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The Effects of Plant Growth Regulators on Cell Growth, Protein, Carotenoid, PUFAs and Lipid Production of *Chlorella pyrenoidosa* ZF Strain

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Abstract: In the present study, eight kinds plant growth regulators—salicylic acid (SA), 1-naphthaleneacetic acid (NAA), gibberellic acid (GA_3), 6-benzylaminopurine (6-BA), 2, 4-epi-brassinolide (EBR), abscisic acid (ABA), ethephon (ETH), and spermidine (SPD)—were used to investigate the impact on microalgal biomass, lipid, total soluble protein, carotenoids, and polyunsaturated fatty acids (PUFAS) production of *Chlorella pyrenoidosa* ZF strain. The results showed the quickest biomass enhancement was induced by 50 mg·L^{-1} NAA, with a 6.3-fold increase over the control; the highest protein content was increased by 0.005 mg·L^{-1} ETH, which produced 3.5-fold over the control; total carotenoids content was induced most effectively by 1 mg·L^{-1} NAA with 3.6-fold higher production than the control; the most efficient elicitor for lipid production was 5 mg·L^{-1} GA_3 at 1.9-fold of the control; 0.2 mg·L^{-1} ETH induced the abundant production of 1.82 ± 0.23% linoleic acid; 0.65 ± 0.01% linolenic acid was induced by 1 mg·L^{-1} NAA; 2.53 ± 0.15% arachidonic acid and 0.44 ± 0.05% docosahexaenoic acid were induced by 5 mg·L^{-1} GA_3. Transcriptional expression levels of seven lipid-related genes, including *ACP*, *BC*, *FAD*, *FATA*, *KAS*, *MCTK*, and *SAD*, were studied by real-time RT-q-PCR. 5 mg·L^{-1} GA_3 was the most effective regulator for transcriptional expressions of these seven genes, producing 23-fold *ACP*, 31-fold *BC*, 25-fold *FAD*, 6-fold *KAS*, 12-fold *MCTK* compared with the controls, respectively.

Keywords: *Chlorella pyrenoidosa*; plant growth regulators; secondary metabolites; gene expression; lipid

1. Introduction

Rapid exploitation of fossil fuels in modern society has been widely recognized as highly unsustainable, and they will eventually run out [1]. Several renewable energy sources are being explored in many countries, especially developed countries. Biodiesel could be a viable alternative to replace conventional petroleum since it is more environmentally friendly and sustainable compared to fossil fuels [2]. Among the various sources of biodiesel, microalgae have attracted significant interest among scientists and policymakers due to the fact that they can be grown almost anywhere, including non-arable lands, using industrial wastes, and the photosynthetic nature of these organisms can be a useful tool to reduce greenhouse gas emissions [3]. However, large-scale microalgae lipid production for biodiesel currently faces cost-related bottlenecks. To improve the economic feasibility of

microalgae-based biofuels, different strategies, including nutrient deprivation, temperature shift, salinity stress and light intensity variation, have been applied to enhance the microalgae lipid productivity [4]. Ho et al. reported that nitrogen (N) limitation/starvation is one of the most effective approaches to enhance lipid accumulation and manipulate fatty acid profiles in various microalgae species [5]. Nevertheless, N limitation/starvation significantly reduces the growth, development and metabolism of algae [6]. Plant hormones have been reported to promote the growth and production of metabolites in plants and microalgae [7]. Microalgae are less morphologically advanced than both seaweeds and vascular plants, but they have many similar metabolic and biochemical processes as well as similar responses to environmental cues [8], which could potentially be attributed to the levels of hormones [9,10]. For this reason, the research into microalgae has become a hot topic in recent years, especially in the field of microalgal biodiesel [11]. However, a series of challenges have to be conquered before commercial application of microalgae-based biodiesel will be possible, one of which is the lack of microalgal strains with both high lipid content and fast growth rate [1,12].

The use of plant growth regulators in microalgae presented new opportunities in developing microalgal lipids for biodiesel production [13]. Along with the classical plant growth regulators including auxins, cytokinins, gibberellins (GAs), abscisic acid (ABA), 1-naphthaleneacetic acid (NAA), 2,4-epibrassinolide (EBR) and brassinosteroids (BRs), other plant growth regulators such as polyamines, salicylic acid (SA), and jasmonic acid (JA) are also recognized [14–18]. It have been reported that plant growth regulators and their analogs have stimulating effects on microalgal growth and metabolite production (i.e., carotenoid, lipid, carbohydrate, and protein) [19–26].

Indole 3-acetic acid (IAA) has been reported to promote growth and lipid production in *Chlamydomonas reinhardtii* [27], *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* [26]. Abscisic acid (ABA) is an effective regulator that affects many aspects of plant growth and developmental processes [16,17]. Earlier reports showed ABA might function as a regulator of carotenogenesis, could be used as effective factors to produce carotenoids in *Dunaliella salina* and *Haematococcus pluvialis* [28,29]. Gao et al. reported that some plant growth regulators (including ABA, EBR, ETH and GA_3) could increase carotenoids and astaxanthin productivity significantly in *H. pluvialis* and stimulate mRNA expressions of the eight carotenogenic genes, with different expression profiles [22–25]. They also reported that SA had positive effects on the expression level of relative genes of the carotenoids and astaxanthin [18].

Brassinosteroids (BRs) are plant growth regulators with significant growth-promoting activity and are involved in multiple developmental processes including the cell cycle and mitosis [15], photosynthesis [30], and modulation of gene expression [31]. Gibberellins are a class of plant growth regulators that are essential for normal growth and development [32] and play a role in the response of plants to stress. Besides the three traditional regulatory molecules, namely JA, SA and ETH, GA_3 has also been shown to function in response to stress in plants [33]. Jasmonic acid (JA), salicylic acid (SA), and ethylene (ETH) serve as regulatory signaling molecules that regulate diverse stress responses in higher plants [34]. The exogenous application of jasmonic acid (JA) promoted *Chlorella vulgaris* growth, with increases of up to 51% in cell density relative to the control and also transiently increases the total oil production of microalgal cells by 54% [35]. Although some plant growth regulators have been studied in microalgae, the effects of plant growth regulators on the growth, lipid production and relative gene expressions have not yet been studied in detail. *Chlorella* has been one of the earliest algae for commercial development. It can produce plenty of protein, an impressive amount of vitamins, minerals, pigments, fatty acids, and growth factors. It was demonstrated that it had potential for biofuel production [36–38]. *Chlorella pyrenoidsa* has been demonstrated as a potential candidate for higher lipid production under a wide range of environmental growth conditions [39,40].

The aim of the present study was to investigate the effect of eight kinds plant growth regulators (ABA, 6-BA, EBR, ETH, GA_3, NAA, SA, SPD) on microalgae growth, lipid and other high value secondary metabolites (carotenoids, total soluble protein and PUFAs composition) production in *C. pyrenoidosa* ZF strain; and to explore the effect of different plant growth regulators on the gene

expression of *C. pyrenoidosa* ZF strain, it would help to understand the underlying mechanism and establish a molecular pathway for further improvement in the biofuel productivity.

2. Results

2.1. Effect of Plant Growth Regulators on Microalgal Growth

The standard curves between cell density and OD_{450nm} as well as growth curves of *C. pyrenoidosa* ZF strain with different laser sources are shown in Figure 1 The regression equation was $y = 6.486x + 1.531$ and R^2 was 0.994 which represented the standard curve between OD_{450nm} and cell number was available (Figure 1).

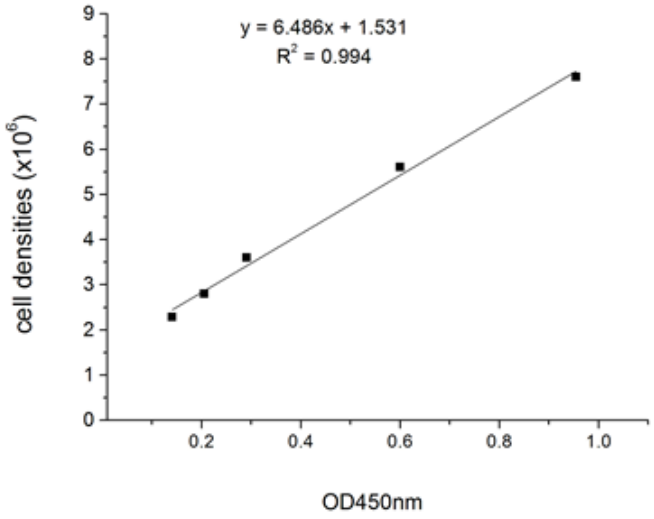


Figure 1. The standard curve between OD450 and *C. pyrenoidosa* ZF strain cell densities.

Figure 2 shows the effect of eight plant growth regulators on growth of *C. pyrenoidosa* ZF strain, respectively. According to the Figure 2, after 12 days of induction, all *C. pyrenoidosa* ZF strain growth curves showed a similar progression: plant growth regulators can promote the growth of *C. pyrenoidosa* ZF strain.

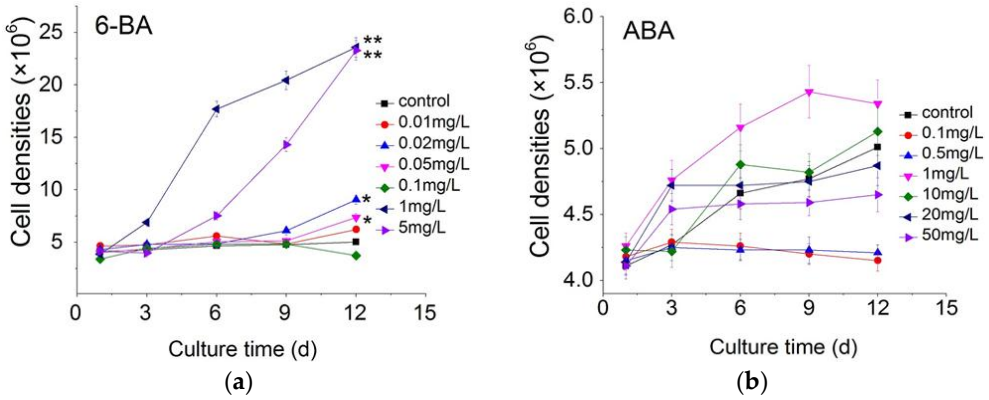


Figure 2. *Cont.*

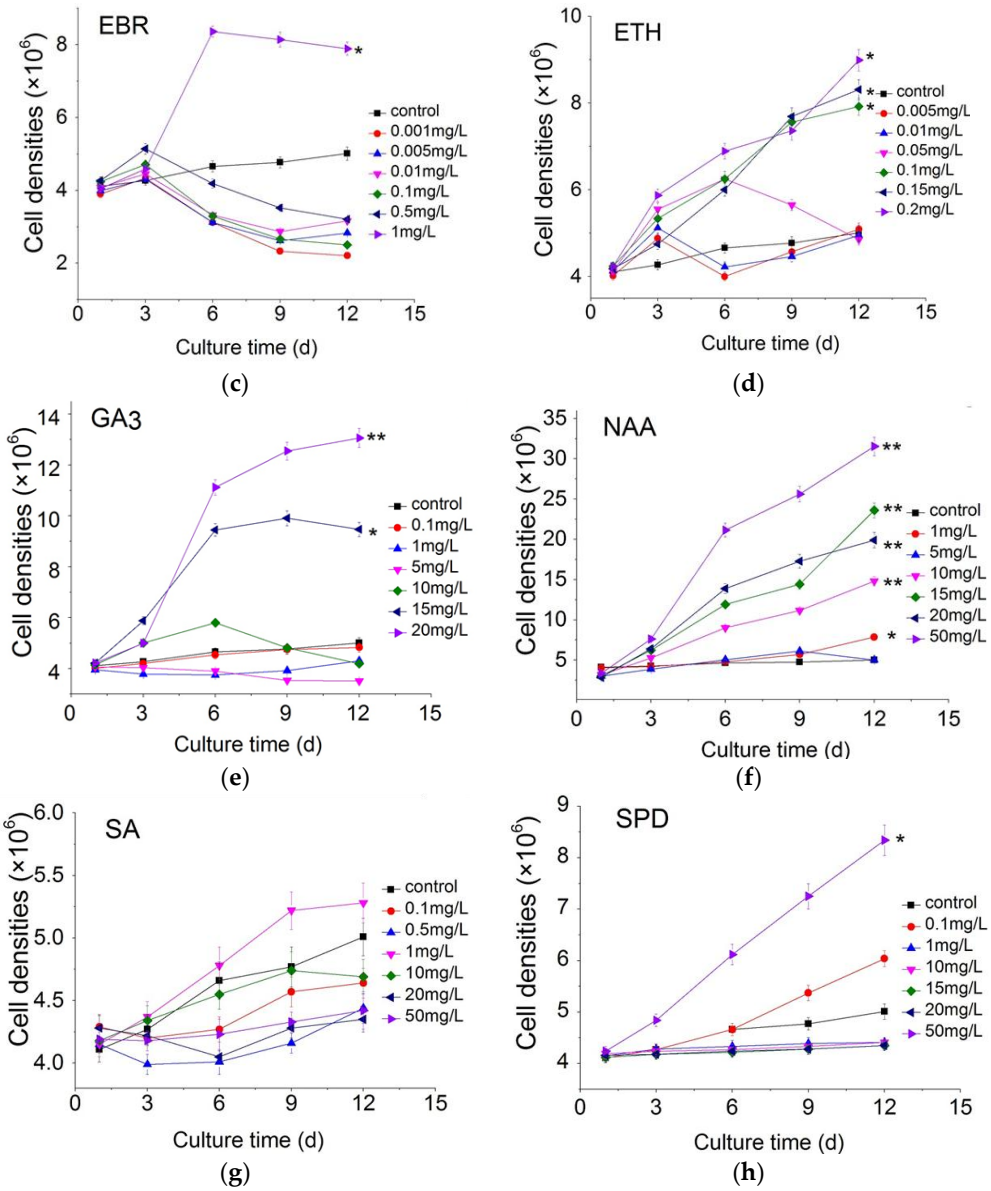


Figure 2. Growth rates of *C. phyrenoidosa* ZF strain with different concentrations of plant growth stimulants: (**a**) 6-BA treatments; (**b**) ABA treatments; (**c**) EBR treatments; (**d**) ETH treatments; (**e**) GA_3 treatments; (**f**) NAA treatments; (**g**) SA treatments; (**h**) SPD treatments.

