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Research Article

Gene expression analysis of a Louisiana native *Chlorella vulgaris* (Chlorophyta)/*Leptolyngbya* sp. (Cyanobacteria) co-culture using suppression subtractive hybridization

A locally isolated co-culture of two photosynthetic species [*Chlorella vulgaris* (Chlorophyta) and *Leptolyngbya* sp. (Cyanobacteria)] displayed enhanced growth when compared to a *Chlorella* monoculture; however, the biological mechanisms driving such improvement are currently not well understood. To investigate these mechanisms, this study examined the differential gene expression in the *Chlorella* between the co-culture and the monoculture. Suppression subtractive hybridization was performed between mRNA from *Chlorella* in the co-culture and in a monoculture, and 105 genes were identified as being putatively differentially expressed. Nine of these genes, corresponding to the key functional categories of energy, metabolism, and protein synthesis, were further examined using quantitative real-time PCR and showed differential regulation of photosystem I and photosystem II and upregulation of stress-response genes and a gene encoding an oil-globule-associated gene in the co-culture *Chlorella*. This differential gene expression study of a *Chlorella*/cyanobacteria co-culture will aid in the development of culture strategies capable of taking advantage of these differences for the production of biomass and bioproducts of interest. Knowledge of the underlying genetic causes of the changes in growth and productivity of the species in co-culture provides insights on possible target genes for optimization of the culture.

Keywords: *Chlorella vulgaris* / Irradiance / *Leptolyngbya* / Native co-culture / subtractive hybridization

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1 Introduction

Since the discovery of petroleum, fossil fuels have grown to be an integral source of energy worldwide. Nonetheless, concerns with greenhouse gas emissions and dependence on petroleum have prompted considerable interest in renewable sources of energy. In particular, microalgal production of lipids for biofuels has been investigated because theoretical energy yields for microalgae are estimated at 10–100 times those of terrestrial crops [1]. Furthermore, algae can be grown in environments not conducive to growth of terrestrial plants [2], using saline or brackish water or even wastewater [3]. Finally, algae can provide added benefit by removing nitrogen and phosphorous from the wastewater [4, 5].

Nonetheless, much work remains to be done to enhance the economic feasibility of microalgal biofuels. One key area for progress is strain selection [6], and an ideal algae strain for biofuels production will display excellent growth physiology (high growth rates and cell densities as well as tolerance to conditions of pH, temperature, and dissolved O_2 and CO_2 encountered in culturing), high lipid productivity, and robustness [3]. One

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Abbreviations: **CC**, *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture; **Chl**, *Chlorella vulgaris* monoculture; **ESTs**, expressed sequence tags; **PCI**, phenol/choloroform/isoamyl alcohol; **PPP**, phosphoprotein phosphatase; **PSI**, photosystem I; **PSII**, photosystem II; **qPCR**, quantitative real-time PCR; **ROS**, reactive oxygen species; **SSH**, suppression subtractive hybridization

particular challenge is that strains possessing high lipid productivity in laboratory studies often do not show the same tendency in large-scale cultures [7]. Additionally, in economical outdoor pond conditions, contamination of laboratory strains by invasive species is a key problem [8]. One solution to finding an ideal algae strain for large-scale outdoor pond cultivation is the identification of robust native species that are already dominant in the environment in which they would be cultivated and that display excellent growth physiology and high lipid productivity.

A native co-culture consisting of *Chlorella vulgaris* and the cyanobacterium *Leptolyngbya* sp. was isolated from a lake contained in Louisiana (Rusch and Gutierrez-Wing, unpublished data). The co-culture has all three of the aforementioned desired traits of good growth physiology, high lipid productivity, and strain robustness. In particular, the co-culture displayed higher biomass levels and superior strain robustness compared to an algal monoculture of the *C. vulgaris* (Rusch and Gutierrez-Wing, unpublished data). Enhancement of *C. vulgaris* growth by co-culturing with bacteria has been widely observed [9–14]. The co-culture in this study is of great interest because it consists of two photosynthetic species rather than an algae and a bacterium. The biological mechanisms behind the enhanced growth of *C. vulgaris* in co-cultures is not well understood, but an understanding of these mechanisms is needed to optimize the growth and lipid productivity of the culture to enhance the economic viability of its cultivation for biofuels production.

