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Elucidating the role of jasmonic acid in oil accumulation, fatty acid composition and gene expression in *Chlorella vulgaris* (Trebouxiophyceae) during early stationary growth phase ☆

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ABSTRACT

Manipulation of microalgae cultivation is an important approach as the growth conditions will determine the quality and compositions of lipids produced by the cells. This study attempts at maximizing the production of lipids in *Chlorella vulgaris* (Trebouxiophyceae) through introduction of the plant hormone jasmonic acid (JA). Exogenous application of JA at early stationary growth phase promoted microalgal growth with increment of up to 51% of cell density relative to the control. JA also transiently increased the total oil production of microalgal cells by 54%. Interestingly, this increment also induced significantly higher production of saturated (SFA) and monounsaturated (MUFA) without compensating the production of polyunsaturated fatty acid (PUFA). Further analysis of fatty acid compositions showed that JA stimulated the production of C16:0 (palmitic acid), C18:0 (stearic acid) and C18:1 (oleic acid) at early stationary growth phase whilst C18:3 n-3 (α-linolenic acid) at later stationary growth phase. This transient modification of fatty acid compositions was correlated with the fatty acid biosynthetic gene expression as quantified by real-time PCR. Taken together, our results indicated that JA significantly increased microalgal lipid accumulation and could be utilized in cultivation to facilitate commercial mass production of microalgae lipids.

1. Introduction

Microalgae-derived biofuels or so-called second generation biofuels are renewable fuels, produced mainly in the form of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) from microalgae [1,2]. The utilization of second generation biofuels from microalgae has increasingly become an interest in the scientific community to replace the first generation biofuels from oilseed crops [3]. The first generation biofuels, since they were introduced in the middle of 1970s, have undoubtedly contributed to the global energy demand, however have encountered numerous socio-economic challenges such as the increase of food prices due to the high demand of food production and the loss of ecosystems due to massive scale plantations [3–5]. Furthermore, as the world population is predicted to increase from 6.6 billion in 2008 to 9.2 billion in 2050 [6], and the current fossil fuel resources that we have been harnessing are only sustainable for another 60 or 80 years before being completely depleted [7], microalgae-derived biofuels are certainly necessary to replace both the current fossil fuels and the first generation biofuels.

Lipids and oils in microalgae are deposited mainly in the form of triacylglycerols (TAGs) which serve as a storage for carbon and energy [8]. These TAGs originated from de novo fatty acid synthesis pathway [9]. However, the biosynthetic pathways, the enzymes and regulatory factors involved in fatty acids and TAGs accumulation in microalgae remain largely unknown. Most of the understandings on microalgae lipid metabolism derived from the knowledge of higher plants based on the evolutionary conserved sequence homology of genes and enzymes involved in lipid metabolism. In most microalgae, TAGs are composed primarily of C14 to C18 fatty acids that are SFAs or MUFAs although in rare occasion, the presence of PUFAs has also been recorded [8,10].

Although the composition of fatty acids and TAGs in microalgae is governed by the genotype of the organism, resulting in species or strain specific, there is now ample evidence that manipulation of growth

Abbreviations: ACP, acyl carrier protein; DAT, days after treatment; JA, jasmonic acid; KAS I, β-ketoacyl ACP synthase I; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ω-3 FAD, omega-3 fatty acid desaturase; ω-6 FAD, omega-6 fatty acid desaturase; SAD, stearoyl-ACP desaturase; SFA, saturated fatty acid.

☆ **Author contribution**: MJ and TSCha conceived and designed the research; MJ conducted experiments; MJ, SHL and TSChuah analysed data. MJ, AA and TSCha wrote the manuscript. All authors read and approved the manuscript.

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Table 1
Real-time PCR primers used in this study.

Gene (accession number)	Primer name	Forward/reverse primer (5′–3′)	Amplicon size (bp)
KAS 1	KasI ForRT	CCATGATTGGTCATTGCTTGGGAGC	166
	KasI RevRT	GCTCTTGCTTCATGTTTGGGACCAC	
SAD (KJ561355)	SAD ForRT	AGTTCTTCAGGCTTGATCCTG	136
	SAD RevRT	TCGTTGAACAGGTTCCTGCC	
ω-6 FAD (KJ561356)	ω-6 FAD ForRT	CTTCACCCACGAAGGCACAGGC	129
	ω-6 FAD RevRT	CCTGCACACTGCTGGGAACG	
ω-3 FAD (EU100100)	ω-3 FAD ForRT	CATGTTGAGAACGACGAGTCCTGGTATC	125
	ω-3 FAD RevRT	GTCAAAGTGGGAGCCAGTCTTGC	
18s rRNA	18s ForRT	CCTGCGGCTTAATTTGACTCAACACG	131
	18s RevRT	TAGCAGGCTGAGGTCACGTTCG	

conditions can influence the fatty acid compositions and TAGs accumulation in microalgae. Several chemical and physical stimuli such as nutrient [11–15], salinity [16,17], temperature [12] and light [14,18] have been extensively used to produce microalgae with desirable fatty acid compositions. Besides these stimuli, microalgae growth also appears to be influenced by plant hormones [19]. However, little is known about the effect of plant hormones on microalgae especially at the gene level.