The experimental results showed that 1 mg·L^{-1} 6-BA, 1 mg·L^{-1}.ABA, 1 mg·L^{-1}. EBR, 0.2 mg·L^{-1}. ETH, 20 mg·L^{-1}. GA_3, 50 mg·L^{-1}. NAA, 1 mg·L^{-1}. SA and 50 mg·L^{-1}. SPD were more effective at increasing cell growth with (23.58 ± 0.92) × 10^6, (5.34 ± 0.18) × 10^6, (7.89 ± 0.18) × 10^6, (8.99 ± 0.25) × 10^6, (13.06 ± 0.38) × 10^6, (31.54 ± 1.15) × 10^6, (5.28 ± 0.16) × 10^6, (8.34 ± 0.30) × 10^6, respectively. Among the plant growth regulators supplemented cultures, the highest biomass (31.54 ± 1.15) × 10^6 in C. *pyrenoidosa* ZF strain were achieved in 50 mg·L^{-1} NAA treatment (Figure 2).

2.2. Effect on Crude Protein Contents

Except for ABA and SPD, which showed no significant effect on the accumulation of crude protein contents, all plant growth regulators used in the present study caused significant increases in crude protein contents in *C. pyrenoidosa* ZF strain biomass when compared to controls after 12 days of treatment, although not in dose-dependent manners (Figure 3). Among the six plant growth regulators exhibiting significant effects on protein contents, ETH was the most effective,

at 0.005–0.2 mg·L^{-1} (0.005 mg·L^{-1}: 99.4 ± 5.2 mg·g^{-1} DW; 0.01 mg·L^{-1}: 57.5 ± 5.8 mg·g^{-1} and 0.15 mg·L^{-1}: 52.5 ± 5.4 mg·g^{-1} vs. 28.2 ± 2.4 mg·g^{-1} in the controls), followed by EBR at 0.01 mg·L^{-1} (64.9 ± 7.1 mg·g^{-1} DW) and 0.1 mg·L^{-1} (43.5 ± 4.0 mg·g^{-1}) and GA_3 at 1 mg·L^{-1} (45.2 ± 5.0 mg·g^{-1}), 10 mg·L^{-1} (42.6 ± 4.8 mg·g^{-1}) and 20 mg·L^{-1} (39.9 ± 4.1 mg·g^{-1}). SA caused a significant increase at 0.1 mg·L^{-1} (53.2 ± 4.8 mg·g^{-1}) and 1 mg·L^{-1} (55.2 ± 4.5 mg·g^{-1}) whereas 6-BA and NAA had effects only at 0.02 mg·L^{-1} (43.3 ± 4.0 mg·g^{-1}) and 10 mg·L^{-1} (46.1 ± 3.5 mg·g^{-1}), respectively.

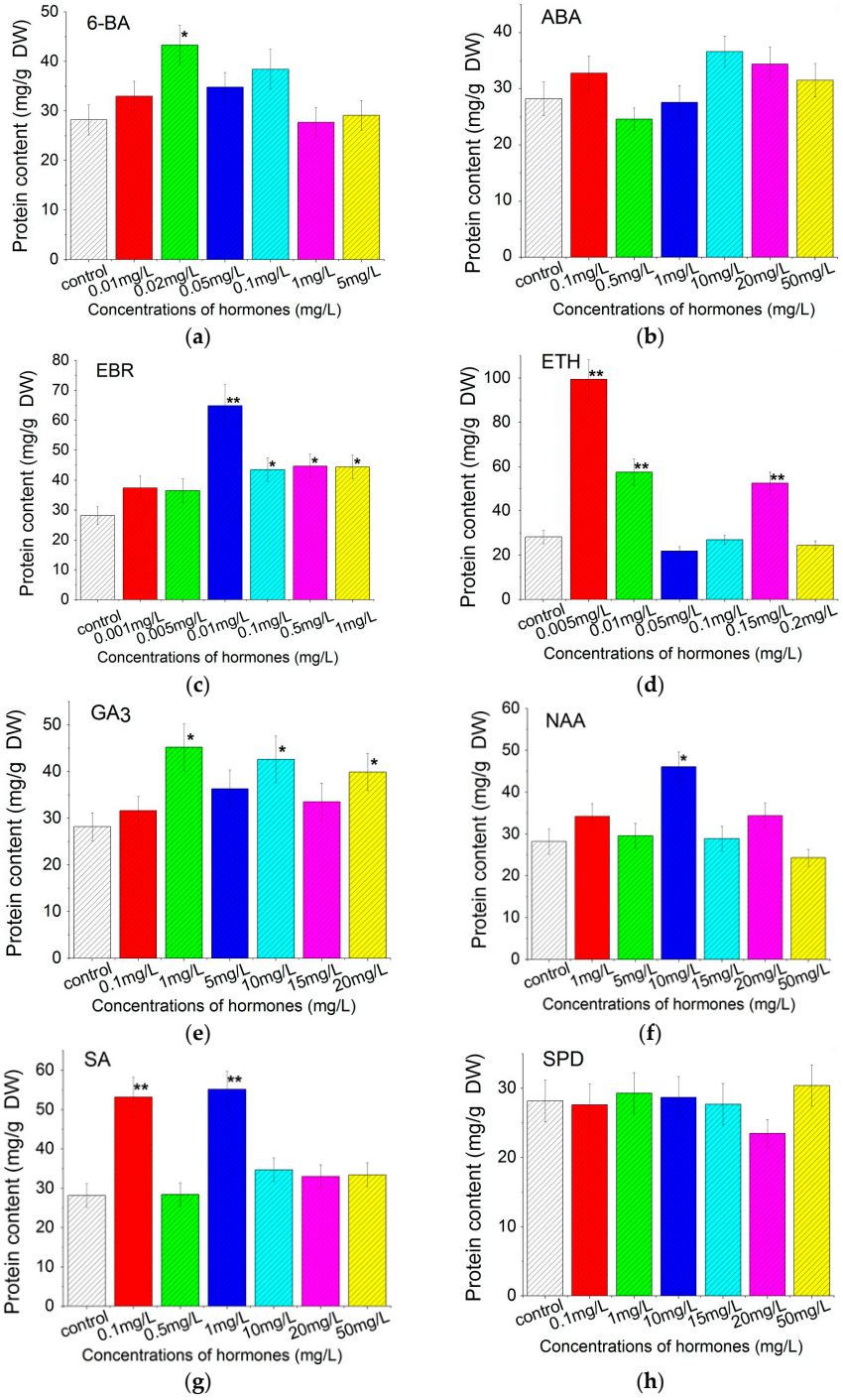
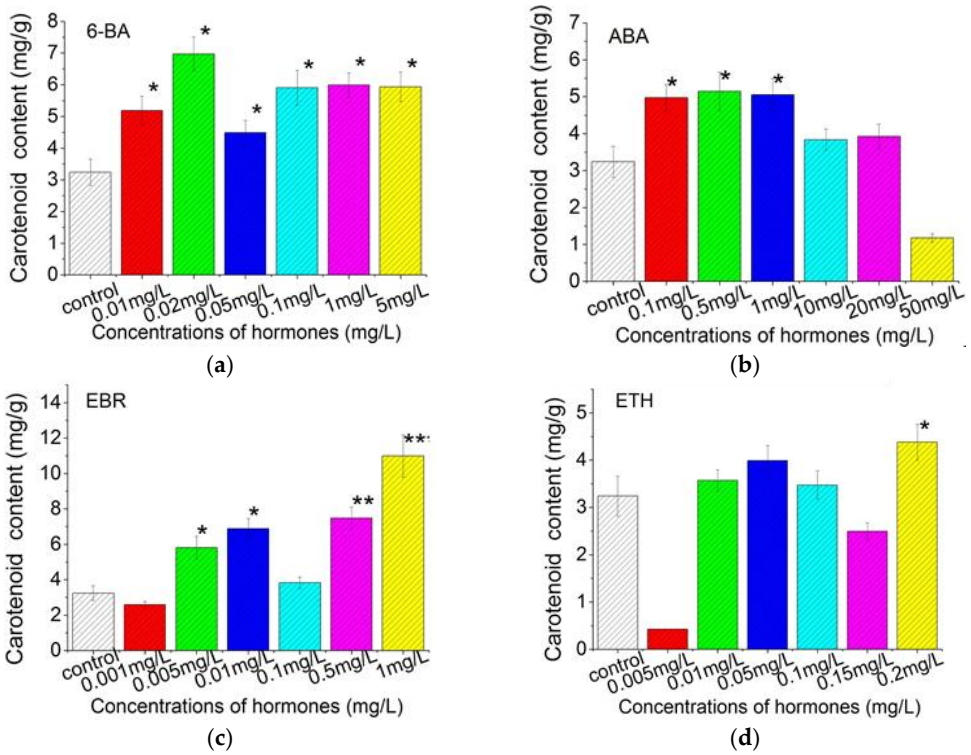


Figure 3. The total soluble protein contents of *C. pyrenodiosa* ZF strain at different concentrations of plant growth regulators: (**a**) 6-BA treatments; (**b**) ABA treatments; (**c**) EBR treatments; (**d**) ETH treatments; (**e**) GA_3 treatments; (**f**) NAA treatments; (**g**) SA treatments; (**h**) SPD treatments.

2.3. Plant Growth Regulators Induced Carotenoids Production in C. Pyrenoidosa ZF Cells

All plant growth regulators used in the present study caused significant increases in total carotenoid contents in *C. pyrenoidosa* ZF strain biomass when compared to controls after 12 days of treatment, although not in dose-dependent manners (Figure 4). Among the eight plant growth regulators used, all concentrations of 6-BA and GA_3 caused increases in total carotenoid contents (6-BA: 0.01 mg·L^{-1} (5.19 ± 0.45 mg·g^{-1}), 0.02 mg·L^{-1} (6.97 ± 0.53 mg·g^{-1}), 0.05 mg·L^{-1} (4.49 ± 0.38 mg·g^{-1}), 0.1 mg·L^{-1} (5.91 ± 0.55 mg·g^{-1}), 1 mg·L^{-1} (5.99 ± 0.39 mg·g^{-1}) and 5 mg·L^{-1} (5.93 ± 0.47 mg·g^{-1}); GA_3: 0.1 mg·L^{-1} (6.13 ± 0.58 mg·g^{-1}), 1 mg·L^{-1} (5.85 ± 0.62 mg·g^{-1}), 5 mg·L^{-1} (7.18 ± 0.77 mg·g^{-1}), 10 mg·L^{-1} (9.36 ± 0.87 mg·g^{-1}), 15 mg·L^{-1} (9.29 ± 0.93 mg·g^{-1}) and 20 mg·L^{-1} (8.74 ± 0.78 mg·g^{-1}) vs 3.24 ± 0.42 mg·g^{-1} DW in the controls. Four concentrations of EBR: 0.005 mg·L^{-1}. (5.82 ± 0.62 mg·g^{-1}), 0.01 mg·L^{-1} (6.88 ± 0.58 mg·g^{-1}), 0.5 mg·L^{-1} (7.48 ± 0.61 mg·g^{-1}) and 1 mg·L^{-1} (10.98 ± 1.20 mg·g^{-1}) and SA: 0.1 mg·L^{-1} (6.51 ± 0.68 mg·g^{-1}), 0.5 mg·L^{-1} (5.39 ± 0.45 mg·g^{-1}), 20 mg·L^{-1} (4.44 ± 0.45 mg·g^{-1}), and 50 mg·L^{-1} (6.48 ± 0.74 mg·g^{-1})) induced higher carotenoids accumulation in the microalgal biomass. Similarly, three concentrations of ABA (0.1 mg·L^{-1} (4.98 ± 0.35 mg·g^{-1}), 0.5 mg·L^{-1} (5.15 ± 0.52 mg·g^{-1}) and 1 mg·L^{-1} (5.05 ± 0.44 mg·g^{-1})) caused increases in carotenoids contents. The other plant growth regulators used in the present study induced carotenoids accumulation due to only one concentration (ETH: 4.38 ± 0.52 mg·g^{-1} at 0.2 mg·L^{-1}, NAA: 11.77 ± 1.27 mg·g^{-1} at 1 mg·L^{-1}, and SPD: 5.22 ± 0.48 mg·g^{-1} at 10 mg·L^{-1}; Figure 4).



