturing system where the culture is maintained and harvested in an active growing state. As such the overall lipid productivity for each strain was assessed for the period of mid exponential growth (Table 1). The elevated lipid content of NBF22-8 was translated into a significant increase in overall productivity despite a decreased growth rate. For NBF22-9 however the reduction in growth rate cancelled out the gains in lipid accumulation and the overall productivity of this transgenic strain was not significantly different from the wild type control.

Despite the *DGA1* gene being silenced early in growth the recombinant strains continued to maintain an elevated lipid content well beyond the point when the *DGA1* gene had been silenced. It has been shown that in non- adipocytes lipid storage can be induced by various stimuli including the presence of long chain unsaturated fatty acids such as oleic acid (C18:1) (Melo et al., 2011). It is therefore plausible that the elevation in C18:1 (Table 2) in the *DGA1+* mutants

caused a feedback loop to continue lipid storage even after the *DGA1* gene had been silenced, and because the cells were grown in an enriched medium the levels of excess lipid was maintained until the culture became nutrient deplete.

		Major Fatty Acids													Saturation profile		
		C14:0	C15:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3γ	C20:0	C20:3	C20:4	C20:5	C24:0	SFA	MUFA	PUFA
DAY 10	wt	3.9 ± 0.1	0.5 ± 0.0	31.6 ± 0.4	29.2 ± 0.2	2.0 ± 0.1	7.1 ± 0.1	4.8 ± 0.1	0.9 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	3.7 ± 0.1	11.5 ± 0.5	0.9 ± 0.0	40.8 ± 0.6	36.8 ± 0.3	22.3 ± 0.7
	NBF22-8	4.0 ± 0.4	0.5 ± 0.1	29.7 ± 1.9	28.7 ± 1.5	2 ± 0.3	7.5 ± 0.1	4.3 ± 0.2	1.1 ± 0.2	0.7 ± 0.2	1.2 ± 0.3	4.2 ± 0.4	12.8 ± 2.6	0.6 ± 0.4	39.2 ± 2.1	36.6 ± 1.2	24.2 ± 3.2
	NBF22-9	3.7 ± 0.5	0.5 ± 0.0	28.9 ± 1.7	28.3 ± 1.2	2 ± 0.3	8.3 ± 0.5	4.4 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	1.2 ± 0.2	4.4 ± 0.3	13.5 ± 1.5	0.7 ± 0.5	37.9 ± 1.4	37.1 ± 0.8	25.0 ± 2.0
Day 16	wt	3.3 ± 0.6	0.6 ± 0.1	34.3 ± 0.9	28.7 ± 0.8	2.6 ± 0.1	9.9 ± 0.4	4.9 ± 0.1	0.7 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	3.6 ± 0.2	8.7 ± 0.6	0.2 ± 0.0	42.3 ± 0.4	38.9 ± 0.8	18.7 ± 1.2