Jasmonic acid (JA) is an oxylipin signalling molecule that plays diverse functions in plant development especially in defence and stress responses [20,21]. Despite extensive studies and findings on the key players in JA signalling in plants, little is known about this hormone in microalgae. Nevertheless, analyses of several microalgae genomes have identified two putative orthologs of JA biosynthesis enzymes: OPDA reductase (OPR) and multifunctional protein (MFP), suggesting that microalgae might synthesize and utilize JA signalling in their development [22]. Several studies have shown the effects of JA on growth, chlorophyll, carotenoids, astaxanthin, and protein content in several microalgae species [23–26]. In addition, Fujii et al. [27] reported the occurrence of JA in *Dunaliella* axenic culture. Given this background information, this study aimed to investigate the effects of JA on growth, total oil content and fatty acid profiles in *Chlorella vulgaris*. Besides, expression of four fatty acid biosynthetic genes was also examined upon exogenous JA application and correlation analysis was carried out to determine the relationship between gene expression and fatty acid profile in *C. vulgaris*. To the best of our knowledge, this is the first study on the effects of JA on oil and fatty acid productions in microalgae.

2. Material and methods

2.1. Microalgal cultivation conditions

Microalgal sample, *C. vulgaris* (strain UMT-M1) was obtained from microalgae stock culture at Universiti Malaysia Terengganu, Malaysia. The algal culture was initiated from a single colony taken from the stock agar plate and cultured in F/2 medium [28] prepared with natural sea water (30 ppt). The cultures were grown at 24 °C (±2 °C) in continuous light (80 μmol m^{-2} s^{-1}) and aerated with 0.22 μm filter sterilized air. The initial cell density of each experiment was standardized at 6×10^5 cells mL^{-1} in 2 L culture medium prepared in 3 L conical flask. The culture growth was monitored by recording the cell density values (using haemocytometer) at two day interval until a constant reading was obtained. The constant readings (three readings) represented an early stationary growth phase. Final concentration of 45 μM JA [(±)-JA, CAS number 77026-92-7, Sigma-Aldrich] was added to the culture at early stationary growth phase and harvested at two day interval. Samples for "days after treatment (DAT)-0" were taken immediately after the culture reaching early stationary growth phase before subjected to JA treatment. For control (without JA), 100% (v/v) ethanol was added to the culture to compensate the solvent used to dilute JA. Final concentration of ethanol in each culture was 0.00435% (v/v). All treatments were conducted in three replicates.

2.2. Quantification of oil content and fatty acid analyses

Microalgae cells were harvested at 4000 ×*g*, rinsed twice with distilled water before dried in an oven at 80 °C until a constant dry weight was obtained. Total oil content was extracted and determined according to the methods of Cha et al. [11]. Fatty acid methyl esters (FAMEs) were prepared using modification of the method described by Cha et al. [11]. One microlitre of the esterified oil sample was analysed using an Agilent 6890 gas chromatograph (Agilent Technologies, USA) fitted with HP-88 capillary column (0.25 mm inner diameter × 30 cm length) and a flame ionization detector. The oven temperature was programmed from 175 °C (10 min hold) to 220 °C (15 min hold) at a rate of 3 °C min^{-1} with helium as the carrier gas at a constant flow rate of 2 mL min^{-1}. The identification of fatty acids was accomplished by comparing peak and retention time of the reference standard, Supelco 37 Component FAME Mix (Sigma-Aldrich). All data were presented as percentage relative to the control.

2.3. Gene expression analysis with real-time polymerase chain reaction (PCR)

Total RNA was isolated with GF-1 Total RNA Extraction Kit (Vivantis) according to the manufacturer's protocol. Approximately, 100 mg of cells was ground in liquid nitrogen to fine powder before adding 400 μL Buffer TR and centrifuged at maximum speed for 3 min. Then, the clear lysate was transferred into a column again was centrifuged to collect the flow-through. Next, 350 μL of 80% ethanol was added to the flow-through before transferring the mixture into a RNA binding column and centrifuged at maximum speed for 1 min. The final RNA was then eluted with 50 μL RNase free water and treated with DNase I (Fermentas) to remove contaminating DNA. Removal of genomic DNA contamination was confirmed by amplification of 18S rDNA gene using RNA as PCR templates. Next, 1 μg RNA was reverse transcribed with iScript Reverse Transcription Supermix (Bio-Rad), in accordance with the manufacturer's instructions. The generated cDNA was used directly for real-time PCR.

Table 2
Effect of JA on cell growth of *C. vulgaris*.

Cultivation time (days)	Days after treatment (DAT)	Cell density (10^6 cells/mL)		Cell density* (% relative to control)
		Control	JA	
22	0	9.25 ± 0.14	9.25 ± 0.14	100.00 ± 1.56[a]
24	2	9.28 ± 0.37	9.33 ± 0.31	100.54 ± 3.29[ab]
26	4	9.55 ± 0.36	10.50 ± 0.40	109.60 ± 4.20[b]
28	6	9.50 ± 0.36	14.40 ± 0.36	151.75 ± 3.73[c]
30	8	7.13 ± 0.15	7.70 ± 0.30	107.94 ± 4.21[ab]
32	10	7.02 ± 0.32	7.62 ± 0.19	108.55 ± 2.70[ab]

* Means followed by the same letter (a, b, c) within the same column are not significantly different according to Tukey's HSD test at $p < 0.05$.