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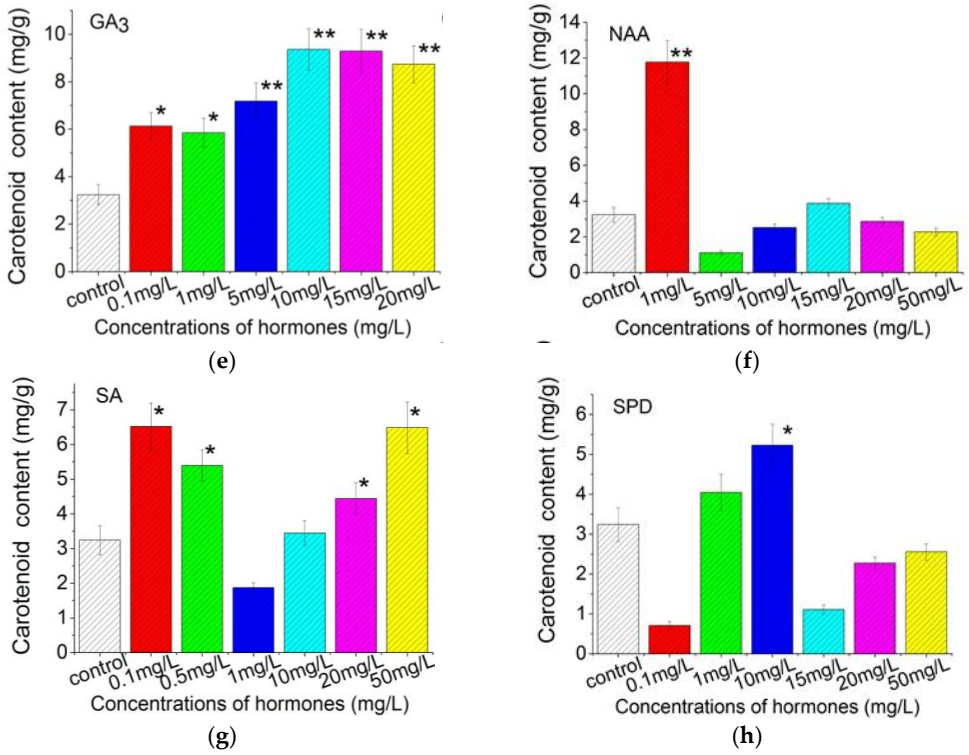
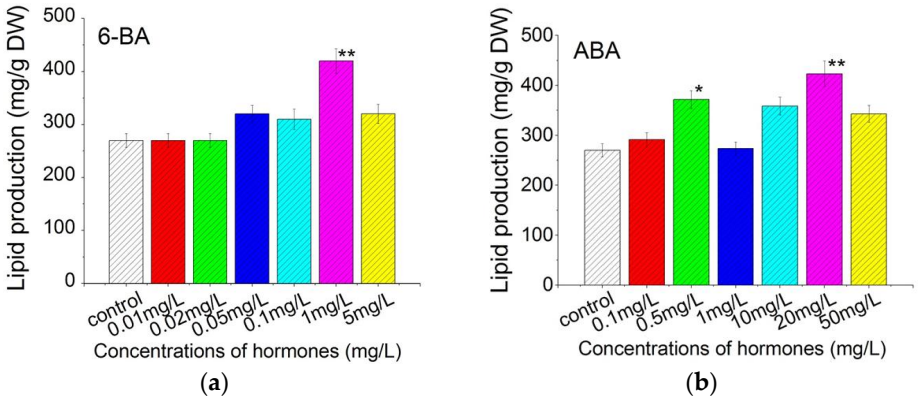


Figure 4. Carotenoid contents of *C. pyrenodiosa* ZF strain at different concentrations of plant growth regulators: (**a**) 6-BA treatments; (**b**) ABA treatments; (**c**) EBR treatments; (**d**) ETH treatments; (**e**) GA_3 treatments; (**f**) NAA treatments; (**g**) SA treatments; (**h**) SPD treatments..

2.4. Plant Growth Regulators Induced Lipid Production in C. Pyrenoidosa ZF Strain Cells

Like proteins, total lipid productions in *C. pyrenoidosa* ZF cells increased significantly in response to plant growth regulator treatment after 12 days of incubation in non-dose-dependent manners (Figure 5), except for ETH which caused no effects on the lipid contents in the microalgal biomass. Among the plant growth regulators used, four concentrations of SA (0.1 $mg \cdot L^{-1}$ (371.2 ± 18.5 $mg \cdot g^{-1}$), 0.5 $mg \cdot L^{-1}$ (390.7 ± 19.3 $mg \cdot g^{-1}$), 10 $mg \cdot L^{-1}$ (389.6 ± 18.7 $mg \cdot g^{-1}$), and 20 $mg \cdot L^{-1}$ (470.5 ± 23.3 $mg \cdot g^{-1}$) and SPD (0.1 $mg \cdot L^{-1}$ (381.2 ± 18.4 $mg \cdot g^{-1}$), 10 $mg \cdot L^{-1}$ (360.3 ± 22.7 $mg \cdot g^{-1}$), 15 $mg \cdot L^{-1}$ (381.2 ± 19.4 $mg \cdot g^{-1}$), and 20 $mg \cdot L^{-1}$ (369.8 ± 17.9 $mg \cdot g^{-1}$) induced higher lipid accumulation compared to controls (270.0 ± 13.2 $mg \cdot g^{-1}$ DW). The treatments by ABA, NAA, 6-BA, EBR, and GA_3 caused higher lipid production at only one or two concentrations used in this study (Figure 5).



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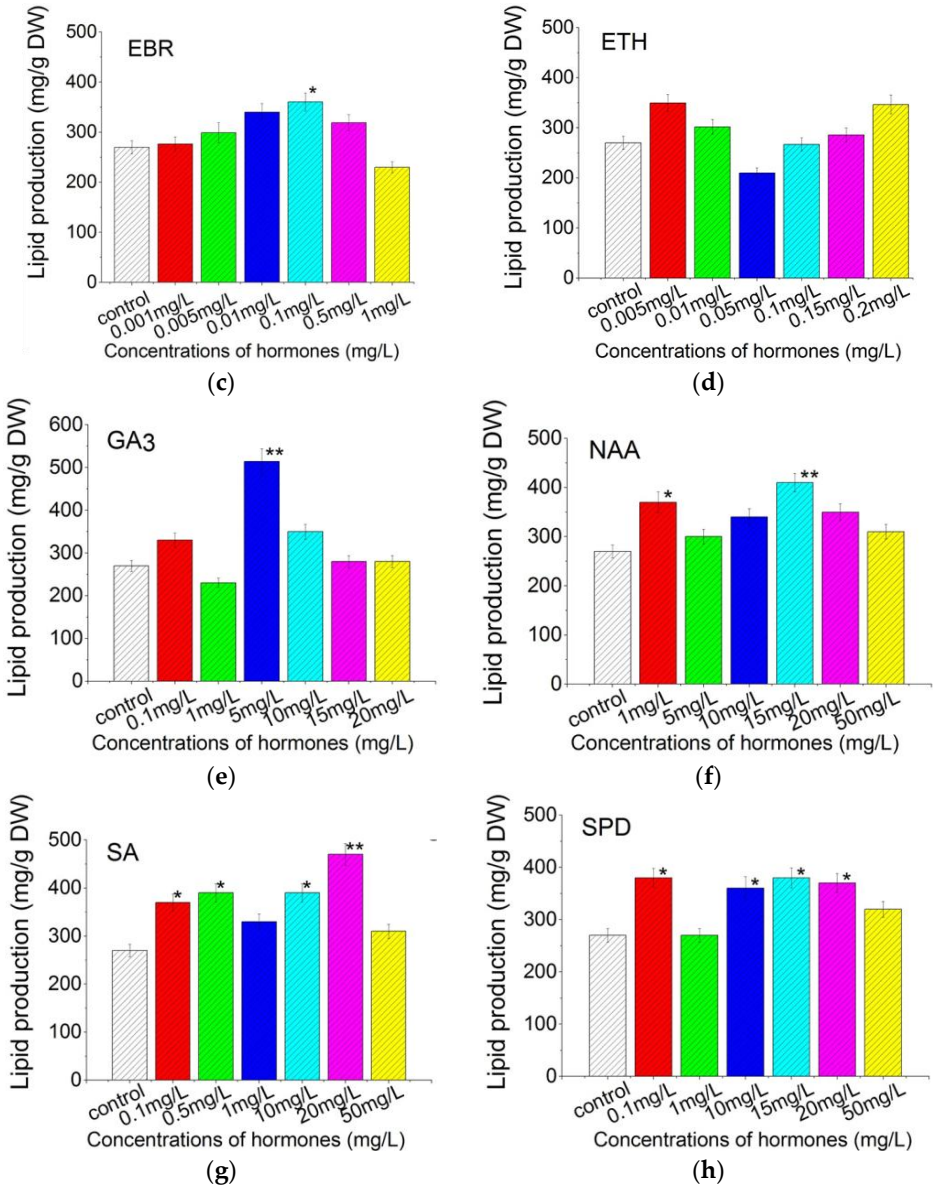

Figure 5. Lipid production by *C. pyrenodiosa* ZF strain at different concentrations of plant growth regulators: (**a**) 6-BA treatments; (**b**) ABA treatments; (**c**) EBR treatments; (**d**) ETH treatments; (**e**) GA_3 treatments; (**f**) NAA treatments; (**g**) SA treatments; (**h**) SPD treatments.

We also calculated lipid productivity of *C. pyrenoidosa* ZF strain treated by plant growth regulators. Increases in lipid productivities were observed due to ABA: 0.1 $mg{\cdot}L^{-1}$ ($8.57 \pm 0.78\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 0.5 $mg{\cdot}L^{-1}$ ($10.99 \pm 1.12\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 10 $mg{\cdot}L^{-1}$ ($9.65 \pm 0.61\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 20 $mg{\cdot}L^{-1}$ ($11.04 \pm 1.08\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) and 50 $mg{\cdot}L^{-1}$ ($8.72 \pm 0.58\ mg{\cdot}L^{-1}{\cdot}d^{-1}$); GA_3: 0.1 $mg{\cdot}L^{-1}$ ($9.21 \pm 1.02\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 5 $mg{\cdot}L^{-1}$ ($15.21 \pm 2.06\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 10 $mg{\cdot}L^{-1}$ ($10.82 \pm 0.99\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 15 $mg{\cdot}L^{-1}$ ($8.89 \pm 0.78\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) and 20 $mg{\cdot}L^{-1}$ ($8.35 \pm 0.59\ mg{\cdot}L^{-1}{\cdot}d^{-1}$); SPD: 0.1 $mg{\cdot}L^{-1}$ ($9.31 \pm 0.66\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 10 $mg{\cdot}L^{-1}$ ($10.29 \pm 1.10\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 15 $mg{\cdot}L^{-1}$ ($9.03 \pm 0.87\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 20 $mg{\cdot}L^{-1}$ ($9.90 \pm 0.63\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) and 50 $mg{\cdot}L^{-1}$ ($8.05 \pm 0.75\ mg{\cdot}L^{-1}{\cdot}d^{-1}$); SA: 0.5 $mg{\cdot}L^{-1}$ ($11.54 \pm 1.22\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 10 $mg{\cdot}L^{-1}$ ($10.01 \pm 0.89\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 20 $mg{\cdot}L^{-1}$ ($14.14 \pm 1.55\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) and 50 $mg{\cdot}L^{-1}$ ($10.85 \pm 0.98\ mg{\cdot}L^{-1}{\cdot}d^{-1}$); NAA: 1 $mg{\cdot}L^{-1}$ ($11.04 \pm 1.11\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 15 $mg{\cdot}L^{-1}$ ($11.05 \pm 1.00\ mg{\cdot}L^{-1}{\cdot}d^{-1}$), 20 $mg{\cdot}L^{-1}$ ($8.11 \pm 0.56\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) and 50 $mg{\cdot}L^{-1}$ ($10.57 \pm 0.99\ mg{\cdot}L^{-1}{\cdot}d^{-1}$); 6-BA: 1 $mg{\cdot}L^{-1}$ ($14.70 \pm 1.56\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) and 5 $mg{\cdot}L^{-1}$ ($9.49 \pm 1.02\ mg{\cdot}L^{-1}{\cdot}d^{-1}$); and ETH: 0.2 $mg{\cdot}L^{-1}$ ($10.25 \pm 1.50\ mg{\cdot}L^{-1}{\cdot}d^{-1}$) compared to controls

with 5.15 ± 0.63 mg·L^{-1}·d^{-1} (Figure 6). However, no significant increase in lipid productivity could be found for EBR treatments.