	NBF22-8	4.1 ± 0.3	0.7 ± 0.2	32.4 ± 0.4	27.8 ± 0.5	2.7 ± 0.1	11.5 ± 0.7	3.6 ± 0.1	0.7 ± 0.0	0.2 ± 0.0	0.9 ± 0.3	4.0 ± 0.3	9.4 ± 0.3	0.1 ± 0.0	41.6 ± 0.9	39.7 ± 0.3	18.8 ± 1.1
	NBF22-9	3.7 ± 0.9	0.5 ± 0.1	34.9 ± 6.7	28.0 ± 10.0	2.4 ± 0.6	5.4 ± 4.9	4.1 ± 0.4	1.1 ± 0.2	0.6 ± 0.1	1.1 ± 0.2	4.8 ± 1.1	10.0 ± 1.9	0.8 ± 0.1	44.8 ± 8.3	33.9 ± 11.9	21.3 ± 4.3
Day 23	wt	2.6 ± 0.0	0.7 ± 0.0	39.7 ± 0.5	32.3 ± 0.2	2.0 ± 0.0	10.0 ± 0.2	3.1 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	2.2 ± 0.1	4.4 ± 0.2	0.0 ± 0.1	46.3 ± 0.3	42.8 ± 0.1	10.9 ± 0.3
	NBF22-8	3.0 ± 0.1	0.6 ± 0.1	36.9 ± 0.2	31.9 ± 0.6	2.0 ± 0.0	12.9 ± 0.8	2.1 ± 0.1	0.7 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	2.4 ± 0.1	4.8 ± 0.1	0.2 ± 0.1	44.1 ± 0.1	45.3 ± 0.2	10.6 ± 0.3
	NBF22-9	2.9 ± 0.1	0.6 ± 0.0	35.4 ± 1.2	31.9 ± 0.5	2.1 ± 0.0	13.1 ± 0.4	2.2 ± 0.1	0.8 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	2.9 ± 0.3	5.4 ± 0.5	0.2 ± 0.1	42.7 ± 1.2	45.5 ± 0.4	11.8 ± 1.1
Day 31	wt	2.4 ± 0.0	0.6 ± 0.0	40.8 ± 0.3	33.7 ± 0.2	1.9 ± 0.0	11.0 ± 0.1	2.5 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	1.7 ± 0.0	2.8 ± 0.1	0.0 ± 0.0	46.9 ± 0.3	45.0 ± 0.2	8.1 ± 0.1
	NBF22-8	2.8 ± 0.2	0.6 ±0.1	38 ± 2.1	33.9 ± 1.9	1.8 ± 0.1	13.0 ± 4.3	1.6 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	1.8 ± 0.1	3.3 ± 0.2	0.0 ± 0.1	44.7 ± 2.3	47.4 ± 2.5	7.9 ± 0.3
	NBF22-9	2.5 ± 0.2	0.5 ±0.0	36.7 ± 0.6	33.8 ± 0.5	1.9 ± 0.1	14.7 ± 0.5	1.6 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	2.0 ± 0.1	3.3 ± 0.2	0.0 ± 0.1	43.0 ± 0.7	48.9 ± 0.2	8.1 ± 0.6
Day 48	wt	2.0 ± 0.2	0.5 ±0.0	43.7 ± 0.6	34.8 ± 0.6	1.5 ± 0.0	11.1 ± 0.4	2.0 ± 0.0	0.8 ± 0.1	0.1 ± 0.2	0.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.0 ± 0.0	48.7 ± 0.7	46.3 ± 0.7	5.1 ± 0.1
	NBF22-8	2.3 ± 0.2	0.6 ± 0.0	39.4 ± 0.8	35.6 ± 0.6	1.6 ± 0.2	14.7 ± 1.0	1.6 ± 0.1	0.8 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	0.0 ± 0.0	44.8 ± 0.9	50.7 ± 0.8	4.5 ± 0.2
	NBF22-9	2.2 ± 0.1	0.6 ± 0.0	39.2 ± 0.8	35.9 ± 0.8	1.6 ± 0.1	14.7 ± 0.4	1.6 ± 0.1	0.8 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	1.1 ± 0.2	1.1 ± 0.2	0.0 ± 0.0	44.5 ± 0.9	50.9 ± 0.7	4.6 ± 0.7

