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Phytohormone Supplementation Significantly Increases Growth of *Chlamydomonas reinhardtii* Cultivated for Biodiesel Production

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Abstract Cultivation is the most expensive step in the production of biodiesel from microalgae, and substantial research has been devoted to developing more cost-effective cultivation methods. Plant hormones (phytohormones) are chemical messengers that regulate various aspects of growth and development and are typically active at very low concentrations. In this study, we investigated the effect of different phytohormones on microalgal growth and biodiesel production in *Chlamydomonas reinhardtii* and their potential to lower the overall cost of commercial biofuel production. The results indicated that all five of the tested phytohormones (indole-3-acetic acid, gibberellic acid, kinetin, 1-triacontanol, and abscisic acid) promoted microalgal growth. In particular, hormone treatment increased biomass production by 54 to 69 % relative to the control growth medium (Tris–acetate–phosphate, TAP). Phytohormone treatments also affected microalgal cell morphology but had no effect on the yields of fatty acid methyl esters (FAMEs) as a percent of biomass. We also tested the effect of these phytohormones on microalgal growth in nitrogen-limited media by supplementation in the early stationary phase. Maximum cell densities after addition of phytohormones were higher than in TAP medium, even when the nitrogen source was reduced to 40 % of that in TAP medium. Taken together, our results indicate that phytohormones significantly increased microalgal growth, particularly in nitrogen-limited media, and have potential for use in the development of efficient microalgal cultivation for biofuel production.

Keywords Plant hormone · Microalgae · Cultivation · Biodiesel · Morphology

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Introduction

Extensive exploitation of fossil fuels over many decades has led to serious environmental problems, most notably the release of substantial amounts of greenhouse gasses, and the need for more advanced and expensive methods for fuel extraction [1]. This has led to increasing interest in the development of various kinds of biofuels. Microalgae are recognized as one of the most feasible biofuel sources because they are a renewable resource and because they sequester atmospheric carbon dioxide [2]. Currently, the major limitation of microalgal biofuel production is the high cost of production. Thus, it is necessary to perform further research to achieve more efficient biofuel production and commercial feasibility [3].

The steps required for production of microalgal biodiesel can be described as four interconnected processes: cultivation, harvest, lipid extraction, and transesterification. Cultivation is the most expensive step and the most fundamental upstream process because it affects harvest, extraction, and transesterification [4]. Moreover, cultivation is vital for determining the quality and quantity of algal lipids because the environmental conditions under which cultivation is performed determine the types and amounts of lipids that microalgae produce [5–7]. Thus, there is an urgent need to develop more efficient microalgal cultivation.

Previous researchers have employed several different microalgal cultivation systems in attempts to achieve efficient cultivation at high cell density [8]. Despite the extensive research on this topic, there have been no significant breakthroughs. Light is considered essential for microalgal growth because photosynthesis is required for the synthesis of biomass. However, there is substantial light attenuation when cells are grown at high density, causing dramatic reduction in light intensity below the surface. This places an upper limit on the microalgal density [9, 10]. Some microalgae species can simultaneously perform photosynthesis and utilize added organic carbon sources. However, organic substrates, such as glucose or sucrose, are probably too expensive for large-scale commercial cultivation, and their use can also increase the susceptibility to bacterial contamination [8, 11]. Therefore, a cultivation process that resolves all of these problems is necessary for the efficient and economical production of biodiesel by microalgae.

Plant hormones (phytohormones) are natural or synthetic chemical messengers that, in conjunction with environmental cues, regulate growth and development and are effective at very low concentrations [12]. There are a wide variety of phytohormones, and they stimulate a variety of processes, such as cell division (cytokinins) and cell growth (auxins). There have been several reports that plant hormones can also stimulate the growth of non-plant microorganisms, including bacteria and fungi [13]. Microalgae are primitive eukaryotic plant cells, so there had been numerous studies of the effects of phytohormones on algal growth and production of metabolites such as chlorophylls and other pigments, carbohydrates, and proteins. One study reported that phytohormones affected cell division and thereby reduced the time to incipient cell division of *Chlorella pyrenoidosa* [14]. Other studies have examined the effect of various phytohormones on the production of starch, chlorophyll and other pigments, and proteins in different species of *Chlorella* [15–17]. When studying the effect of an experimental treatment on lipid accumulation in microalgae, it is necessary to measure starch, protein, and lipid contents because these are all synthesized by carbon sources [18].

The best-known and most important phytohormones are indole-3-acetic acid (IAA), gibberellic acid (GA3), kinetin (KIN), 1-triacontanol (TRIA), and abscisic acid (ABA). IAA is the most abundant naturally occurring auxin, and it stimulates plant cell enlargement, bud formation, and root initiation in higher plants. GA3 is a type of gibberellin and is widely used to promote cell elongation and seed germination in higher plants. KIN is a type of cytokinin, a class of hormones that promote cell division and shoot formation. KIN and IAA

affect growth synergistically. TRIA is a fatty alcohol that acts as a growth stimulant for many plants. Finally, ABA is a stress regulator in plant cells, and it induces protective reactions to abiotic environmental stress such as osmotic shock, desiccation, and low temperature [19]. However, the functions of these phytohormones in microalgae have not yet been firmly established [12, 20]. Previous research has reported that *Chlamydomonas reinhardtii* produces cytokinins and has enzymes need for the synthesis of IAA, gibberellins, and ABA, but their roles in regulating growth and development have not yet been established [21, 22].