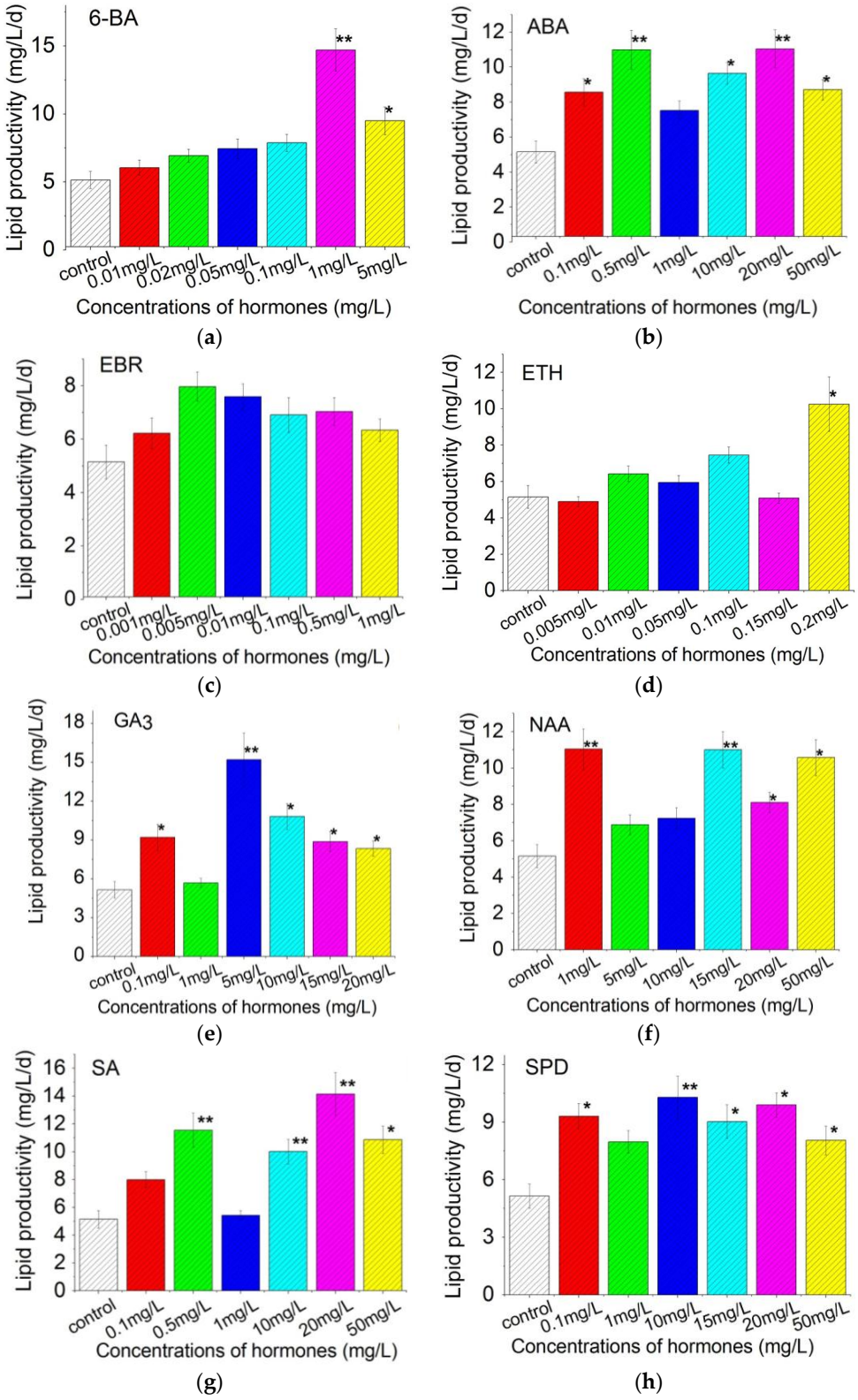

Figure 6. Lipid productivities of *C. pyrenodiosa* ZF strain at different concentrations of plant growth regulators: (**a**) 6-BA treatments; (**b**) ABA treatments; (**c**) EBR treatments; (**d**) ETH treatments; (**e**) GA_3 treatments; (**f**) NAA treatments; (**g**) SA treatments; (**h**) SPD treatments.

2.5. Effect on Fatty Acid Compositions

We detected 11 individual fatty acid methyl esters (FAME) in the treated *C. pyrenoidosa* ZF strain biomass which was higher than that of in the controls (Table 1). Among the FAMEs detected in the control and treatments, C17:1 was the most dominant fatty acid with a few exceptions. Additionally, significant increases in the percentage composition of C17:1 were found after all treatments (34.97 ± 3.48% for 0.1 mg·L^{-1} SPD to 58.45 ± 4.82% for 0.5 mg·L^{-1} EBR) compared to control (26.35 ± 3.27%) with a few exceptions (Table 1). Similarly, C17:0 was also significantly increased in the treatments compared to the controls (8.47 ± 0.58 to 33.26 ± 3.78% vs. 6.57 ± 0.62% in the controls) with a few exceptions.

The other less dominant FAME C16:0 was also induced to accumulate at higher percentages due to all hormone treatments except ETH. Increases in the contents of C12:0 were observed only due to ETH and SA treatments (ETH: 7.38 ± 0.98 to 15.49 ± 1.02%; SA: 7.56 ± 0.53 to 15.94 ± 1.05% vs. 4.59 ± 0.57% in the controls). Similarly, the treatments by ABA (50 mg·L^{-1}), ETH (0.005 and 0.15 mg·L^{-1}) and NAA (10 and 20 mg·L^{-1}) and SA (0.1 mg·L^{-1}) induced higher production of C15:1 compared to the controls (Table 1). Although detected in the control biomass, C16:1 was at undetectable levels in many treatments, with significant increases observed only due to GA_3 treatment (10.62 ± 1.32 to 12.83 ± 0.98% at 0.1–5 mg·L^{-1} vs. 3.82 ± 0.48% in the controls). The other detected FAME (Oleic acid C18:1n9, Linoleic acid C18:2n6, Linolenic acid C18:3n3, Arachidonic acid C20:4n6, Docosahexaenoic acid C22:6n3) were at low concentrations (<5% of total fatty acids) in the controls and treatments and therefore were not tested for statistical significance (Table 1).

2.6. Expression of Fatty Acid Synthesis Related Genes

The expression of FA synthesis genes were explored using quantitative RT-qPCR. Among the seven genes investigated in our study, all except *KAS* were up-regulated in *C. pyrenoidosa* ZF biomass in response to ABA treatments (Figure 7). The treatment by GA_3 also up-regulated all studied genes except *FATA* and *SAD*. Similar patterns were observed for the treatments with SA, NAA, 6-BA and SPD where same genes (*FAD*, *MCTK*) were up-regulated. 6-BA and NAA treatments also up-regulated the *ACP* gene whereas SA, NAA, EBR treatments caused the up-regulation of *BC* and *FAD* genes (Figure 7). No gene was up-regulated in response to the treatments with ETH.

Table 1. Fatty acid profile (%) in control and plant growth regulators; "-" represent this polyunsaturated fatty acid does not exist or is below the instrument detection concentration range. Shown are mean values and SEs from three separately-grown cultures, each. "*" represented increased significantly ($p < 0.05$) and "**" increased different significantly ($p < 0.01$), respectively.

Components (%)	C12:0	C15:1	C16:1	C16:0	C17:1	C17:0	C18:1n9t	C18:2n6	C18:3n3	C20:4n6	C22:6n3	Others
Control	4.59 ± 0.57	8.02 ± 1.10	3.82 ± 0.48	6.28 ± 0.52	26.35 ± 3.27	6.57 ± 0.62	-	1.31 ± 0.22	-	0.71 ± 0.08	-	42.35 ± 3.86
0.5 mg·L^{-1} ABA	3.67 ± 0.28	1.32 ± 0.10	-	9.54 ± 1.02 *	51.30 ± 5.34 **	9.28 ± 1.02 *	-	-	-	-	-	24.89 ± 2.73
10 mg·L^{-1} ABA	3.15 ± 0.33	0.85 ± 0.04	-	8.89 ± 0.54 *	43.11 ± 3.89 **	11.40 ± 0.86 **	-	-	-	-	-	32.60 ± 4.05
20 mg·L^{-1} ABA	2.88 ± 0.30	1.26 ± 0.10	-	7.49 ± 0.64	32.59 ± 4.02	15.28 ± 1.32 **	3.54 ± 0.22	-	-	-	-	36.96 ± 4.52
50 mg·L^{-1} ABA	1.74 ± 0.23	11.83 ± 1.02 *	-	13.04 ± 1.02 **	51.45 ± 4.85 **	9.35 ± 1.00 *	-	-	-	-	-	12.59 ± 1.23
0.05 mg·L^{-1} 6-BA	2.96 ± 0.31	7.75 ± 0.52	-	7.62 ± 0.55	43.23 ± 3.99 **	6.20 ± 0.38	-	-	-	-	-	32.24 ± 2.88
0.1 mg·L^{-1} 6-BA	2.55 ± 0.25	-	-	6.45 ± 0.63	33.48 ± 3.48	8.47 ± 0.58 *	-	-	-	-	-	49.05 ± 4.36
1 mg·L^{-1} 6-BA	4.26 ± 0.18	0.82 ± 0.13	-	12.08 ± 0.87 **	35.23 ± 2.36 *	10.28 ± 0.72 **	3.06 ± 0.11	-	-	-	-	34.27 ± 2.96
5 mg·L^{-1} 6-BA	3.07 ± 0.15	2.23 ± 0.23	-	6.77 ± 0.74	44.56 ± 3.89 **	3.45 ± 0.31	-	-	-	-	-	39.92 ± 4.03
0.005 mg·L^{-1} EBR	2.48 ± 0.22	-	-	13.55 ± 1.24 **	38.13 ± 3.56 *	5.24 ± 0.35	4.16 ± 0.21	-	-	0.77 ± 0.05	-	35.67 ± 2.86
0.01 mg·L^{-1} EBR	2.47 ± 0.19	-	-	7.83 ± 0.56	42.87 ± 0.23 **	-	-	-	-	-	-	46.83 ± 4.21
0.1 mg·L^{-1} EBR	4.78 ± 0.55	0.87 ± 0.05	-	5.52 ± 0.36	47.37 ± 2.53 **	8.07 ± 0.50	-	-	-	-	-	33.39 ± 2.89
0.5 mg·L^{-1} EBR	1.79 ± 0.22	1.61 ± 0.11	-	11.38 ± 1.22 **	58.45 ± 4.82 **	2.15 ± 0.15	-	-	-	-	-	24.62 ± 3.08
0.005 mg·L^{-1} ETH	15.49 ± 1.02 **	16.67 ± 2.10 **	3.78 ± 0.44	4.49 ± 0.42	16.83 ± 1.83	33.26 ± 3.78 **	-	1.24 ± 0.12	-	-	-	8.24 ± 0.58
0.01 mg·L^{-1} ETH	10.27 ± 0.89 **	7.11 ± 0.36	3.05 ± 0.25	3.12 ± 0.21	53.74 ± 5.62 **	14.74 ± 1.23 **	-	0.76 ± 0.03	-	-	-	7.21 ± 0.32
0.1 mg·L^{-1} ETH	9.16 ± 0.92	8.56 ± 1.00	4.02 ± 0.45	3.03 ± 0.18	54.76 ± 7.23 **	0.46 ± 0.05	-	0.62 ± 0.07	-	-	-	19.39 ± 175
0.15 mg·L^{-1} ETH	11.61 ± 1.22 **	13.02 ± 1.52 *	3.18 ± 0.30	5.10 ± 0.45	52.75 ± 4.78 **	1.30 ± 0.10	-	1.46 ± 0.13	-	-	-	11.58 ± 1.05
0.2 mg·L^{-1} ETH	7.38 ± 0.98 *	8.30 ± 0.89	3.19 ± 0.26	4.56 ± 0.45	45.75 ± 4.55 **	18.59 ± 2.92 **	-	1.82 ± 0.23	-	-	-	10.41 ± 1.02
0.1 mg·L^{-1} GA3	4.73 ± 0.38	8.93 ± 1.08	10.62 ± 1.32 **	8.11 ± 0.75	38.57 ± 4.31 *	15.57 ± 2.57 **	-	1.74 ± 0.21	-	-	-	11.73 ± 1.51
5 mg·L^{-1} GA3	2.37 ± 0.11	6.47 ± 0.32	12.83 ± 0.98 **	6.81 ± 0.45	32.25 ± 1.88	12.28 ± 0.86 **	0.93 ± 0.02	0.87 ± 0.12	0.57 ± 0.03	2.53 ± 0.15 *	0.44 ± 0.05	21.65 ± 1.42
10 mg·L^{-1} GA3	6.78 ± 0.30	10.23 ± 1.02	-	9.18 ± 1.02 *	39.27 ± 2.88 *	10.35 ± 1.02 **	-	1.25 ± 0.18	-	-	-	22.94 ± 1.98
15 mg·L^{-1} GA3	4.64 ± 0.35	6.51 ± 0.39	-	2.79 ± 0.23	56.43 ± 6.15 **	18.62 ± 1.53 **	-	0.53 ± 0.05	-	-	-	10.48 ± 1.12
20 mg·L^{-1} GA3	4.85 ± 0.41	9.81 ± 0.83	-	1.51 ± 0.21	55.32 ± 4.86 **	8.77 ± 1.02 *	-	0.39 ± 0.02	-	-	-	19.35 ± 2.14
1 mg·L^{-1} NAA	3.17 ± 0.38	8.25 ± 1.02	-	3.80 ± 0.41	4 .35 ± 3.02 **	7.47 ± 0.65	1.21 ± 0.07	0.57 ± 0.03	0.65 ± 0.01	1.64 ± 0.12*	-	27.89 ± 2.76
10 mg·L^{-1} NAA	1.59 ± 0.15	12.77 ± 1.21 *	-	8.05 ± 0.26	36.71 ± 2.23 *	5.28 ± 0.32	-	-	-	-	-	35.60 ± 3.76
15 mg·L^{-1} NAA	2.35 ± 0.22	9.26 ± 0.41	-	11.84 ± 1.08 **	26.61 ± 3.03	4.67 ± 0.03	-	-	-	-	-	45.27 ± 4.16
20 mg·L^{-1} NAA	2.02 ± 0.18	11.19 ± 0.28 *	-	8.31 ± 0.38 *	45.87 ± 2.30 **	1.81 ± 0.26	-	-	-	-	-	30.80 ± 2.57
0.1 mg·L^{-1} SA	8.42 ± 0.76 *	11.36 ± 1.32 *	2.54 ± 0.25	3.12 ± 0.25	40.09 ± 4.87 *	27.26 ± 2.85 **	-	1.30 ± 0.09	-	-	-	5.91 ± 0.43
0.5 mg·L^{-1} SA	2.25 ± 0.18	2.73 ± 0.23	-	8.23 ± 0.48 *	25.00 ± 2.87	19.87 ± 1.48 **	-	-	-	-	-	41.92 ± 3.94
1 mg·L^{-1} SA	12.62 ± 2.35 **	7.47 ± 0.92	2.46 ± 0.44	3.33 ± 0.26	45.55 ± 6.95 **	16.17 ± 1.98 **	-	0.89 ± 0.04	-	-	-	11.51 ± 1.22
10 mg·L^{-1} SA	13.73 ± 1.12 **	8.73 ± 1.22	3.19 ± 0.15	5.53 ± 0.75	41.10 ± 6.01 *	15.25 ± 3.59 **	-	1.07 ± 0.11	-	-	-	11.40 ± 1.24
20 mg·L^{-1} SA	15.94 ± 1.05 **	9.78 ± 1.02	3.09 ± 0.30	3.08 ± 0.26	42.07 ± 4.98 **	18.22 ± 3.02 **	-	0.96 ± 0.5	-	-	-	6.86 ± 0.84
50 mg·L^{-1} SA	7.56 ± 0.53 *	2.26 ± 0.12	-	5.82 ± 0.36	30.36 ± 2.36	15.24 ± 1..63 **	-	-	-	-	-	38.76 ± 4.15
0.1 mg·L^{-1} SPD	-	2.67 ± 0.21	-	3.82 ± 0.22	34.97 ± 3.48 *	12.29 ± 1.12 **	-	-	-	-	-	46.25 ± 5.82
10 mg·L^{-1} SPD	1.30 ± 0.18	8.13 ± 0.56	2.77 ± 0.27	11.89 ± 0.98 **	54.56 ± 4.99 **	2.44 ± 0.15	2.76 ± 0.22	0.46 ± 0.02	-	-	-	15.69 ± 1.28
20 mg·L^{-1} SPD	2.93 ± 0.15	5.23 ± 0.22	-	10.21 ± 0.86 *	38.28 ± 3.26 *	1.95 ± 0.23	-	0.37 ± 0.05	-	-	-	41.03 ± 4.53
50 mg·L^{-1} SPD	5.34 ± 0.37.	5.39 ± 0.66	-	8.98 ± 1.03 *	38.55 ± 4.02 *	2.40 ± 0.23	-	-	-	-	-	39.34 ± 3.64