To understand the enhanced growth of the *C. vulgaris* in the Louisiana strain co-culture, a comparison of the underlying genetics of the algae in the co-culture compared to the algae in the monoculture is essential. Suppression subtractive hybridization (SSH) is a widely used technique for identification of differentially expressed genes between tissues or cells in two different environmental conditions. The technique has been applied in the past to study differentially expressed genes in algae involved in tolerance of hyperosmotic shock [15], predator avoidance [16], and acquisition of freezing tolerance [17]. In this study, SSH was used to test the hypothesis that the gene expression of *C. vulgaris* in the monoculture and in the co-culture with *Leptolyngbya* sp. was different.

The present work identified genes differentially expressed in *C. vulgaris* in the monoculture and in the co-culture. cDNA clones corresponding to differentially expressed genes in the monoculture and co-culture were isolated by SSH. DNA sequences identified by SSH were compared to those in databases, and the genes were grouped by functional classification. Finally, quantitative gene regulation for selected genes representing functional categories prevalent in the genes identified by SSH was measured by quantitative real-time PCR (qPCR).

2 Materials and methods

2.1 Algal cultures

A mixed algal culture of *C. vulgaris* and *Leptolyngbya* sp. was isolated from College Lake (30°24′24.1308″N, 91°10′12.486″W) in Baton Rouge, LA, USA (Rusch and Gutierrez-Wing, unpublished data). A *Chlorella vulgaris* monoculture (Chl) was isolated from the mixed culture by serial dilution and plating on nutrient agar (BD Difco, Franklin Lakes, NJ, USA). Although neither the *Chlorella vulgaris*/*Leptolyngbya* sp. co-culture (CC) nor the Chl is axenic, *Leptolyngbya* sp. is the sole cyanobacterium in the former and *C. vulgaris* the sole eukaryote in both cultures. The CC and Chl cultures were maintained under continuous fluorescent lighting and aerated using 0.3 μm filtered and UV-sterilized ambient air in Bold's Basal Medium with Vitamins (http://epsag.netcity.de/pdf/media_and_recipes/26_Bold_Modified_Basal_Medium.pdf.).

The CC and Chl cultures for the SSH experiments were grown under high-pressure sodium lamps in a circulating water bath maintained at 29 ± 1°C. The CC and Chl cultures grown according to a 2 × 2 factorial experimental design with initial scalar irradiance levels of 180 and 400 μmol m^{-2} s^{-1} PAR (corresponding to incident irradiance levels of 87 and 204 μmol m^{-2} s^{-1} PAR, respectively) and starting nitrate levels in the media of 1.47 mmol N L^{-1} and 2.94 mmol N L^{-1} (50 and 100% the nitrate levels of Bold's Basal Medium). Five-liter cultures at each condition were continuously aerated at 46 L h^{-1} using 0.3 μm filtered ambient air. CO_2 was injected for 1 min every 72 min for a total of 9.4 L d^{-1} to provide a carbon source and control the culture pH. Optical density values were collected twice daily at 664 nm and 750 nm to construct growth curves.

2.2 Isolation of total RNA

Cultures were collected in the late exponential growth phase for total RNA extraction. The late exponential phase was chosen because cell state changes from rapid growth and cell division to slowed growth and lipid accumulation during this phase. Three liters of each culture were collected by centrifugation at 6000 × *g* for 5 min at 4°C [18]. Five milliliter aliquots of the cell slurry were poured into 50-mL polypropylene screw top tubes, flash-frozen in liquid nitrogen, and ultimately stored at −80°C until RNA extraction was performed. Total RNA was extracted by modifying two procedures developed for RNA extraction from green algae [17, 19], using a solution of phenol, chloroform, and isoamyl alcohol (PCI) (25:24:1, pH 5.2) and extraction buffer: 100 mM Tris-HCl (pH 8.2), 1.4 M NaCo, 20 mM EDTA (pH 8.0), and 2% w/v CTAB [19]. To each 5 mL frozen sample, 1 mL of beta-mercaptoethanol, 5 mL of extraction buffer, 5 mL of PCI, and 5 mL of 0.5 mm diameter acid-washed glass beads (Ken-Ichi Honjoh, personal communication) were added. Samples were vortexed for 10 cycles of 1 min vortexing at maximum speed followed by 1 min on ice (Ken-Ichi Honjoh, personal communication).