In this study, we examined the effects of the addition of exogenous phytohormones on growth and production of fatty acid methyl esters (FAMEs) (biodiesel precursors) in *C. reinhardtii*. In particular, we attempted to maximize biomass and lipid production in *C. reinhardtii* by the addition of five different plant hormones—IAA, GA3, KIN, TRIA, and ABA. We also observed changes in cell morphology, cell size, and production of daughter cells in response to these different plant hormones and identified the cellular growth phase when exogenous phytohormones provide the most benefit.

Materials and Methods

Strain and Materials

C. reinhardtii strain CC124 was obtained from the culture collection of algae at the Biological Resource Center, Korea. The phytohormones (IAA, GA3, KIN, TRIA, and ABA) were obtained from MB Cell (Los Angeles, CA, USA). Other chemicals were purchased from Sigma-Aldrich. Stock solutions of plant hormones were prepared in 1 M KOH (IAA, KIN), distilled water (GA3), or hot DMSO (TRIA, ABA).

Culture Conditions and Medium

Algae were routinely maintained in Tris–acetate–phosphate (TAP) agar plates, and inoculums were prepared from 6-day-old exponentially growing seed cultures. Algae were cultivated in 250-mL Erlenmeyer flasks with 200 mL of sterile TAP medium [23]. The TAP medium was supplemented with hormones at appropriate times, and the same amount of blank solvent was added to controls. Cultivation was performed at 28 °C with continuous illumination (180–200 μmol m^{-2} s^{-1}). Air was supplied continuously at a constant rate of 1 vvm, and the flasks were shaken at 180 rpm to ensure sufficient aeration. There were three or four replicates for each experimental treatment. Two separate culture media were tested: normal TAP medium with 100 ppm of ammonium chloride and modified TAP medium with 40 ppm of ammonium chloride.

Measurement of Growth

The dry cell weight was measured by filtering the algal suspension through a pre-dried and pre-weighed, 0.45-μm cellulose nitrate membrane filter (Whatman, USA) and drying in an oven at 80 °C for 10 h. The number of cells was determined by direct counting with an improved Neubauer hemocytometer. Optical density at 750 nm, measured with a UV/Vis spectrophotometer (Optizen 3220UV, Mecasys, Korea) was also used to estimate microalgal dry weight (DW) based on the following equation:

$$\mathrm{DW} = 0.5337 \times \mathrm{OD}_{750} - 0.02856, \quad R^2 = 0.9869 \tag{1}$$

Chlorophyll Analysis

Chlorophyll was measured after extraction with MeOH. Cells were centrifuged, washed twice with deionized (DI) water, and the pellet was freeze-dried at −40 °C for 3 days. Then, 10 mg of dried cells was resuspended in 5 mL of 100 % MeOH and stored in a refrigerator in the dark at 4 °C for 30 min. After chlorophyll extraction, samples were centrifuged at 7,000 rpm for 10 min and absorbance was measured at 652 and 665 nm. Chlorophyll concentration was calculated by Ritchie's method [24]:

$$\mathrm{Chl\,a}(\mu\mathrm{g/mL}) = -8.0962 \times \mathrm{OD}_{652} + 16.5169 \times \mathrm{OD}_{665} \quad (2)$$

$$\mathrm{Chl\,b}(\mu\mathrm{g/mL}) = 27.4405 \times \mathrm{OD}_{652} - 12.1688 \times \mathrm{OD}_{665} \quad (3)$$

Protein and Starch Analysis

The absolute analysis of 2 mg of each dry algal sample was performed using an Elements analyzer [18] and the amount of starch was determined after glucose conversion using the anthrone method [25].

$$\text{Glucose concentration}\,(\mathrm{mg}) = 0.0422 \times (\mathrm{OD}_{625})2 + 0.5138 \times (\mathrm{OD}_{625}) + 0.0162 \quad (4)$$

Fatty Acid Methyl Esters Analysis

After cultivation, algal biomass was harvested by centrifugation (7,000 rpm, 3 min) and then washed twice with DI water. The cell pellet was freeze-dried at −40 °C for 3 days and used for lipid extraction. Total lipids were extracted from 10 mg of lyophilized biomass with a chloroform–methanol (2:1*v*/*v*) solvent mixture according to the modified Folch method [26]. Extracted lipids were then converted into FAMEs via the transesterification reaction (with methanol and sulfuric acid as a catalyst) at 105 °C for 20 min. The FAMEs in the organic phase were analyzed by gas chromatography (HP6890, Agilent, USA), with a flame ionized detector and an INNOWAX capillary column (Agilent, USA, 30 m×0.32 mm×0.5 μm). The identification and quantification of fatty acids were determined by comparison with the retention times and peak areas of standards.

Microscopy

Observations of cell size, developmental stage, and cell division were made using a compound microscope (Leica Microsystems CMS GmbH, Germany). Images were analyzed using Leica Application Suite V3 software and a Leica Up-Right Microscope DM2500 equipped with a Leica DFC425 C digital camera.