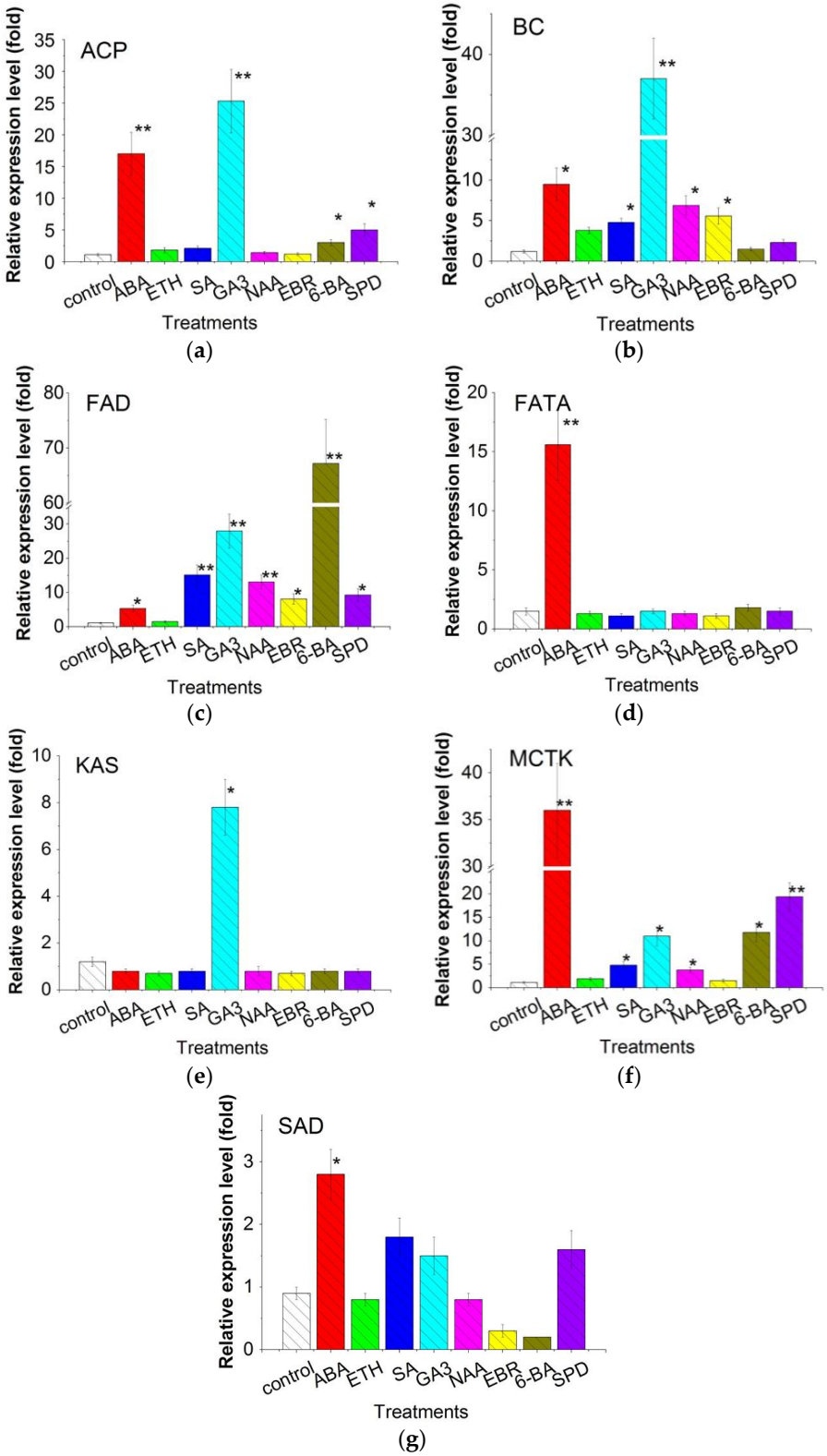

Figure 7. Gene expression detected by real-time qPCR at different concentrations of plant growth regulators: (**a**) relative expression of *ACP*; (**b**) relative expression of *BC*; (**c**) relative expression of *FAD*; (**d**) relative expression of *FATA*; (**e**) relative expression of *KAS*; (**f**) relative expression of *MCTK*; (**g**) relative expression of *SAD*.

3. Discussion

For biodiesel production from microalgae, increasing the growth rate and lipid content are the main goals. In our present study, we have studied the growth, protein content, carotenoids content and lipid content and productivity of *C. pyrenoidosa* ZF strain treated with eight kinds of plant growth regulators. Our results indicated that almost all of treatments selected in this study could promote cell growth, protein content, carotenoids content, lipid content, lipid productivity and related gene expression in *C. pyrenoidosa* ZF strain.

3.1. The Cell Growth Analysis in C. Pyrenoidosa ZF Strain

In the present study, all plant growth regulators we selected stimulated the growth of the *C. pyrenoidosa* ZF strain. NAA is the most commonly used synthetic plant growth regulator, with higher activity [41], and it can obviously affect algal growth, which was consistent with results from [12]. In our study, 50 mg·L^{-1} NAA were the most effective for *C. pyrenoidosa* ZF strain growth, with a 6.3-fold increase compared to the control. Liu et al. reported that cell cycle composed of growth phase 1 (G_1), DNA synthetic phase (S), growth phase 2 (G_2) and mitosis (M) with two main check points-the G_1/S and G_2/M transitions were prone to ambient environmental factors [12]. Stirk et al. suggested that auxins are the most influential cell cycle hormones as auxins affect the re-entry into the cell cycle as well as most other phases, such as G_1, S, G_2, or M [42]. Many hormone (e.g., NAA) signaling pathways converge at the G_1/S and G_2/M transitions to promote the normal progression of the cell cycle [41,42]. In our study, all the plant hormones except ABA and SA can significantly increase *C. pyrenoidosa* ZF strain cell growth. Collectively, plant hormone and its analogs were characterized by the potential superiority in improving biomass productivity.

3.2. Total Soluble Protein and Carotenoids Production

In the present study, plant growth regulators (6-BA, EBR, ETH, GA_3, NAA and SA) significantly increased protein accumulation in *C. pyrenoidosa* ZF strain after 12 days of cultivation. Czerpak et al. reported that 10^{-4} M SA increased the *C. vulgaris* protein content (about 60%) cultured 8–12 days [19]. Gao et al. reported that 25 mg·L^{-1} SA could up-regulate the protein levels in *H. pluvialis* for 123 proteins, our study also indicated that 1 mg·L^{-1} SA induced *C. pyrenoidosa* ZF to produce protein [18]. Hunt et al. found that GA_3, NAA and SPD had no effect on *C. Sorokiniana* protein accumulation [41]. Our results also showed SPD didn't induce *C. pyrenoidosa* ZF strain to produce protein efficiently, while GA_3 and NAA could induce our microalgae to produce protein efficiently. Although not all plant growth regulators promoted the production of proteins by our alga, most of those studied can be used for this purpose. This study provides us with a good direction for the production of microalgal proteins by plant growth regulators, and also has broad application prospects.

Our results showed that the use of plant growth regulators could be a useful tool to induce carotenoid accumulation in *C. pyrenoidosa* ZF strain cells. During 12 days of cultivation, the carotenoid content were increased significantly with the addition of ABA (0.1, 0.5 and 1 mg·L^{-1}), EBR (0.005, 0.01, 0.5 and 1 mg·L^{-1}), ETH (0.2 mg·L^{-1}), NAA (1 mg·L^{-1}), SA (0.1, 0.5, 20 and 50 mg·L^{-1}) and SPD (10 mg·L^{-1}) treatments, respectively. Besides, the treatments of 6-BA and GA_3, the whole concentrations we studied can promote the carotenoids accumulation efficiently. However, both ETH (except 0.2 mg·L^{-1}) and SPD (except 10 mg·L^{-1}) had no obvious influence on carotenoid accumulation in *C. pyrenoidosa* ZF strain. Carotenoids act as accessory light-harvesting pigments, and they perform an essential photo protective role by quenching triplet state chlorophyll molecules and scavenging toxic oxygen radicals formed within the chloroplast [43]. Cowan and Rose reported that ABA might function as a regulator of carotenogenesis in salt-stressed cells of the β-carotene-producing *Dunaliella salina* [28]. Kobayashi et al. reported analogs of ABA could be used as effective regulators to produce carotenoids in *H. pluvialis* cells [29]. Earlier, Gao et al. also had reported that addition of 25 mg·L^{-1} ABA, 25 mg·L^{-1} EBR, 12 mg·L^{-1} ETH, 40 mg·L^{-1} GA_3 could increase the astaxanthin accumulation with 1.49 mg·L^{-1},

2.26 mg·L^{-1}, 3.3 mg·L^{-1} and 2.39 mg·L^{-1} in *H. pluvialis*, respectively [22–25]. The addition of exogenous plant growth regulators can promote the accumulation of carotenoids in *C. pyrenoidosa* ZF strain, which provides a possibility for its commercialization.

3.3. The Lipids and High Value PUFAs Analysis

Some unadvisable environmental conditions for microalgae growth, such as nitrogen depletion [44,45], high salinity [46], high light intensity [45,47] as well as extreme temperatures [48], and so forth, were reported to induce microalgae to accumulate fatty acids (FAs). Earlier study reported that auxin is involved in control of many developmental processes in plants [49]. Exogenous application of phytohormone could not only stimulate microalgae growth but also trigger intracellular lipid biosynthesis at different levels [13,35,50,51].

This study catered to this conclusion by the results that all tested additives (ABA, 6-BA, EBR, ETH, GA_3, NAA, SA and SPD) induced the lipid accumulation within 12 days cultivation in *C. pyrenoidosa* ZF strain. Our results indicated that the highest lipid content (514.3 ± 25.5 mg·g^{-1}) was induced by 5 mg·L^{-1} GA_3, approximately 1.9-fold compared to the controls. Moreover, the addition of 20 mg·L^{-1} ABA, 1 mg·L^{-1} 6-BA, 0.1 mg·L^{-1} EBR, 15 mg·L^{-1} NAA, 20 mg·L^{-1} SA and 15 mg·L^{-1} SPD also led to higher lipid contents, respectively. According to our result, GA_3 plays the most significant increase on lipid accumulation, and other plant growth regulators also can promote lipid production (Figure 5). Hu et al. reported that some algal species can accumulate lipid as much as 60–70% dry weight [52]. Liu et al. reported that 1.0 mg·L^{-1} ABA-treatment can achieve the highest lipid content of 50%, which was almost equal to the highest lipid content (47.84%) of NAA-treated in *C. vulgaris* [12]. Our results also showed that 20 mg·L^{-1} ABA treatment induced the lipid content to 42.31% in *C. pyrenoidosa*. Earlier study reported ABA functioned as a stress molecule in cyanobacteria under salt stress [53] and under salt, osmotic, oxidative, drought and nutrient stresses in unicellular eukaryotic algae [54–58]. The superiority of ABA in promoting lipid biosynthesis was also reported by Park et al. [27], where ABA-treatment favored energy storage in the form of either starch or lipid. However, ABA had little effect on algal growth in our results. From our study, using exogenous plant growth regulators (6-BA, EBR, GA_3, NAA and SPD) can not only promote the growth of *C. pyrenoidosa* ZF strain, but also induce it to accumulate lipid efficiently. Therefore, considering both of biomass and lipid content, plant growth regulators might be a suitable supplementation in developing algal lipids for biodiesel production.