Following homogenization, the lysate was transferred to a 50-mL Nalgene Oak Ridge 3119 centrifuge tube (Thermo Fisher Scientific, Rochester, NY, USA). For maximal recovery of the cell lysate, the glass beads were washed with 2 mL of PCI solution followed by 2 mL of the extraction buffer, and these solutions were added to the same 3119 tube. The lysate-PCI mixture was vortexed for 1 min, then centrifuged at 2300 × *g* for 10 min at 4°C. The aqueous phase was transferred to a new tube and 5 mL of fresh PCI was added. Three PCI extractions and centrifugations were performed. After the third centrifugation, the aqueous phase was transferred to a new tube. The remainder of the RNA extraction was performed according to Machida et al. [17], starting with nucleic acid precipitation by NaCl and ethanol, with the exception that centrifugation following the addition of NaCl and ethanol was performed for 40 min at 3000 × *g*. Following LiCl

precipitation of RNA, the samples were DNase treated with the TURBO DNA free kit (Ambion, Grand Island, NY, USA) and further purified using the RNA Clean & Concentrator-5 columns (Zymo Research, Irvine, CA, USA).

2.3 cDNA synthesis and subtractive hybridization

cDNA synthesis and amplification was performed with 1 μg of total RNA from each of the eight samples using the SMARTer cDNA Synthesis Kit (Clontech, Mountain View, CA, USA). The SMARTer kit used oligo(dT) primers, which only reverse transcribed the poly-$(A)^+$ RNA, corresponding to the mRNA of the *Chlorella*, from the total RNA samples. This factor allowed total RNA samples, rather than poly-$(A)^+$ samples of the *Chlorella*, to be used for the downstream experiments. SSH was conducted using the PCR-Select cDNA Subtraction Kit (Clontech). Both cDNA synthesis and amplification and SSH were performed according to the manufacturer's protocol. For each culture condition of irradiance (180 and 400 μmol m^{-2} s^{-1} PAR) and starting media nitrate level (1.47 and 2.94 mmol N L^{-1}), a subtraction using the CC as tester and the Chl as driver (to identify genes putatively upregulated in the CC culture) was performed. Additionally, a subtraction using the Chl as driver and the CC as tester (to identify genes putatively upregulated in the Chl culture) was done, for a total of eight subtractions.

2.4 DNA sequencing and identification of homologous genes

Following the completion of SSH, subtracted cDNAs were subcloned into the pGEM-T Easy Vector and grown in JM109 transformed *Escherichia coli* cells (Promega, Madison, WI, USA). Blue/white selection was performed using LB agar plates supplemented with 100 μg mL^{-1} ampicillin and ChromoMax IPTG/X-Gal solution (Thermo Fisher Scientific, Waltham, MA, USA). Ninety-six white colonies from each subtracted library were picked and grown in deep well blocks in Terrific Broth overnight in an incubated shaker. Cell pellets were submitted to Functional Biosciences (Madison, WI, USA) for plasmid minipreps and DNA sequencing, which was done on the Applied Biosystems 3730×l DNA Sequencer (Carlsbad, CA, USA). Sequences of the expressed sequence tags (ESTs) were compared to database sequences using the BLASTx algorithm [20] through the Blast2Go software [21]. The protein sequences corresponding to the ESTs were compared to proteins in the non-redundant protein sequences database (nr) using a BLASTx threshold value of 1×10^{-6}, as previously used by others [22]. Homologous genes were grouped into functional categories using a standard method [23].

2.5 Gene expression analysis by qPCR

Nine genes identified in the subtracted libraries were selected for expression analysis by qPCR. Primers for these genes were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) [24]. Primer sequences are provided in Table 1. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA) with standard desalting purification. Reverse transcription was performed using 3 μg of total RNA from each culture in a 40 μL reaction using an oligo$(dT)_{18}$ primer and the SMARTScribe RT enzyme (Clontech) according to the manufacturer's protocol. Each reaction was diluted with the addition of 80 μL of water.

Twenty microliter qPCR reactions were performed in triplicate with 1 μL of the diluted reverse transcription product and a 200 nM concentration of each primer using the SYBR FAST Universal 2X qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA). Reactions were performed using the MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in GeneMate 8-Strip UltraFlux white PCR tubes (Catalog Number T-3224-1; BioExpress, Kaysville, UT, USA). Thermal cycling conditions were 3 min at 95°C followed by 40 cycles of 3 s at 95°C, 20 s at 60°C, and 2 s and a plate read to measure fluorescence