Economic Feasibility Analysis of Phytohormones for Microalgal Culture

The price of each added phytohormone at the Sigma-Aldrich website was recorded on March 15, 2012. American Chemical Society (ACS)-grade plant hormones were not available, so we used the prices for 98 % IAA and TRIA and the prices for bioreagent grade GA3, KIN, and ABA. The prices of all other chemicals were based on ACS grade products. For

comparison of bulk chemicals, the price of each chemical, including the phytohormones, was determined from the Alibaba.com website (March 15, 2012). Cost was calculated using Eqs. 4 and 5:

$$\text{Cost}(\$\text{US}/\text{g biomass}) = \frac{(A \times B) + C}{D} \tag{5}$$

$$\text{Cost}(\$\text{US}/\text{g FAME}) = \frac{(A \times B) + C}{E} \tag{6}$$

where A is the amount of each phytohormone added to a unit of culture medium (in milligrams per liter), B is the price of the substrate (US dollars per milligram), C is the price of the unit medium (US dollars per liter), and D is the FAME yield (grams per liter).

Results and Discussion

Effect of Phytohormones on Yields of Biomass, FAME, Chlorophyll, Protein, and Starch

We first examined the effect of various concentrations (0.1 to 10 ppm) of five different phytohormones on biomass production in *C. reinhardtii* (Fig. 1). The results indicate that each hormone stimulated biomass production in a dose-dependent manner. The optimum concentrations for growth stimulation were about 3, 1, 1, 3, and 10 ppm for IAA, GA3, KIN, TRIA, and, ABA, respectively. For each hormone, except for ABA, there was a slight decline in biomass production when the hormone was above the optimum dose (Fig. 1). At the optimal concentrations, the maximal biomass production was 1.69, 1.76, 1.77, 1.61, and 1.06 g/L for IAA, GA3, KIN, TRIA, and ABA, respectively. This corresponds to 61, 68, 69, and 54 % increased biomass production due to addition of IAA, GA3, KIN, and TRIA (Fig. 1).

Previous studies reported positive effects of plant hormones on the growth of microorganisms. In contrast, the functional or physiological effects of phytohormones on microalgal species are not well established, although some research has indicated the potent operation of phytohormones for enhancing microalgal growth. For example, IAA produced by *Azospirillum* spp. increased the growth of *Chlorella vulgaris* [27, 28]. Therefore, limited information is available to elucidate the activities of phytohormones in microalgae or to confirm the relationship between specific hormones and the growth of microalgae. In this regard, our results are noteworthy because our data indicate that all four tested hormones, except for ABA, stimulate the growth of *C. reinhardtii*, and that there appears to be an optimum concentration for each hormone.

Next, we studied the effect of various concentrations of the same hormones on FAME production in *C. reinhardtii* (Fig. 1). No significant effect of IAA, GA3, KIN, TRIA, on FAME yields were observed within the range of concentrations tested in this study. However, interestingly, ABA treatments led to the increase of FAME yield, though no significant changes of biomass productivities were observed. More than 10 % (10, 3, 1, 0.1 ppm) and even 13 % (5 ppm) increases of FAME yields were achieved via the treatment of ABA (Fig. 1). Therefore, we concluded that IAA, GA3, KIN, and TRIA significantly increase algal biomass, whereas ABA enhances FAME production.

We also tested whether phytohormones affected the contents of chlorophyll, protein, and starch of *C. reinhardtii* cells (Fig. 2). All the hormones increased the concentration of