Lipid productivity is also of particular importance in large scale microalgal biodiesel production processes and it concerns both lipid content and biomass [1]. In our present study, 5 mg·L^{-1} GA_3 induced the highest lipid production (15.21 ± 2.06 mg·L^{-1}/d), while, the other plant growth regulators, except EBR, were also significant in promoting *C. pyrenoidosa* ZF strain lipid productivity, compared to the controls. Fan also found that the regular patterns of cell growth and lipid accumulation usually go against the grain [1]. One possible reason is that plant growth regulators provide a disadvantageous environment, and this special circumstance, similar to nitrogen starvation and osmotic stress, guided lipid biosynthesis. Therefore, considering the data derived from both of cell growth and lipid content, the exogenous plant growth regulators except EBR might be the most suitable supplementation in developing lipids for biodiesel production in the *C. pyrenoidosa* ZF strain.

Fatty acids were pivotal component of lipids as well as the fatty acid profiles directly related to biofuel properties [59]. Fatty acids are the building blocks for the formation of various types of lipids including TAGs and all other cellular lipids. Most TAGs are the source for biofuel [52]. As we all know, the main raw material of biodiesel is triglycerides. Most of the bulk of the molecule (as well as the energy contained therein) is contained in the long hydrocarbon chains called fatty acid chains that are attached to it. As long as an oil or fat has this basic structure, it is a good candidate for being turned into biodiesel. The primary factor of an oil or fat that determines if it can be turned into biodiesel is if it was initially composed of triglycerides. Saturated fats are simply a specific type of triglyceride that has no double bonds along the carbon chain, which can make excellent

biodiesel, One drawback of biodiesel made from saturated fats is that it typically has a high melting point; Unsaturated fats are fats or oils in which one or more of the carbon to carbon bonds in the fatty acid chain, is a double bond, which are excellent for cold weather biodiesel production and use, and the downside of unsaturated fats is that they will often be more prone to oxidation and rancidification than their saturated counterparts. In our study, the fatty acid profiles were shown in Table 1, and were mainly composed of C16 and C17 FAs. Such fatty acid profiles were basically the same as those reported in [35,51], where JA-treated and IAA-treated *C. vulgaris* cells produced a high proportion of C16–C18 fatty acids. Mu et al. found that fatty acid (C16–C18) compositions were also indispensable to endow excellent cold filter plugging point for fuels produced by algal biomass [60]. In our study, C16–C17 fatty acids contents were significantly in 50 mg·L^{-1} ABA (73.84 ± 6.87%), 0.5 mg·L^{-1} EBR (79.90 ± 7.99%), 15 mg·L^{-1} GA_3 (83.98 ± 7.23%), which was higher than results from Liu et al. with NAA-treated (72%) and ABA-treated (61%) strains [26]. In general, the peak percentage of C17 fatty acids (mainly C17:0, C17:1) achieved in 50 mg·L^{-1} ABA, 0.05 mg·L^{-1} 6-BA, 0.5 mg·L^{-1} EBR, 0.01 mg·L^{-1} ETH, 15 mg·L^{-1} GA_3, 1 mg L^{-1} NAA, 0.1 mg·L^{-1} SA and 10 mg·L^{-1} SPD with about 60.80%, 49.43%, 60.60%, 68.48%, 75.05%, 52.82%, 67.35% and 57.00%, respectively, which was significantly higher than the control (32.92%). Nevertheless the content of C16 (mainly C16:0, C16:1) only in 0.1 mg·L^{-1} GA_3, 5 mg·L^{-1} GA_3 (19.64%) and 10 mg·L^{-1} SPD (14.66%) was more than control (10.10%). Our study also showed that some valuable polyunsaturated fatty acid (i.e., linoleic acid, linolenic acid, arachidonic acid and docosahexaenoic acid) were produced in *C. pyrenoidosa* ZF strain treated by supplementation of various plant growth regulators. However, its content was very low so that required further exploration. Therefore, we can induce the *C. pyrenoidosa* ZF strain by adding exogenous plant growth regulators to produce saturated fatty acids and unsaturated fatty acids which are beneficial to us.

3.4. Transcriptional Expression of 7 Genes Related Fatty Acid Synthesis

The transcriptional expression of seven different genes (*ACP, BC, AD, FATA, KAS, CTK, SAD*) related to lipid synthesis in *C. pyrenoidosa* ZF strain was analyzed in samples cultured with various plant growth regulators. Khozingoldberg et al. thought detailed characterization of crucial genes in FA biosynthesis should be studied in depth for further information due to the high potential of microalgae as a biodiesel feedstock [61]. Many experts have reported correlations between gene expression in the FA synthesis pathway and FA profiling in higher plants [62–65], but there was no detailed investigation in connection with FA synthesis genes in microalgae, though the FA profiles under different treatments were reported [45,66,67]. In our study, almost all selected genes were up-regulated with differential mechanisms under various plant growth regulators stresses, which indicated that the FA biosynthesis be up-regulated at a transcriptional level in *C. pyrenoidosa* ZF strain.

The present study evaluated both FA profile and expression patterns of seven genes involved in FA biosynthesis, which was coupled with an increase of lipid content in *C. pyrenoidosa* ZF strain. In our study, all genes we selected were significantly upregulated, especially in 5 mg·L^{-1} GA_3, where all genes were up-regulated, and five genes were significantly or very significantly increased. What's more, the maximum transcriptional level of *ACP* reached 23-fold in 5 mg·L^{-1} GA_3 treatment, which was higher than that of reported by Lei et al. under HT with 8.7-fold [68]. The highest expression of *BC, FAD, FATA, KAS, MCTK* and *SAD* gene was 31-, 61-, 11-, 7-, 33- and 3-fold with 5 mg·L^{-1} GA_3, 1 mg·L^{-1} 6-BA, 20 mg·L^{-1} ABA treatments, respectively. It is intriguing that different genes had various expression levels under different plan growth regulators (Figure 7). The transcriptional expression levels of these seven genes are very crucial [69,70] and were up-regulated with differences under various plant growth regulators in *C. pyrenoidosa* ZF strain. Lei also reported that some of genes coding for FA synthesis were up-regulated, coupled with an increase of FA content in *H. pluvialis* under stress conditions [68]. Gao et al. also reported that JA and SA regulated the mRNA levels of *ACP, BC, FAD, FATA, KAS, MCTK* and *SAD* genes in *H. pluvialis* [18]. In a word, the eight plant growth regulators we studied might

up-regulate the related genes for the FA biosynthesis and affect lipid production in *C. pyrenoidosa* ZF strain, in which lipid production had significant positive correlations with all selected seven genes.

4. Materials and Methods

4.1. Culture of C. pyrenoidosa ZF Strain

The *C. pyrenoidosa* ZF strain was obtained from the Institute of Oceanology, Chinese Academy of Sciences. The algal culture was initiated from a single colony taken from the stock agar plate and cultured in BG11 medium. The composition of BG11 culture medium was as follows (per litre): $NaNO_3$ 75 g, K_2HPO_4 2 g, $MgSO_4{\cdot}7H_2O$ 3.75 g, Na_2CO_3 1 g, citric acid 0.3 g, $CaCl_2{\cdot}2H_2O$ 1.8 g, ferric ammonium citrate 0.3 g, $EDTANa_2$ 50 mg, H_3BO_3 2.86 g, $MnCl_2{\cdot}4H_2O$ 1.86 g, $ZnSO_4{\cdot}7H_2O$ 0.22 g, $CuSO_4{\cdot}5H_2O$ 80 mg, $Na_2MoO_4{\cdot}2H_2O$ 0.39 g, $Co(NO_3)_2{\cdot}6H_2O$ 50 mg. The pH of the culture medium was adjusted to 7.1 with 1 M HCl. The BG11 growth medium and all erlenmeyer flasks used for growing *C. pyrenoidosa* ZF strain were sterilized at 121 °C for 30 min. The microalgal cultures were illuminated with cool-white fluorescent light at 36 µmol photons/m^2/s on a 12:12 h light/dark cycle at 24 ± 2 °C. The culture flasks were shaken manually (three times at fixed time every day) and nutrients were added (2-fold of BG11concentration of above) to the flasks once a week.

4.2. Treatment of C. Pyrenoidosa ZF Strain Cultures

The plant growth regulators used in this study were dissolved in water, made into 1 g·L^{-1} stock solutions and stored at 4 °C. Six different concentrations of EBR, GA_3, ETH, SPD, 6-BA, NAA, SA and ABA were prepared from the stock and used to treat *C. pyrenoidosa* ZF cultures (separately grown cultures; n = 3) at exponential growth phase (Table 2).

Table 2. The final concentrations gradients of plant growth regulators were added into algae solution.

PGR Kinds	Concentrations of Plant Growth Regulators (mg·L^{-1})					
EBR	0.001	0.005	0.010	0.100	0.500	1.000
GA_3	0.100	1.000	5.000	10.000	15.000	20.000
ETH	0.005	0.010	0.050	0.100	0.150	0.200
SPD	0.100	1.000	10.000	15.000	20.000	50.000
6-BA	0.010	0.020	0.050	0.100	1.000	5.000
NAA	1.000	5.000	10.000	15.000	20.000	50.000
SA	0.100	0.500	1.000	10.000	20.000	50.000
ABA	0.100	0.500	1.000	10.000	20.000	50.000

Microalgae cultures grown in BG11 media only were used as controls. Medium was added regularly (2 × concentration of BG11) to avoid any effect of nutrient starvation in the microalgal cultures. Biomass was collected after 12 days of cultivation and harvested by centrifugation at 7100× g for 10 min (GTR16-2, Beijing Era Beili, Beijing, China). The supernatants were discarded and the pallets were dried at −40 °C in a freeze-dryer (LGJ-10, Beijing Sihuan Technology, Beijing, China) and then stored at −20 °C until used for further analyses.

4.3. Determination of Growth of C. Pyrenoidosa ZF Cultures

The growths of treated and untreated (control) *C. pyrenoidosa* ZF cultures were determined by measuring their optical densities (OD) at 450 nm in a UV/VIS spectrophotometer (T6, Puxitongyong Company, Beijing, China). The cell densities of the cultures were determined by plotting a calibration curve of OD readings against various cell numbers as previously described in [71].

4.4. Determination of Total Soluble Protein Contents

In order to measure total soluble protein, 50 mg freeze-dried microalgal biomass (n = 3) was dissolved in 20 mL 0.2 M PBS pH 7.4. The PBS stock was prepared by dissolving 8 g NaCl, 0.2 g KCl, 3.63 g $Na_2HPO_4 \cdot 12H_2O$ and 0.24 g KH_2PO_4 in 1 L distilled water. Proteins were extracted by ultrasonic fragmentation (VC605, SONICS, Oklahoma City, OK, USA) in an ice-water bath and the conditions were: effective power of 240 W, work 15 S, interval 5 S, cycle 2 min. Supernatants were harvested by centrifugation at 7100× g for 10 min and were used for measurement of protein contents. The protein contents in the treated and untreated microalgal biomass were determined according to Bradford's method [72].

The soluble protein contents were determined using the following Equation:

$$\text{Total protein (mg/g DW)} = \frac{m(\text{protein})}{m(\text{biomass})} \quad (1)$$

where m (protein) = weight of protein (mg), m (biomass) = weight of biomass (g DW).

4.5. Measurement of Total Carotenoid Contents

Freeze-dried microalgal biomass (50 mg, n = 3) from treated and control cultures were dissolved in 90% acetone (10 mL) and kept in dark for 24 h at 4 °C. Total carotenoids in the extracts were measured by recording optical densities at 663.6 nm, 646.6 nm and 470 nm with a UV/VIS spectrophotometer (T6, Puxitongyong Company) and using the following equations as described by [73]:

$$\begin{aligned} Ca &= 12.25\text{A}663.6 - 2.79\text{A}646.6 \\ Cb &= 21.50\text{A}646.6 - 5.10\text{A}663.6 \\ \text{Total carotenoids (g/L)} &= \frac{1000A470 - 1.82Ca - 85.02Cb}{198} \end{aligned} \quad (2)$$

where Ca = Chlorophyll a content; Cb = Chlorophyll b content; A = Absorbance value.