Table 1. Primers used for qPCR for each of the nine genes analyzed

Gene	Primers (forward then reverse; each listed 5′ to 3′)	GenBank accession number	Amplicon length (bp)
PSII apoprotein CP47	AGGTGTAGCAGCAGCCCACATTG TGTCGCGTCCCATGACGGAG	JK815816	283
Cytochrome b559 alpha subunit	TGTCTGGTGCTACAGGAGAACGC ACGGTCTGTAATAAGCGGGGTTTCT	JK815930	206
PSI Reaction Center XI	GGACGCACTCTCTCCCCGGAT GCCCAGGCAACTCCGGAAAGT	JK816118	98
Light-harvesting chlorophyll-a/b binding	CAGGTCACCCGCGCCTCTGT CACCGAGCATAGCCCAGCGTG	JK816321	202
ATP synthase subunit alpha	AAACAGTCTTCGTCGCCCACGC GAGACACGGCCCAGACGCTT	JK815952	199
Phosphoprotein phosphatase	TGCATTCACGGTGGTCTTTCCCCTG CCAGCCAGCACCTCTTGGACTGA	JK816253	162
Oil-globule-associated protein	ATTGATGGAAGCTGCACGCCCC AGACGCTACCACGCTGTCGTGA	JK816424	79
60S ribosomal protein L23a	AAGCCCCGCTATTCGGTGGT GGCACGGATGTCGACGATGAACA	JK816403	198
Molecular chaperone (HSP family)	AGCTGTGTCCCGAGTGGATGGG GTGGAGCGGTAGCGCAGCAAA	JK816244	268

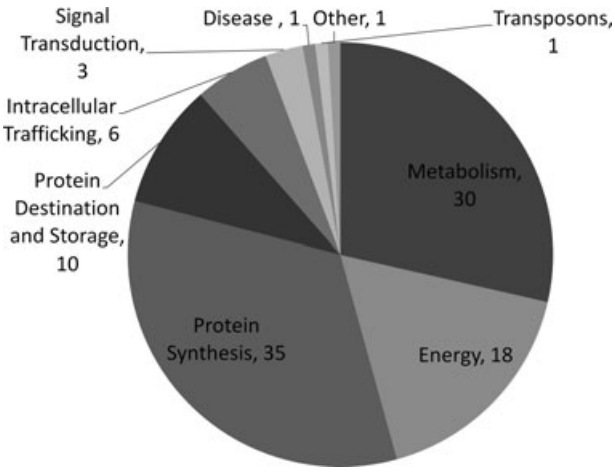

Figure 1. Functional categories of cDNA clones identified by suppression subtractive hybridization (SSH). Clones were homologous to 105 genes in the BlastX database as identified by the Blast2Go software. Functional groupings are according to the method of Bevan et al. [23].

at 72°C. Immediately following thermal cycling, a melting curve was generated from 50°C to 95°C to guarantee that only the desired product had been amplified. Quantification cycle data were collected with the MJ Opticon Monitor software (version 3.1.32; Bio-Rad).

Statistical analyses were performed using SAS (version 9.3; SAS Institute, Cary, NC, USA). Raw quantification cycle values from qPCR were adjusted using the efficiency corrected model [25] and normalized using the amount of total RNA that was used in the reverse transcription products used to generate the template cDNA [26–28]. ANOVA was performed with the least significant difference post-hoc test and Tukey adjustment for multiple comparisons to test pairwise significance of gene expression levels between the *C. vulgaris* in the Chl and the CC cultures at the same irradiance and nitrate level [29]. To test the effects of irradiance or nitrate level across the four Chl, four CC, or eight cultures, the Wilcoxon two-sample test was used [29,30]. A p-value of less than 0.05 was considered to be statistically significant. In the case that the mean expression level exceeded 10 000 times relative to the lowest expression level among the eight cultures, the value is reported as 10 000 in the expression plots (Fig. 2). This phenomenon occurs because transcripts for some genes would be nearly undetectable in some samples and grossly upregulated in others.

3 Results

3.1 Isolation of co-culture-related genes

A total of eight cDNA libraries were constructed from SSH. For each of the four combinations of irradiance and nitrate level, one cDNA library representing genes putatively upregulated in the Chl culture and one cDNA library representing genes putatively upregulated in the CC culture were created. Six hundred and ninety-four cDNA clones were obtained following nucleotide sequencing, and their sequences were deposited into GenBank (accession numbers JK815763–JK816456). An explanation of the eight SSH libraries and their corresponding accession numbers is provided in Supporting information, Table S1. Three hundred and seventy-seven clones (54.3%) showed homology to genes in the BLASTx non-redundant proteins (nr) database, representing a total of 105 unique homologous genes. The 105 homologous genes were grouped according to functional classification [23] (Fig. 1).