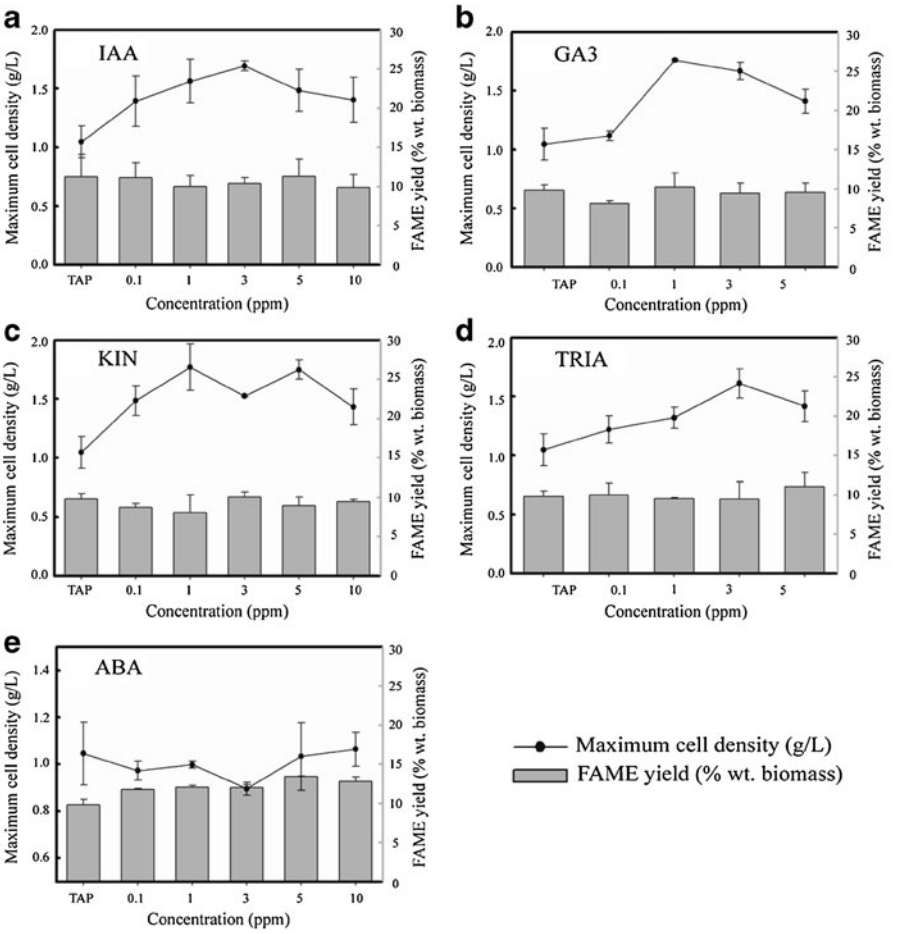


Fig. 1 Effect of different concentrations of **a** Indole-3-acetic acid (*IAA*), **b** Gibberellic acid (*GA3*), **c** Kinetin (*KIN*), **d** 1-triacontanol (*TRIA*), and **e** Abscisic acid (*ABA*) on maximal cell density (*filled circles*) and yield of fatty acid methyl esters (*FAMEs*) (*bars*). FAME yield is expressed as percent biomass

chlorophyll a + b in the given concentration. Compared with that in the control, the concentrations of chlorophyll a + b were increased by 81, 68, 59, 43, and 39 % at 3 ppm IAA, 1 ppm GA3, 0.1 ppm KIN, 1 ppm TRIA, and 5 ppm ABA, respectively. Previous research also indicated that cytokinins stimulate photosynthetic processes, including accumulation of photosynthetic pigments, in higher plants [21]. Our results indicate that the physiological response upon KIN, a type of cytokinin, is well conserved between microalgae and higher plant.

The similar results were also observed in protein contents. Compared with that in the control, the protein content increased by 35, 26, 25, 44, and 32 % at 0.1 ppm IAA, 1 ppm GA3, 0.1 ppm KIN, 0.1 ppm TRIA, and 3 ppm ABA. The results indicate that all the hormones increased content of chlorophyll a + b as well as proteins. Generally, protein contents are well correlated with the metabolic activity in the cells. Therefore, the accumulation of proteins occurred when the microalgal cells displayed the maximal algal metabolic and mitotic activity as shown in the Piotrowska's research [17]. Based on that, we speculated

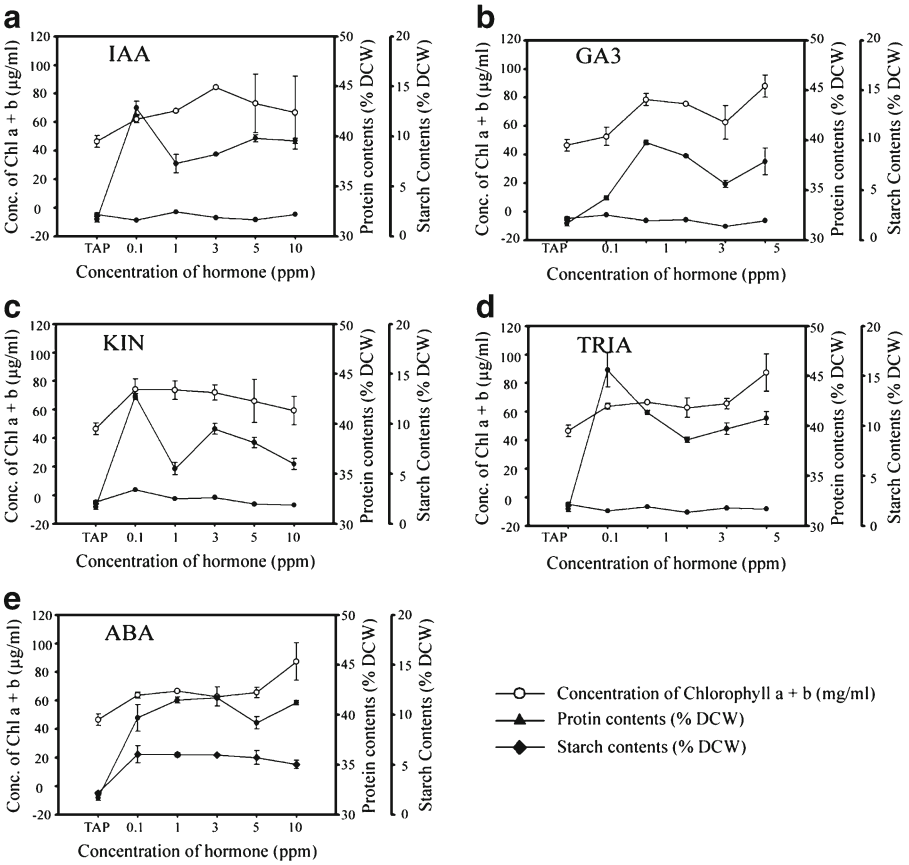


Fig. 2 Effect of different concentrations of **a** IAA, **b** GA3, **c** KIN, **d** TRIA, and **e** ABA on the concentration of chlorophyll a+b (*open circles*), protein (*filled triangle*), and starch (*filled diamond*) contents

that increase in protein content via the treatment of phytohormones reflects enhanced metabolic or mitotic activities in *C. reinhardtii*.