4.6. Measurement of Total Lipid Contents

Measurement of total lipid contents in the freeze-dried biomass from treated and control microalgal cultures was conducted according to [74] with minor modifications. In brief, dry biomass (10 mg) was added to a chloroform-methanol (2:1) mixture (volume) and placed in an ultrasonic crusher (VC605, SONICS, Bloomington, MN, USA) in ice bath (effective power of 200 W, cycle 1 min, work 15 s, interval 5 s). The mixture was centrifuged at 10,000× g for 10 min at 4 °C and then the supernatants were added to a 1:3 chloroform and water mixture. The clear layers on the top were dried at 61 °C. The total lipid contents, lipid production and lipid productivity were calculated using the following Equations:

$$\begin{aligned} \textit{Total} \text{ lipid content}(\%) &= \frac{m_1}{m_0} \times 100\% \\ \text{Lipid production}(\text{mg/g DW}) &= \frac{m_1}{m_0} \\ \textit{Lipid} \text{ productivity (mg/L/d)} &= \frac{m_1}{V \times D} \end{aligned} \quad (3)$$

where m_0 =the weight of biomass (g); m_1 = the weight of lipid layers DW (mg); V = the volume of cultures (L); D = the duration of culture (d).

4.7. Analysis of Fatty Acid Methyl Esters (FAMEs)

The analysis of FAME was conducted following the method described by [75] with some modifications. In brief, the dried lipid layers from the previous assay were mixed with 0.5 M potassium hydroxide/methanol solution and incubated at 60 °C for 15 min; and the internal standard was methyl nonadecanoate (Sigma, Ronkonkoma, NY, USA). The mixture was cooled to room temperature and mixed with 2 mL 14% boron trifluoride/methanol solution for incubation at 60 °C for 2 min to

facilitate transesterification. Then, 1 mL saturated NaCl and 1 mL of hexane was added and mixed for 20 s. To separate the phase, anhydrous Na_2SO_4 was added to the samples and the mixture was then centrifuged at 16,000× g for 3 min. A total of 1 µL of the hexane layer was injected into a GC-2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) fitted with a sp-2560 column (100 m × 0.25 mm × 0.20 µm, Supelco, Bellefonte, PA, USA) and a flame ionization detector. Nitrogen was used as carrier gas at a constant flow rate of 30 mL·min^{-1}. Split injections at 1:18 were performed at 300 °C and the oven temperatures applied were 120 °C for 1 min, and then increased to 210 °C at 6 °C/min and held for 10 min. Identification of fatty acids was accomplished by comparing the peaks and retention times of the FAMEs to that of the reference standard Supelco 37 Component FAME Mix (Sigma Aldrich, Shanghai, China).

4.8. Expression Profiling of Fatty Acid Biosynthesis Genes

Frozen fresh microalgal biomass was ground into a fine powder using liquid nitrogen. The Trizol reagent was used to extracted total RNA according to the manufacturer's instructions. The RNA samples was digested with DNaseI. The cDNA used for real-time PCR was synthesized from total RNA using SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA).

Using gene sequences retrieved from NCBI database (HM560033, HM560034, HM560035, HM560036, and HM560037), primers were designed according to [68] (Table 3). Primers were synthesized using Nanjing Genscript. Seven known FA synthesis genes, biotin carboxylase (*BC*), 3-ketoacyl carrier protein synthase gene (*KAS*), acyl-acyl carrier protein (*ACP*), acyl carrier protein thioesterase (*FATA*), ω-3 fatty acid desaturase (*FAD*), malonyl-CoA: *ACP* transacylase (*MCTK*) and stearoyl-ACP-desaturase (*SAD*), were investigated in this study.

Table 3. Genes and primer sequences.

Name	Primer Sequence (5′-3′)
BC	F: CAAGAAGGTGATGATCGCCA R: GACGTGCAGCGAGTTCTTGTC
FATA	F: AGACTCGTTCAGCGAGGAGC R: CATGCCCACAGCATGGTTC
ACP	F: CAGCTCGGCACTGACCTTG R: CAAGGGTCAGCTCGAACTTCTC
SAD	F: CCGAGCCCAAGCTTCTAGTG R: TTTGCCTCCATGTAATCCCC
MCTK	F: GGTGAGGACAAGGCGGTG R: TCATCCTGGCCTTGAAGCTC
FAD	F: GTAGGTCACCACGTCCAGCC R: CTTGATAGGCATGCTGGGTGT
KAS	F: CACCCCACTCTGAACCAGGA R: GACCTCCAAACCCGAAGGAG

Real-time qPCR analysis was conducted on an ABI-7900HT System (Applied Biosystems, Foster City, CA, USA) using SYBR green fluorescence (Applied Biosystems), using actin gene as the internal control. Each PCR reaction consisted of 10 µL SYBR green, 0.5 µL dNTP, 1 µL Taq DNA polymerase, 7.5 µL diH_2O, 1 µL of primer mixture (forward and reverse, 0.5 µL each) and 2 µL of cDNA (2 µg·µL^{-1}). The thermal cycles were set as follows: stage 1—95 °C for 10 min; stage 2—45 cycles of 95 °C for 15 s and 60 °C for 1 min; stage 3—1 cycle of 95 °C for 2 min, 60 °C for 15 s and 95 °C for 15 s. The $2^{-\triangle\triangle CT}$ method was used to analyze quantitative real-time qPCR data.

4.9. Statistical Analysis

All experiments were performed in triplicate. The effects of various plant growth regulators on the accumulation of lipids, fatty acid methyl ester (FAMEs), proteins, carotenoids and the expressions of fatty acid biosynthesis relevant genes in *C. pyrenoidosa* ZF strain were analyzed by one-way analysis of variance (ANOVA) and LSD multiple comparison tests were used to detect differences among the groups of different trials. *p*-values of less than 0.05 and 0.01 were considered to be different statistically significantly and very significantly, respectively. Results were analyzed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and data were expressed as mean ± standard error (SE).

5. Conclusions

In this study, cultivation with eight kinds of plant growth regulator was able to produce high cell number and other secondary metabolites, as well as improvement of oil yield. The highest biomass of *C. pyrenoidosa* ZF strain was achieved with 50 mg·L^{-1} NAA (31.54 ± 1.15) × 10^6; the highest protein content was increased by 0.005 mg·L^{-1} ETH (99.4 ± 5.2 mg·g^{-1}); the carotenoids were up-regulated most effectively by 20 mg·L^{-1} NAA (11.77 ± 1.27 mg·g^{-1}); the most efficient elicitor for lipid content was 5 mg·L^{-1} GA_3 (514.3 ± 25.5 mg·g^{-1}). Regarding the influence on levels of four kinds of high value polyunsaturated fatty acids, ETH induced linoleic acid with 1.82 ± 0.23% of total fatty acids; while NAA increased linoienic acid with 0.65 ± 0.01%; GA_3 induced the production of both arachidonic acid and docosahexaenoic acid with 2.53 ± 0.15% and 0.44 ± 0.05%, respectively. In this study, seven genes related with FA biosynthesis were studied in *C. pyrenoidosa* ZF strain, and the expression level of each gene varied differentially under diverse plant growth regulators. With 5 mg·L^{-1} GA_3, all seven genes were up-regulated, and the FA accumulation was also promoted. The part of this study showed that plant growth regulators might promote lipid accumulation as well as cell growth in *C. pyrenoidosa* ZF strain. Future work on plant growth regulators and gene expression availability provide new insight in the algal cell.

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References

1. Fan, J.H.; Cui, Y.B.; Wan, M.X.; Wang, W.L.; Li, Y.G. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. *Biotechnol. Biofuel* **2014**, *7*, 17–30. [CrossRef] [PubMed]
2. Wan, M.X.; Liu, P.; Xia, J.L.; Rosenberg, J.L.; Oyler, G.; Betenbaugh, M.; Nie, Z.Y.; Qiu, G.Z. The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in *Chlorella sorokiniana*. *Appl. Microbiol. Biotechnol.* **2011**, *91*, 835–844. [CrossRef] [PubMed]
3. Rosenberg, J.N.; Oyler, G.; Wilkinson, L.; Betenbaugh, M.J. A green light for engineered algae: Redirecting metabolism to fuel a biotechnology revolution. *Curr. Opin. Biotechnol.* **2008**, *19*, 430–436. [CrossRef] [PubMed]
4. Singh, P.; Kumari, S.; Guldhe, A.; Misra, R.; Rawat, I.; Bux, F. Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. *Renew. Sustain. Energy Rev.* **2016**, *55*, 1–16. [CrossRef]
5. Ho, S.H.; Chen, C.Y.; Chang, J.S. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. *Bioresour. Technol.* **2012**, *113*, 244–252. [CrossRef] [PubMed]

6. Hockin, N.L.; Mock, T.; Mulholland, F.; Kopriva, S.; Malin, G. The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants. *Plant Physiol.* **2012**, *158*, 299–312. [CrossRef] [PubMed]
7. Yu, X.H.; Chen, L.; Zhan, W.W. Chemicals to enhance microalgal growth and accumulation of high-value bioproducts. *Front. Microbiol.* **2015**, *6*, 56. [CrossRef] [PubMed]
8. Evans, L.V.; Trewavas, A.J. Is algal development controlled by plant growth substances? *J. Phycol.* **1991**, *27*, 322–326. [CrossRef]
9. Bradley, P.M. Plant hormones do have a role in controlling growth and development of algae. *J. Phycol.* **1991**, *27*, 317–321. [CrossRef]
10. Tarakhovskaya, E.R.; Maslov, Y.I.; Shishova, M.F. Phytohormones in algae. *Russ. J. Plant Physiol.* **2007**, *54*, 186–194. [CrossRef]
11. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **2007**, *25*, 294–306. [CrossRef] [PubMed]
12. Liu, T.T.; Liu, F.; Wang, C.; Wang, Z.Y.; Li, Y.Q. The boosted biomass and lipid accumulation in *Chlorella vulgaris* by supplementation of synthetic phytohormone analogs. *Bioresour. Technol.* **2017**, *232*, 44–52. [CrossRef] [PubMed]
13. Lu, Y.D.; Xu, J. Phytohormones in microalgae: A new opportunity for microalgal biotechnology? *Trends Plant Sci.* **2015**, *20*, 273–282. [CrossRef] [PubMed]
14. Bajguz, A.; Tretyn, A. The chemical characteristics and distribution of brassinosteroids in plants. *Phytochemistry* **2003**, *62*, 1027–1046. [CrossRef]
15. Howell, W.M.; Keller, G.E., III; Kirkpatrick, J.D.; Jenkins, R.L.; Hunsinger, R.N.; Mclaughlin, E.W. Effects of the plant steroidal hormone, 2,4-epibrassinolide, on the mitotic index and growth of onion (*Allium cepa*) root tips. *Genet. Mol. Res.* **2007**, *6*, 50–58. [PubMed]
16. Brock, A.K.; Willmann, R.; Kolb, D.; Grefen, L.; Lajunen, H.M.; Bethke, G.; Lee, J.; Nürnberger, T.; Gust, A.A. The *Arabidopsis* mitogen-activated protein kinase phosphatase PP_2C_5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. *Plant Physiol.* **2010**, *153*, 1098–1111. [CrossRef] [PubMed]
17. Yang, X.; Yang, Y.N.; Xue, L.J.; Zou, M.J.; Liu, J.Y.; Chen, F.; Xue, H.W. Rice ABI_5-Like1 regulates abscisic acid and auxin responses by affecting the expression of ABRE-containing genes. *Plant Physiol.* **2011**, *156*, 1397–1409. [CrossRef] [PubMed]
18. Gao, Z.Q.; Miao, X.X.; Zhang, X.W.; Wu, G.X.; Guo, Y.Y.; Wang, M.M.; Li, B.; Li, X.B.; Gao, Y.H.; Hu, S.; et al. Comparative fatty acid transcriptomic test and iTRAQ-based proteomic analysis in *Haematococcus pluvialis*, upon salicylic acid (SA) and jasmonic acid (JA) inductions. *Algal Res.* **2016**, *17*, 277–284. [CrossRef]
19. Czerpak, R.; Bajguz, A.; Gromek, M.; Kozłowska, G.; Nowak, I. Activity of salicylic acid on the growth and biochemism of *Chlorella vulgaris* Beijerinck. *Acta Physiol. Plant.* **2002**, *24*, 45–52. [CrossRef]
20. Falkowska, M.; Pietryczuk, A.; Piotrowska, A.; Bajguz, A.; Grygoruk, A.; Czerpak, R. The effect of gibberellic acid ga(3) on growth, metal biosorption and metabolism of the green algae *Chlorella vulgaris* (Chlorophyceae) Beijerinck exposed to cadmium and lead stress. *Pol. J. Environ. Stud.* **2011**, *20*, 53–59.
21. Raman, V.; Ravi, S. Effect of salicylic acid and methyl jasmonate on antioxidant systems of *Haematococcus pluvialis*. *Acta Physiol. Plant.* **2011**, *33*, 1043–1049. [CrossRef]
22. Gao, Z.Q.; Meng, C.X.; Gao, H.Z.; Ye, N.H.; Zhang, X.W.; Su, Y.F.; Zhao, Y.R.; Wang, Y.Y. Effect of exogenous ethylene (ET) on regulating carotenogenesis expression and astaxanthin accumulation in *Haematococcus pluvialis*. *J. Pure Appl. Microbiol.* **2013**, *7*, 2503–2511.
23. Gao, Z.Q.; Gao, H.Z.; Meng, C.X. Effects of Abscisic acid (ABA) carotenogenesis expression and astaxanthin accumulation in *Haematococcus pluvialis*. *Res. J. Biotechol.* **2013**, *8*, 9–15.
24. Gao, Z.Q.; Meng, C.X.; Gao, H.Z.; Li, Y.; Zhang, X.W.; Xu, D.; Zhou, S.T.; Liu, B.H.; Su, Y.F.; Ye, N.H. Carotenoid genes transcriptional regulation for astaxanthin accumulation in fresh water unicellular alga *Haematococcus pluvialis* by gibberellin A_3 (GA_3). *Indian J. Biochem. Biophys.* **2013**, *50*, 548–553. [PubMed]
25. Gao, Z.Q.; Meng, C.X.; Gao, H.Z.; Zhang, X.W.; Xu, D.; Su, Y.F.; Wang, Y.Y.; Zhao, Y.R.; Ye, N.H. Analysis of mRNA expression profiles of carotenogenesis and astaxanthin production of *Haematococcus pluvialis* under exogenous 2,4-epibrassinolide (EBR). *Biol. Res.* **2013**, *46*, 201–206. [CrossRef] [PubMed]
26. Liu, J.Y.; Qiu, W.; Song, Y.M. Stimulatory effect of auxins on the growth and lipid productivity of *Chlorella pyrenoidosa*, and *Scenedesmus quadricauda*. *Algal Res.* **2016**, *18*, 273–280. [CrossRef]