The functional categories of energy (18 genes, 186 ESTs), protein synthesis (35 genes, 112 ESTs), and metabolism (30 genes, 49 ESTs) comprised 79% of the genes and 71.6% of the ESTs corresponding to known genes. The other categories, in decreasing order of the number of genes represented, were protein destination and storage (10 genes, 13 ESTs), intracellular trafficking (six genes, seven ESTs), signal transduction (three genes, three ESTs), transposons (one gene, five ESTs), disease (one gene, one EST), and other (one gene, one EST). The categories of energy, protein synthesis, and metabolism represented 79% of the genes and 71.6% of the ESTs of known gene function.

3.2 Gene expression investigated by qPCR

Genes were selected for qPCR analysis to verify upregulation and obtain quantitative gene expression data (Fig. 2). Eight of the nine genes chosen represented the three main functional categories of energy (Fig. 2A–E), metabolism (Fig. 2F–G), and protein synthesis (Fig. 2H, I); the remaining gene represented the protein destination and storage category, the fourth most prevalent functional group in the cDNA libraries. Four genes, photosystem II (PSII) apoprotein CP47 (Fig. 2A), cytochrome b559 alpha subunit (Fig. 2B), photosystem I (PSI) reaction center subunit XI (Fig. 2C), and chl-a/b-binding protein (Fig. 2D) are part of photosynthesis. The remaining genes span a variety of roles, including cellular energy production (ATP synthase subunit alpha, Fig. 2E), lipid storage (oil-globule-associated protein, Fig. 2G), cell signaling, and cytoskeletal structure (phosphoprotein phosphatase (PPP) 1/2A, Fig. 2F), protein synthesis (60S ribosomal protein L23a, Fig. 2H), and protein repair and stress response (molecular chaperone, heat shock protein family) (Fig. 2I).

For all nine genes, expression levels between the Chl and CC cultures at each of the four combinations of irradiance and nitrate level were significantly different at the 99% or greater confidence level. Furthermore, qPCR of these putatively upregulated genes validated their upregulation. Neither the effect of irradiance nor the effect of nitrate level on gene expression was significant at the 95% confidence level across all eight cultures for any of the nine genes (Supporting information, Table S2). Among the Chl cultures, the effect of irradiance on expression was significant for chlorophyll a/b-binding protein ($p = 0.0173$); the effect of nitrate level on expression was significant for PSII apoprotein CP47 ($p = 0.0152$), PSI reaction center subunit XI ($p = 0.0022$), 60S ribosomal protein L23a ($p = 0.0303$), and molecular chaperone (HSP family) ($p = 0.0260$). Among the CC cultures, the effect of irradiance on expression was significant for PSII apoprotein CP47 ($p = 0.0130$) and PPP 1/2A ($p = 0.0022$); the effect of nitrate level on expression was significant for ATP