Unlike chlorophyll and protein, starch contents did not show any difference or alteration upon the treatment with IAA, GA3, KIN, and TRIA. Remarkably, however, ABA increased the starch contents more than two times (from 2.2 to 6 %). It has been known that microalgal cells store energy in the forms of both starch and lipid. It is likely that microalgal cells upon ABA treatment favor further energy storage in the form of either starch or lipid, though it does not have any significant effect on microalgal growth. Taken together, our results indicate that each of plant hormones including IAA, GA3, KIN, TRIA, and ABA plays a unique role in the algal metabolisms or cell physiology.

Effect of Phytohormones on Microalgal Morphology

Phytohormones regulate the morphological development of specific cells in higher plants [29]. Thus, we investigated the effect of phytohormones on morphological changes in microalgae. In particular, we performed microscopic observations at the early stationary stage of growth, a stage in which phytohormones might be expected to have the greatest

effect. Our results indicate that the different phytohormones had significant effects on the cultures (Fig. 3). The controls (no hormone treatment) exhibited typical cell division, and the cells were normal in size, typically less than 10 μm in diameter (Fig. 3f). However, phytohormone treatments resulted in marked increase of cell size containing multiple

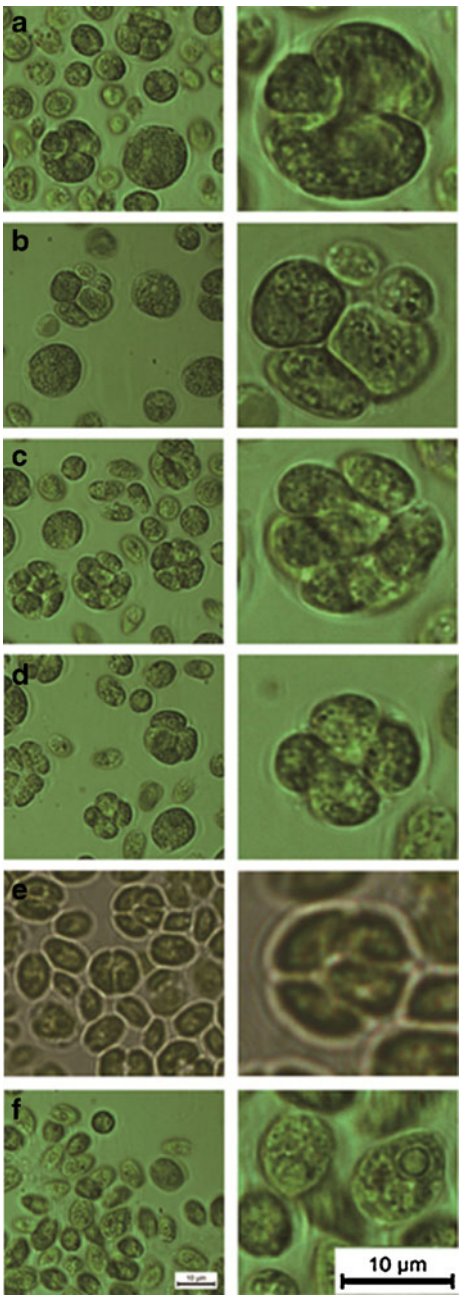


Fig. 3 Effect of **a** IAA, **b** GA3, **c** KIN, **d** TRIA, **e** ABA, and **f** control (TAP medium) on cell morphology. 10 ppm IAA, 3 ppm GA3, 10 ppm KIN, 10 ppm TRIA, 5 ppm ABA was applied respectively

daughter cells retained in the single cell before the release (Fig. 3a–e). Interestingly, all five tested phytohormones had similar effects.

We also performed statistical analysis of the effect of phytohormones on cell size and the percentage of cells undergoing cell division (Fig. 4). The results indicate that only about 4.1 % of the control cells were enlarged (Fig. 4a), but that the percentages of enlarged cells was 42.5 % with IAA, 39 % with GA3, 38 % with KIN, 46 % with TRIA, and 18 % with ABA (Fig. 4b–f). Additional analysis indicated that the percentage of cells in division were about 0 % (control) 4.6 % (IAA), 4.3 % (GA3), 25.7 % (KIN), 10.5 % (TRIA), and 8.8 % (ABA) (Fig. 4g). Phytohormone treatment also affected the number of daughter cells retained in a single cell (Fig. 4g). About 70 % of the cells in all four groups (IAA, GA3, KIN, and TRIA) consisted of four daughter cells. Up to 10 % of dividing cells consist of eight daughter cells under KIN treatment. Similar patterns were also observed in the treatment of ABA. These data clearly indicate that the five tested phytohormones had significant effects on microalgal cell division, and these changes may explain the increased biomass production (Fig. 1).