Table 2. Changes in fatty acid profile and levels of saturation over a 48 day period with (±SD). Significant changes in the transgenic lines given as a % change from the WT control

Alterations in the fatty acid profiles of both NBF22-8 and NBF22-9 saw a reduction in palmitic acid (C16:0) and linoleic acid (18:2) and a corresponding increase in C18:1 content (Table 2). Arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5) were also elevated compared to the control and the overall level of unsaturation was increased in the transition and stationary phases. For both *DGA1+* strains, the overall level of stored lipid returned to levels comparable with the unmodified control once the transgene had been silenced but interestingly changes to the FA profile arising from transgene expression were maintained throughout the time course.

Very long chain polyunsaturated fatty acids (VL-PUFAs) are known to play important roles in sustaining membrane structure and function including maintaining optimal membrane fluidity and providing an antioxidative function facilitating protection against biotic and abiotic reactive oxygen species (Okuyama et al., 2008). In the wild type control cells the levels of EPA and ARA started at relatively high levels, 3.7 % and 11.5% respectively, but gradually declined as the culture aged. This fall is likely a response to change in cell culture activity from vigorous respiration and growth (high levels of membrane production and maintenance required) through to energy storage and cell quiescence. Both NBF22-8 and NBF22-9 showed the same fall in EPA and ARA over time as the wild type, although with the exception of day 48, the levels of both theses VL-PUFAs were significantly elevated compared to the wild type controls throughout the time course. This elevation is unlikely to be as a result of *DGA1* activity and probably the result of the mutant cultures lagging behind the wild type in terms of culture progression; the slower doubling time of the mutants mean that the cultures had a slower transition from exponential to stationary phase and as such the turnover of ARA and EPA was slower leading to the appearance of elevated levels.

It had been anticipated that the sequestration of more C18:1 in TAG could mean less resource available for chain lengthening which might ultimately reduce the overall PUFA content and thereby improve suitability for use in biodiesel. As we have seen this was not the case, with the maintenance of VL-PUFAs levels being tightly controlled. The reduction in C16:0 and C18:2 is likely a consequence of the change in flux with more C16:0 being diverted to chain lengthening to maintain the levels of PUFAs combined with a reduction in the level of C16:0 being sequestered in TAG. Competition for the C18:1-acyl-CoAs between the DAG1p and the Δ12 desaturase responsible for the conversion of C18:1 to C18:2 combined with the need to maintain flux in to the VL-PUFAs is likely responsible for the reduction in C18:2 seen in both NBF22-8 and NBF22-9. That the levels of C20:4 and C20:5 are maintained whilst the levels of 18:2 declined indicates that the Δ12 desaturase is likely the rate-limiting step in this biosynthetic pathway, and also indicates that both the KasII and Δ9 desaturase enzymes responsible for the conversion of C16:0 to C18:1 and the DAG1p enzyme have a higher turnover than the Δ12 desaturase.

3.4 Conclusion

We have demonstrated that increasing the availability of DGAT by introducing an additional copy of the transgene can be useful in over stimulating accumulation of lipids in *N. salina*. It is well known that an increase in lipid accumulation is frequently accompanied by a reduction in growth rates as more resources are diverted to energy storage and away from propagation. Choice of transgene is therefore important both for the level of activity and substrate specificity– we chose a DGAT2 (*DGA1*) for its well defined mode of action and steady activity. We have demonstrated here that "less is more" with the *DGA1* gene generating only a modest increase in

393 lipid storage which had only a low level impact on overall growth rates and hence generated a
394 significant increase in overall lipid productivity. Use of *Agrobacterium*-mediated T-DNA vector
395 technology proved to have unforeseen consequences arising from the mode of *Agrobacterium*
396 action – integration into a transcriptionally-active region of the genome which was only active
397 during a short phase of the culture life cycle. The silencing observed has implications for the
398 expression of the selection marker and may be a reason for the frequent failure of selection and
399 apparent low transformation efficiency observed. It may also account for the loss of
400 transformants when maintained in selective media over an extended period due to the
401 intermittent expression of resistance markers undermining selection.
402 It is interesting to note that the duration of transient expression appears to be longer for NBF22-8
403 compared with NBF22-9 (Fig. 2A) and that the growth rate is also faster (Fig. 1). NBF22-8 also
404 demonstrates greater mid-exponential productivity (Table 1). This suggests that whilst both
405 strains display similar transient gene expression patterns the local conditions for gene expression
406 may not be the same. It is possible that transgene insertion has occurred in the same general
407 region of the genome but that the sites of insertion are distinct resulting in localised variation in
408 transgene expression. Another possibility is that if the site of insertion is indeed identical then the
409 construct has inserted in opposite orientations relative to the surrounding DNA, again resulting in
410 localised variation in transgene expression.
411 This environmentally-controlled conditional silencing whilst not the intended outcome, could be
412 an advantageous way of naturally controlling transgene expression in the heterologous host,
413 especially if the product is toxic to the host, unstable or energetically expensive to synthesise.
414 Host -regulated gene expression could provide for maximal output and no loss of energetic
415 resources during the growth phases when the product is not required allowing for a more

efficient production strategy. Further analysis of the transgene insertion site will provide new insights into the complex, but little understood, mechanisms of gene regulation adopted by microalgae which is essential if microalgae are to deliver on their promise as photsynthetically-driven biofactories.

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