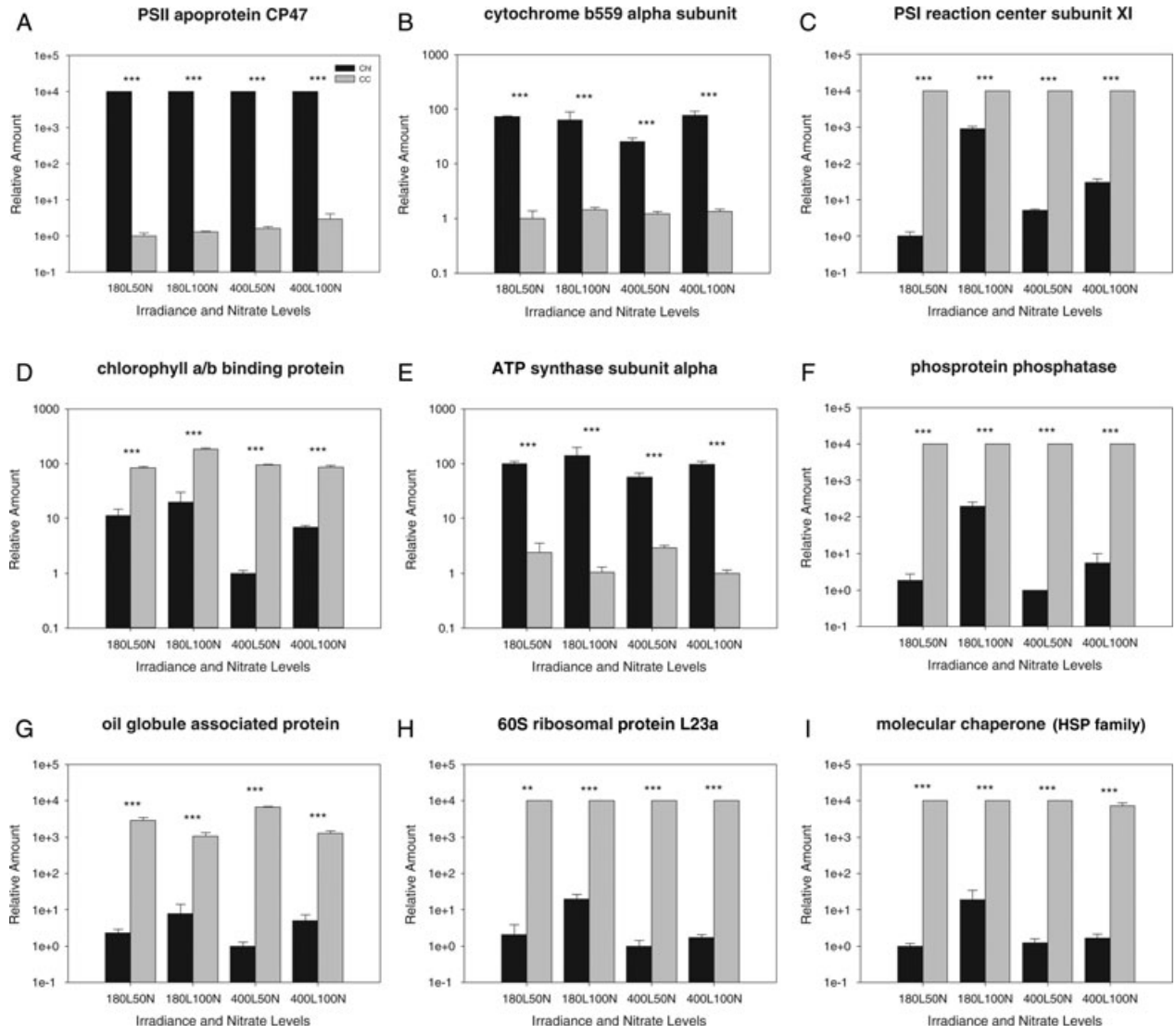

Figure 2. Quantitative PCR analysis of gene expression levels. Relative amounts are normalized to the lowest mean transcript level among the eight cultures. Bars represent mean ± SE for three technical replicates. Mean values above 10 000 are reported as 10 000. *P*-values for significantly different expression level between the *Chlorella vulgaris* in the monoculture and in the co-culture at the same irradiance and nitrate level are represented by ** ($p<0.01$) and *** ($p<0.001$).

synthase subunit alpha ($p = 0.0216$), oil-globule-associated protein ($p = 0.0022$), and 60S ribosomal protein L23a ($p = 0.0087$). Thus, the main effect on gene expression level was co-culturing, not irradiance or nitrate level.

PSII apoprotein CP47 and cytochrome b559 alpha subunit, the two genes representing PSII, show substantial upregulation in the Chl cultures compared to the CC cultures. On the other hand, PSI reaction center subunit XI, was upregulated in the CC cultures. The remaining two genes in the energy category, chlorophyll a/b-binding protein and ATP synthase subunit alpha, showed moderate upregulation in the CC and Chl cultures, respectively. The two genes representing the functional classification of metabolism, PPP 1/2A, and oil-globule-associated protein, both showed gross upregulation in the CC cultures. Finally, the two genes representing protein synthesis (60S ribosomal protein L23a) and protein destination and storage (molecular chaperone, HSP family) both displayed gross upregulation in the CC cultures.

4 Discussion

4.1 Genes in the functional category of energy

Energy-related genes identified by SSH and analyzed by qPCR included PSII apoprotein CP47, cytochrome b559 subunit alpha, PSI reaction center subunit XI, chlorophyll a/b-binding protein, and ATP synthase subunit alpha. The first two are part of PSII, and both were upregulated in the Chl cultures. CP47 transmits excitation energy in PSII from the antenna proteins

to the reaction center [31] In the green algae *Chlamydomonas*, new PSII complexes are assembled stepwise through a process called control by epistasy of synthesis [32]. The control occurs at the translational level, so accumulation of mRNA transcripts encoding CP47 in the Chl cultures may indicate that previous proteins in the assembly process, cytochrome b559, D1, and D2, have not yet been assembled [32]. The role of cytochrome b559, on the other hand, is not well understood, although it is believed to have a role in protection against light stress [33]. Nonetheless, the expression level of cytochrome b559 alpha subunit is not significantly higher at the higher level of irradiance. In any case, a deeper understanding of the upregulation of these two PSII genes in the Chl cultures would be elucidated through examination of additional PSII genes, such as the reaction center proteins D1 and D2.