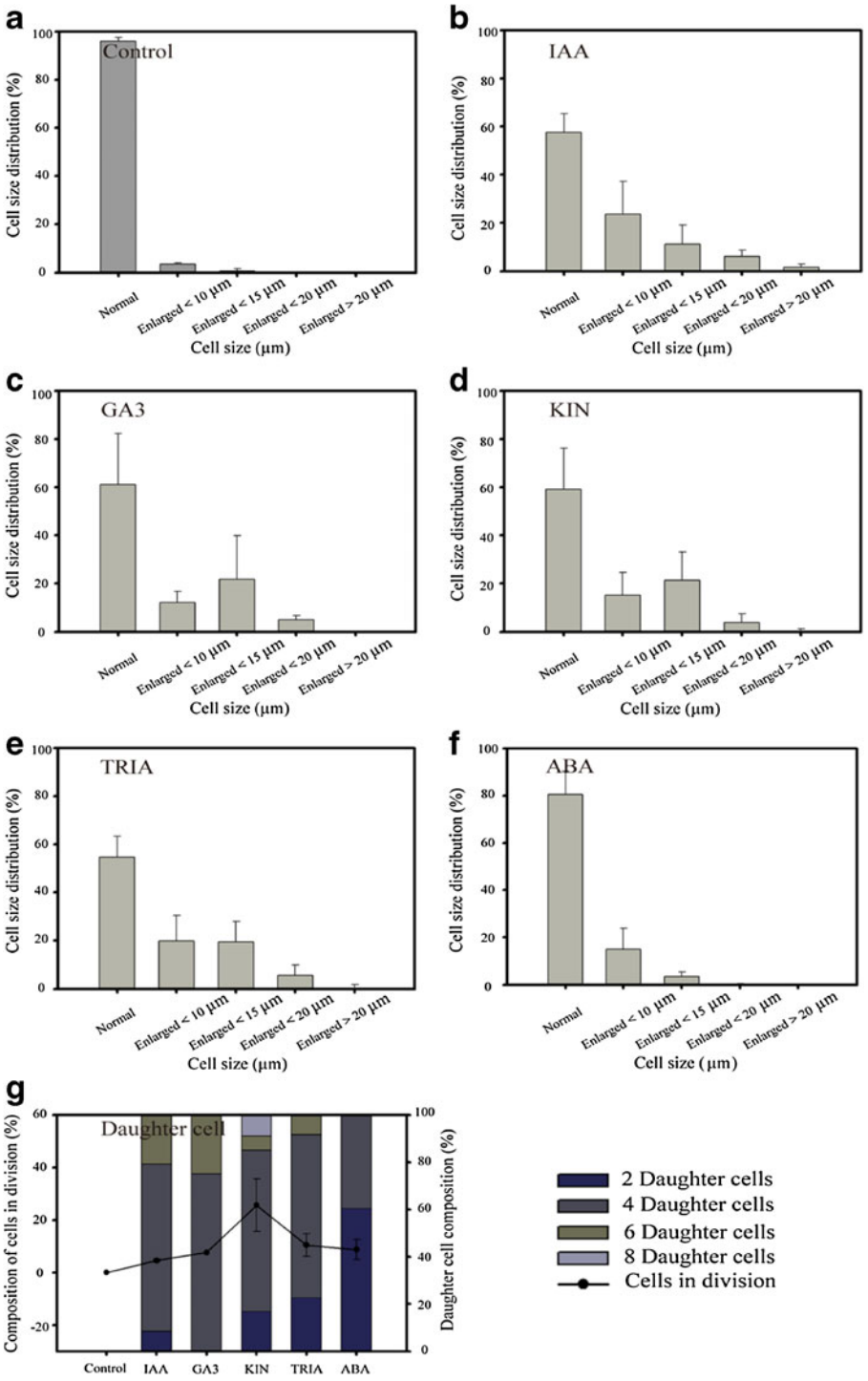
Each plant hormone has a unique role in regulating a variety of cellular processes. However, the four phytohormones selected in this study have similar functional roles in promoting cell enlargement and growth [12, 20]. In particular, IAA (an auxin) promotes growth, cell enlargement, and bud formation in higher plants. GA3, KIN, and TRIA all promote cell elongation, cell division, or growth. ABA maintains bud dormancy and functions in the response to environmental stressors in higher plants. Considering the functional roles of these five phytohormones in plants, it is reasonable to hypothesize that these phytohormones might have similar effects in microalgae. Our results indicated that these phytohormones significantly increased the size of microalgal cells and also increased the number of daughter cells produced during cell division. These results are consistent with our previous observation of increased biomass production after treatment with phytohormones [30]. Microalgal cell size depends on environmental conditions, such as the light/dark cycle [31]. We provided continuous light in these experiments ruling out the possibility of involvement of the light/dark cycle. Therefore, we speculate that plant hormones might affect growth in a manner similar to the light/dark cycle. Further study will be necessary to identify the exact effects and biological activities of these individual phytohormones on *C. reinhardtii*.

Economic Feasibility Analysis of Using Plant Hormones for Mass Production of Microalgae

The economic impact of using phytohormones for large-scale cultivation must be considered before implementation of industrial production. The current prices of available phytohormones depend on the purity, although the use of phytohormones with different purities had no different effects on microalgal responses. Expensive phytohormones are available in ACS grade, and bulk low purity phytohormones are inexpensive. Thus, we performed economic feasibility analyses for ACS grade and bulk grade phytohormones.