27. Park, W.K.; Yoo, G.; Moon, M.; Kim, C.W.; Choi, Y.E.; Yang, J.W. Phytohormone supplementation significantly increases growth of *Chlamydomonas reinhardtii* cultivated for biodiesel production. *Appl. Biochem. Biotechnol.* **2013**, *171*, 1128–1142. [CrossRef] [PubMed]
28. Cowan, A.K.; Rose, P.D. Abscisic acid metabolism in salt-stressed cells of *Dunaliella salina*. *Plant Physiol.* **1991**, *97*, 798–803. [CrossRef] [PubMed]
29. Kobayashi, M.; Todoroki, Y.; Hirai, N.; Kurimura, Y.; Ohigashi, H.; Tsuji, Y. Biological activities of abscisic acid analogs in the morphological change of the green alga *Haematococcus pluvialis*. *J. Ferment. Bioeng.* **1998**, *85*, 529–531. [CrossRef]
30. Anuradha, S.; Rao, S.S.R. Effect of 2,4-epibrassinolide on the photosynthetic activity of radish plants under cadmium stress. *Photosynthetica* **2009**, *47*, 317–320. [CrossRef]
31. Ding, J.; Shi, K.; Zhou, Y.H. Effects of root and foliar applications of 24-epibrassinolide on fusarium wilt and antioxidant metabolism in cucumber roots. *Hortscience* **2009**, *44*, 1340–1345.
32. Swain, S.M.; Olszewski, N.E. Genetic analysis of gibberellin signal transduction. *Plant Physiol.* **1996**, *112*, 11–17. [CrossRef] [PubMed]
33. Navarro, L.; Bari, R.; Achard, P.; Lison, P.; Nemri, A.; Harberd, N.; Jones, J.D. Dellas control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Curr. Biol.* **2008**, *18*, 650–655. [CrossRef] [PubMed]
34. Anerson, J.P.; Badruzsaufari, E.; Schenk, P.M.; Manners, J.M.; Desmond, O.J.; Ehlert, C.; Maclean, D.J.; Ebert, P.R.; Kazan, K. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* **2004**, *16*, 3460–3479. [CrossRef] [PubMed]
35. Jusoh, M.; Loh, S.H.; Chuah, T.S.; Azizc, A.; Cha, T.S. Elucidating the role of jasmonic acid in oil accumulation, fatty acid composition and gene expression in *Chlorella vulgaris*, (Trebouxiophyceae) during early stationary growth phase. *Algal Res.* **2015**, *9*, 14–20. [CrossRef]
36. Illman, A.M.; Scragg, A.H.; Shales, S.W. Increase in Chlorella strains calorific values when grown in low nitrogen medium. *Enzym. Microb. Technol.* **2000**, *27*, 631–635. [CrossRef]
37. Xu, H.; Miao, X.L.; Wu, Q.Y. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. *J. Biotechnol.* **2006**, *126*, 499–507. [CrossRef] [PubMed]
38. Yeh, K.L.; Chang, J.S. Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: Implications for biofuels. *Biotechnol. J.* **2011**, *6*, 1358–1366. [CrossRef] [PubMed]
39. Fan, J.H.; Huang, J.K.; Li, Y.G.; Han, F.F.; Wang, J.; Li, X.W.; Wang, W.L.; Li, S.L. Sequential heterotrophy-dilution-photoinduction cultivation for efficient microalgal biomass and lipid production. *Bioresour. Technol.* **2012**, *112*, 206–211. [CrossRef] [PubMed]
40. Han, F.F.; Huang, J.K.; Li, Y.G.; Wang, W.L.; Wang, J.; Fan, J.H.; Shen, G.M. Enhancement of microalgal biomass and lipid productivities by a model of photoautotrophic culture with heterotrophic cells as seed. *Bioresour. Technol.* **2012**, *118*, 431–437. [CrossRef] [PubMed]
41. Hunt, R.W.; Chinnasamy, S.; Bhatnagar, A.; Das, K.C. Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*. *Appl. Biochem. Biotechnol.* **2010**, *162*, 2400–2414. [CrossRef] [PubMed]
42. Stirk, W.A.; Bálint, P.; Tarkowská, D.; Novák, O.; Maróti, G.; Ljung, K.; Turečková, V.; Strnad, M.; Ördög, V.; Staden, J. Effect of light on growth and endogenous hormones in *Chlorella minutissima*, (Trebouxiophyceae). *Plant Physiol. Biochem.* **2014**, *79*, 66–76. [CrossRef] [PubMed]
43. Young, A.J. The photoprotective role of carotenoids in higher plants. *Physiol. Plant.* **1991**, *83*, 702–708. [CrossRef]
44. Rodolfi, L.; Zittelli, G.C.; Bassi, N.; Padovani, G.; Biondi, N.; Bonini, G.; Tredici, M.R. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.* **2009**, *102*, 100–112. [CrossRef] [PubMed]
45. Damiani, M.C.; Popovich, C.A.; Constenla, D.; Leonardi, P.I. Lipid analysis in *Haematococcus pluvialis* to assess its potential use as a biodiesel feedstock. *Bioresour. Technol.* **2010**, *101*, 3801–3807. [CrossRef] [PubMed]
46. Roessler, P.G. Environmental control of glycerol lipid metabolism in microalgae: Commercial implications and future research directions. *J. Phycol.* **1990**, *26*, 393–399. [CrossRef]

47. Zhukova, N.V.; Titlyanov, E.A. Effect of light intensity on the fatty acid composition of dinoflagellates symbiotic with hermatypic corals. *Bot. Mar.* **2006**, *49*, 339–346. [CrossRef]
48. Norman, H.A.; Thompson, G.A. Effects of low-temperature stress on the metabolism of phosphatidylglycerol molecular species in *Dunaliella salina*. *Arch. Biochem. Biophys.* **1985**, *242*, 168–175. [CrossRef]
49. Zažímalová, E.; Petrášek, J.; Benková, E. *Auxin and Its Role in Plant Development*; Springer: Vienna, Australia, 2014.
50. Cho, K.; Kim, K.N.; Lim, N.L.; Kim, M.S.; Ha, J.C.; Shin, H.H.; Kim, M.K.; Roh, S.W.; Kim, D.; Oda, T. Enhanced biomass and lipid production by supplement of myo-inositol with oceanic microalga *Dunaliella salina*. *Biomass. Bioenergy* **2015**, *72*, 1–7. [CrossRef]
51. Jusoh, M.; Loh, S.H.; Chuah, T.S.; Aziz, A.; Cha, T.S. Indole-3-acetic acid (IAA) induced changes in oil content, fatty acid profiles and expression of four fatty acid biosynthetic genes in *Chlorella vulgaris* at early stationary growth phase. *Phytochemistry* **2015**, *111*, 65–71. [CrossRef] [PubMed]
52. Hu, Q.; Sommerfeld, M.; Jarvis, E.; Ghirardi, M.; Posewitz, M.; Seibert, M.; Darzins, A. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *Plant J.* **2008**, *54*, 621–639. [CrossRef] [PubMed]
53. Maršálek, B.; Zahradníčková, H.; Hronková, M. Extracellular Abscisic acid produced by cyanobacteria under salt stress. *J. Plant Physiol.* **1992**, *139*, 506–508. [CrossRef]
54. Tominaga, N.; Takahata, M.; Tominaga, H. Effects of NaCl and KNO_3, concentrations on the abscisic acid content of *Dunaliella* sp. (Chlorophyta). *Hydrobiologia* **1993**, *267*, 163–168. [CrossRef]
55. Kobayashi, M.; Hirai, N.; Kurimura, Y.; Ohigashi, H.; Tsuji, Y. Abscisic acid-dependent algal morphogenesis in the unicellular green alga *Haematococcus pluvialis*. *Plant Growth Regul.* **1997**, *22*, 79–85. [CrossRef]
56. Yoshida, K.; Igarashi, E.; Mukai, M.; Hirata, K.; Miyamoto, K. Induction of tolerance to oxidative stress in the green alga, *Chlamydomonas reinhardtii*, by abscisic acid. *Plant Cell Environ.* **2003**, *26*, 451–457. [CrossRef]
57. Yoshida, K.; Igarashi, E.; Wakatsuki, E.; Miyamoto, K.; Hirata, K. Mitigation of osmotic and salt stresses by abscisic acid through reduction of stress-derived oxidative damage in *Chlamydomonas reinhardtii*. *Plant Sci.* **2004**, *167*, 1335–1341. [CrossRef]
58. Lu, Y.D.; Arkowska, D.; Tureckova, V.; Luo, T.W.; Xin, Y.; Li, J.; Wang, Q.T.; Jiao, N.Z.; Strnad, M.; Xu, J. Antagonistic roles of abscisic acid and cytokinin during response to nitrogen depletion in oleaginous microalga *Nannochloropsis oceanica* expand the evolutionary breadth of phytohormone function. *Plant J.* **2014**, *80*, 52–68. [CrossRef] [PubMed]
59. Salama, E.S.; Kabra, A.N.; Ji, M.K.; Kim, J.R.; Min, B.; Jeon, B.H. Enhancement of microalgae growth and fatty acid content under the influence of phytohormones. *Bioresour. Technol.* **2014**, *172*, 97–103. [CrossRef] [PubMed]
60. Mu, J.X.; Li, S.T.; Chen, D.; Xu, H.; Han, F.X.; Feng, B.; Li, Y.Q. Enhanced biomassand oil production from sugarcane bagasse hydrolysate (SBH) by heterotrophic oleaginous microalga *Chlorella protothecoides*. *Bioresour. Technol.* **2015**, *185*, 99–105. [CrossRef] [PubMed]
61. Khozingoldberg, I.; Cohen, Z. Unraveling algal lipid metabolism: Recent advances in gene identification. *Biochimie* **2011**, *93*, 91–100. [CrossRef] [PubMed]
62. Cheesbrough, T.M. Changes in enzymes for fatty acid synthesis and desaturation during acclimation of developing soybean seeds to altered growth temperature. *Plant Physiol.* **1989**, *90*, 760–764. [CrossRef] [PubMed]
63. Iba, K. Acclimative response to temperature stress in higher plants: Approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant Biol.* **2002**, *53*, 225–245. [CrossRef] [PubMed]
64. Vega, S.E.; Rio, A.H.D.; Bamberg, J.B.; Palta, J.P. Evidence for the up-regulationof stearoyl-ACP (D9) desaturase gene expression during cold acclimation. *Am. J. Potato Res.* **2004**, *81*, 125–135. [CrossRef]
65. Upchurch, R.G. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol. Lett.* **2008**, *30*, 967–977. [CrossRef] [PubMed]
66. Thompson, G.A. Lipids and membrane function in green algae. *Biochim. Biophys. Acta* **1996**, *13*, 17–45. [CrossRef]
67. Griffiths, M.J.; van Hille, R.P.; Harrison, S.T. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. *Lipids* **2010**, *45*, 1053–1060. [CrossRef] [PubMed]

68. Lei, A.P.; Chen, H.; Shen, G.M.; Hu, Z.L.; Chen, L.; Wang, J.X. Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors. *Biotechnol. Biofuels* **2012**, *5*, 18–28. [CrossRef] [PubMed]
69. Harwood, J.L.; Guschina, I.A. The versatility of algae and their lipid metabolism. *Biochimie* **2009**, *91*, 679–684. [CrossRef] [PubMed]
70. Riekhof, W.R.; Sears, B.B.; Benning, C. Annotation of genes involved in glycerol lipid biosynthesis in *Chlamydomonas reinhardtii*: Discovery of the betaine lipid synthase BTA1Cr. *Eukaryot. Cell* **2005**, *4*, 242–252. [CrossRef] [PubMed]
71. Battah, M.; El-Ayoty, Y.; Abomohra, A.E.F.; El-Ghany, S.A.; Esmael, A. Effect of Mn^{2+}, Co^{2+} and H_2O_2 on biomass and lipids of the green microalga Chlorella vulgaris as a potential candidate for biodiesel production. *Ann. Microbiol.* **2015**, *65*, 155–162. [CrossRef]
72. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
73. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods Enzymol.* **1987**, *148*, 350–382.
74. Folch, J.M.L.; Lees, M.P.; Stanley, G.H.A.S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [PubMed]
75. Cha, T.S.; Chen, J.W.; Goh, E.G.; Aziz, A.; Loh, S.H. Differential regulation of fatty acid biosynthesis in two *Chlorella* species in response to nitrate treatments and the potential of binary blending microalgae oils for biodiesel application. *Bioresour. Technol.* **2011**, *102*, 10633–10640. [CrossRef] [PubMed]