While two PSII genes were upregulated in the Chl cultures, PSI reaction center subunit XI is upregulated in the CC cultures. PSI is involved in the production of NADPH for the cell. Upregulation of ATP synthase subunit alpha in the Chl cultures and PSI reaction center subunit XI in the CC cultures may indicate a different balance between ATP and NADPH production in the Chl and CC cultures, a balance that is important to photosynthetic productivity [34]. Cells in the Chl cultures may be attempting to produce more ATP, whereas cells in the CC cultures are attempting to produce more NADPH. Another possible explanation for the upregulation of PSI in the CC cultures may be that the cyanobacteria absorb the wavelengths of light preferred by PSI, whereas this does not occur in the Chl cultures because cyanobacteria are not present. The upregulation of chlorophyll a/b-binding protein in the CC cultures, perhaps in an attempt to capture the wavelengths of light absorbed by the cyanobacteria, would support this hypothesis. On the other hand, levels of reactive oxygen species (ROS) are known to correlate with levels of light harvesting proteins in *C. vulgaris* [35], so upregulation of chlorophyll a/b-binding protein in the CC cultures may be an indicator of stress caused by ROS. Another explanation for the upregulation of chlorophyll-binding protein in the CC cultures may be an attempt to capture more blue light, which is known to influence respiratory oxygen uptake as well as nitrate, amino acid, and ammonia in *Chlorella* [36, 37].

A better understanding in differential regulation of photosynthesis-related genes in the Chl and CC cultures would be aided by examining expression levels of other genes in both PSII and PSI. Additionally, comparing the absorbance spectra of the two culture types would provide information regarding the absorption of light by the *Leptolyngbya* sp. and yield more insight on how it affects photosynthesis in the CC cultures.

4.2 Genes in the functional category of metabolism

Two metabolism genes, PPP 1/2A and oil-globule-associated protein, were analyzed using qPCR. PPPs are involved in a variety of cell processes, including apoptosis, RNA splicing, and the regulation of DNA replication and gene expression [38]. PPPs are a known target of the cyanobacterial produced toxins, microcystins and nodularins [39, 40], which might explain the upregulation of a gene encoding a PPP in the CC cultures. Additionally, cyanobacteria of the genus *Leptolyngbya* are known to produce microcystins in a freshwater setting [41]. ATP synthase is also inhibited by microcystins [42]. However, the gene for ATP synthase subunit alpha was upregulated in the Chl cultures, not the CC cultures as would be expected if ATP synthase activity was inhibited by microcystins that could be produced by *Leptolyngbya* sp. in the CC cultures. Thus, another cause than microcystins or nodularins for the upregulation of the PPP gene in the CC cultures is likely.

The other metabolism gene examined, oil-globule-associated protein, is homologous to genes encoding other lipid droplet proteins identified in green algae [43]. These proteins have been identified in *Chlamydomonas reinhardtii* [44–46], *Haematococcus pluvialis* [47], and *Dunaliella salina* [43]. In *Haematococcus*, the droplet protein was barely present until the cells were exposed to nitrogen depletion and high light conditions, which are known to promote enhanced lipid production in cells [47]. Nitrogen limitation has been linked to increased lipid content in *Chlorella* in a variety of studies (R. Bai, et al., Louisiana State University, Baton Rouge, LA, unpublished results) [48, 49]. A better understanding of the involvement of the oil-globule-associated protein and its role in lipid accumulation in the *C. vulgaris* in the CC cultures would be merited, and this gene may provide a target for genetic engineering in future work. The genetic engineering could focus on designing cells that would partition more of the fixed carbon into the oil globules, as opposed to competing pathways for the carbon, such as starch synthesis.

4.3 Genes in the functional categories of protein synthesis and protein destination and storage

The final two genes examined by qPCR were 60S ribosomal protein L23a, in the category of protein synthesis, and molecular chaperone (heat shock protein family), in the category of protein destination and storage. The 60S ribosomal protein L23a is believed to be involved in protein translation and secretion, due to its location in the ribosome complex near where the newly assembled polypeptide exits the complex [50]. Thus, upregulation of the gene for L23a may be related to signaling in the CC cultures. However, genes related to protein synthesis are known to be upregulated in stressed conditions in *C. vulgaris* [17], so the upregulation of the L23a gene may be due to stress response. Examining gene expression levels of other ribosomal proteins in the CC cultures would provide a better picture of the regulation of protein synthesis.