Table 1 summarizes our economic feasibility analysis of using ACS grade phytohormones. This analysis demonstrated that supplementation with three phytohormones (IAA, GA3, and KIN) reduced the cost of biomass production and the cost of FAME production. Other additives, such as acetate, increased biomass and FAME production, but also increased the cost, making this reagent unsuitable for large-scale applications.

We obtained similar results in analysis of the feasibility of using bulk grade phytohormones, although the cost of biomass and FAME production were only about 5 % of that from ACS grade phytohormones (Table 2). These cost reductions are quite impressive when


a
Control
Cell size distribution (%)
Normal
Enlarged < 10 μm
Enlarged < 15 μm
Enlarged < 20 μm
Enlarged > 20 μm
Cell size (μm)
b
IAA
c
GA3
d
KIN
e
TRIA
f
ABA
g
Daughter cell
Composition of cells in division (%)
Daughter cell composition (%)
Control
IAA
GA3
KIN
TRIA
ABA
2 Daughter cells
4 Daughter cells
6 Daughter cells
8 Daughter cells
Cells in division

◀ **Fig. 4** Effect of **a** control medium (TAP), **b** IAA, **c** GA3, **d** KIN, **e** TRIA, and **f** ABA on cell size distribution (*bars*) and **g** the percent of cells in division (*filled circles*) and number of daughter cells (*bars*). Data were processed with 0.1 to 10 ppm each hormone supplemented into the cultivation

compared with the cost of using an additional carbon source (acetate). Moreover, unlike phytohormones, substantial inputs of organic carbon compounds, such as acetate, increase the susceptibility to contamination, a significant limitation for biotechnological applications [11]. Although phytohormone except for ABA treatments did not increase the percentage of FAME in the algal biomass (Fig. 1), there was a significant increase in the overall FAME yield due to the increased microalgal biomass (Tables 1 and 2). Therefore, our results indicate that treatment with phytohormones may help to increase the efficiency and reduce the cost of biofuel production.

Application of Phytohormones in Low-Nitrogen Medium and Identification of Optimal Growth Stage for Phytohormone Supplementation

It is well known that nitrogen starvation initiates lipid biosynthesis in microalgae [32]. In particular, previous research showed that growth in low-nitrogen medium can increase the yield of FAMEs by more than 2-fold relative to growth in the presence of abundant nitrogen [33]. Therefore, microalgal growth and lipid production require different conditions for optimization. Nitrogen is expensive and indispensable for microalgal growth, but low levels of nitrogen trigger lipid biosynthesis. Thus, phytohormone addition under low-nitrogen conditions may lead to a high growth rate and a high rate of FAME production in microalgae, and thereby provide an inexpensive method for commercial microalgal cultivation. In addition, phytohormones may have different effects on cell growth when added at different stages in the life cycle, such as the lag phase or the early stationary phase.

First, we assessed the effect of phytohormone addition during the lag phase in medium that had a low level of ammonium (40 ppm), designated as modified TAP medium (MT) (Fig. 5). Controls were grown in normal TAP medium without phytohormone additions. Consistent with previous reports of increased microalgal lipid biosynthesis under limited

Table 1 Economic feasibility analysis of plant hormone supplementation based on use of ACS grade chemicals

ACS	Additive	Price			Maximum cell density (g/L)	FAME yield (g/L)	Cost (US$/g biomass)	Cost (US$/g FAME)
	Hormone (mg/L) Substrate (g/L)	Hormone (US$/mg) Substrate (US$/g)	TAP (US$/L)	Total (US$/L)				
Control	0	–	0.471	0.471	1.05	0.10	0.45	4.69
IAA	3	0.002	0.471	0.477	1.69	0.18	0.28	2.72
GA3	1	0.031	0.471	0.502	1.76	0.18	0.28	2.79
Kinetin	1	0.016	0.471	0.487	1.77	0.14	0.28	3.43
TRIA	3	1.274	0.471	4.293	1.61	0.15	2.66	28.10
ABA	10	0.522	0.471	5.691	1.06	0.14	5.35	41.75
Acetate	10	0.108	0.471	1.551	2.00	0.34	0.78	4.56

Table 2 Economic feasibility analysis of plant hormone supplementation based on use of bulk chemicals

Bulk	Additive	Price			Maximum cell density (g/L)	FAME yield (g/L)	Cost (US$/g biomass)	Cost (US$/g FAME)
	Hormone (mg/L) Substrate (g/L)	Hormone (US$/mg) Substrate (US$/g)	TAP (US$/L)	Total (US$/L)				
Control	0	–	0.0247	0.0247	1.05	0.10	0.024	0.25
IAA	3	0.0001	0.0247	0.0250	1.69	0.18	0.015	0.14
GA3	1	0.00015	0.0247	0.0248	1.76	0.18	0.014	0.14
Kinetin	1	0.0001	0.0247	0.0248	1.77	0.14	0.014	0.17
TRIA	3	0.00032	0.0247	0.0256	1.61	0.15	0.016	0.17
ABA	10	0.000077	0.0247	0.0255	1.06	0.14	0.024	0.19
Acetate	10	0.00095	0.0247	0.0342	2.00	0.34	0.017	0.10

nitrogen, we observed that the FAME yield increased more than 30 % in the MT medium relative to the TAP medium (Fig. 5). The addition of IAA, GA3, KIN, and TRIA during the lag phase had no significant effect on maximum cell density as well as FAME yield (Fig. 5). However, unlike the other phytohormones, ABA had a significant effect in that it led to decrease in cell density and increase in FAME yield (Fig. 5). Considering the effect of ABA as a stress regulator in higher plant, ABA-mediated stress regulation in the microalgal cells might also impact on lipid biosynthesis directly or indirectly, particularly when added in a lag phase [19].