Molecular chaperones (HSP family) are a family of stress-response proteins. These proteins play a role in response to heat, salinity, and photoinhibition stresses in algae, including *Ulva fasciata* [51], *Dunaliella* [52], and *Chlamydomonas* [53, 54]. Heat shock proteins stabilize proteins by promoting proper folding of and maintaining the structural integrity of proteins [51]. The upregulation of an HSP may be a response in the CC cultures to oxidative stress in the form of ROS, to which HSPs are known to respond [51].

4.4 Synopsis of gene expression analysis

Several trends emerge in the gene expression analysis of the nine genes examined by qPCR. First, two genes (PSII apoprotein

CP47 and cytochrome b559 alpha subunit) encoding proteins in PSII are upregulated in the Chl cultures, and one gene encoding a PSI protein (PSI reaction center subunit XI) is upregulated in the CC cultures. Second, the regulation of several genes suggests a response to oxidative stress in the CC cultures. These genes include chlorophyll a/b-binding protein, molecular chaperone (HSP family), 60S ribosomal protein L23a (due to the role of protein synthesis in stressed conditions). Third, the upregulation of the PPP gene may indicate the possible production of microcystins or nodularins by the *Leptolyngbya* sp. in the CC cultures, although upregulation of the gene for ATP synthase subunit alpha would be expected as well if this were the case.

4.5 Concluding remarks

For microalgal biofuels to be economically viable, a deeper understanding of algal growth and lipid productivity is important. Furthermore, robust strains such as the co-culture investigated in this study are essential for cultivation of mass cultures. The robustness of this co-culture, which would not be expected in a monoculture, represents an advancement toward the viability of outdoor mass cultivation of microalgae. This work represents a crucial step toward these goals by examining the underlying gene expression in a co-culture of algae that possesses good growth, lipid productivity, and robustness. In this study, SSH was used to identify 105 genes putatively differentially regulated in the Chl cultures compared to the CC cultures. Genes were categorized by functional classification, with energy, metabolism, and protein synthesis genes comprising the majority of the 105 genes. Nine genes were further analyzed using qPCR, providing additional insight into the effect of the co-culture. This work represents the first known gene expression study of a co-culture consisting of a green alga and a cyanobacterium. This work should greatly enhance the bioprocess engineering strategies employed in the biofuel industry. The differences observed in the gene expression in Chl versus in co-culture with the *Leptolyngbya* sp. can be used in the development of culture strategies that take advantage of these differences for improved production of biomass and bioproducts of interest. This is particularly valuable in outdoor cultures where co-culture is often inevitable.

The next step in understanding the Louisiana strain co-culture consisting of *C. vulgaris* and *Leptolyngbya* sp. will be to further explore other genes identified by SSH, gaining an even better understanding of this co-culture of two photosynthetic species. Knowledge of the underlying genetic causes of the changes in growth and productivity of the species in co-culture provides insights on possible target genes for culture optimization. Future analysis should explore the interaction between PSI and PSII in the Chl and CC cultures and the possible presence of ROS-induced stress in the CC cultures. Second, testing the culture media in the CC cultures for microcystins or nodularins would reveal whether upregulation of the PPP gene is connected to these cyanobacterial toxins. Third, the oil-globule-associated protein should be investigated further, and it may prove to be a useful target for genetic engineering. Finally, other native co-cultures can be screened using the techniques used in this study, gaining additional insight into the effect of co-culturing.

Practical application

This work has the potential to greatly impact microalgae utilization for the production of biofuels and other commodity products. First, understanding the genetics driving the enhanced biomass accumulation observed for the co-culture of two photosynthetic species will enhance the bioprocessing of such organisms. More robust cultures can be designed, leading to larger commodity product yields without increasing culture resources, thereby improving the economics of algal-based biofuels. Second, the genes identified as being differentially regulated in either co-culture or monoculture are prime targets for expression manipulation studies. By over or underexpressing these genes, it will be possible to enhance microalgal biomass accumulation in response to multiple environmental conditions, including variations in irradiance, nitrogen availability, and carbon availability.

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The authors have declared no conflict of interest.

5 References

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