Finally, we assessed the effect of phytohormone addition during the early stationary phase (Fig 6). In contrast to the results of phytohormone supplementation in the lag phase (Fig. 5), the addition of phytohormone during this growth phase significantly increased biomass production and FAME biosynthesis (Fig 6). Interestingly, maximum cell densities with phytohormones added to the MT medium were greater than that in the TAP medium, even though the nitrogen content was significantly lower. GA3 and KIN were the most effective phytohormones in

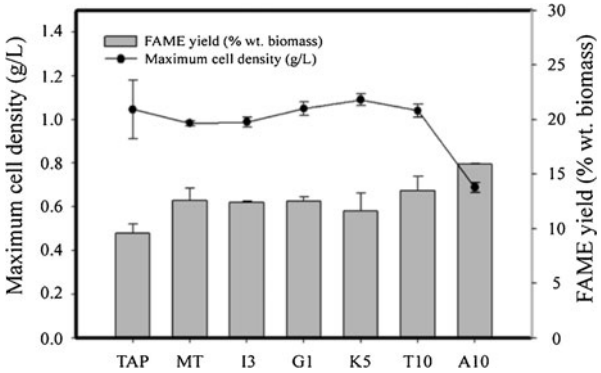


Fig. 5 Effect of phytohormone supplementation during the lag phase on maximum cell density (*filled circles*) and FAME production (*bars*) in nitrogen-limited medium (*MT*). Controls were growth in regular TAP medium, and 3 ppm IAA (I3), 1 ppm GA3(G1), 5 ppm KIN (K5), 10 ppm TRIA (T10), 10 ppm ABA (A10) was applied respectively

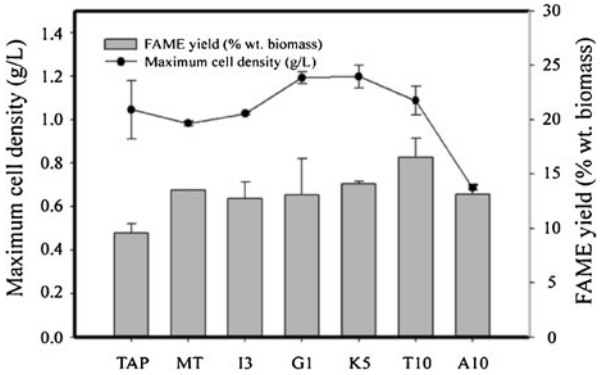


Fig. 6 Effect of phytohormone supplementation during the early stationary phase on maximum cell density (*filled circles*) and FAME production (*bars*) in nitrogen-limited medium (*MT*). Controls were growth in regular TAP medium, and 3 ppm IAA (I3), 1 ppm GA3(G1), 5 ppm KIN (K5), 10 ppm TRIA (T10), 10 ppm ABA (A10) was applied respectively

increasing biomass (Fig. 6). Furthermore, microalgae supplemented with phytohormones in the early stationary phase produced much higher levels of FAMEs (Figs. 5 and 6). Unexpectedly, the extent of influence of ABA during the early stationary phase was not high enough, compared to that supplemented during the lag phase. We speculated that the effect of ABA could be maximized, only when microalgal cell was not under the stress condition such as the lag phase. Taken together, our results clearly indicate that phytohormones are most effective when added at the appropriate growth stage and that they also have great potential for increasing microalgal growth, particularly in nitrogen-limiting media, and the production of FAMEs.

Conclusion

In this study, we examined the effects of supplementation with four different phytohormones—IAA, GA3, KIN, TRIA, and ABA—on growth and FAME production in microalgae. Our results indicate that phytohormone supplementation increased microalgal growth, biodiesel production, chlorophyll concentration, and protein contents. These positive effects were accompanied by increases in algal cell size and related morphological changes. Importantly, phytohormone supplementation promoted growth in nitrogen-limited medium as well as the normal TAP medium. Phytohormones had the greatest effect on growth and FAME production when added during the early exponential growth phase, though the effects of phytohormones varied depending on each hormone. For example, ABA has markedly influenced on microalgal lipid biosynthesis, compared to the others. With these results, further development of sophisticate protocol of phytohormones supplemented with appropriate amount and at proper timing might be possible for enhanced the biomass production as well as lipid accumulation. These experiments employed *C. reinhardtii* as a model organism to study the effect of phytohormone supplementation on promotion of biodiesel production, and provide a foundation for the study of this effect in other oleaginous microalgae species. The results presented here will help to overcome the current bottleneck in the development of efficient microalgal cultivation methods and will facilitate commercial and biotechnological progress in microalgal biofuel production.

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