Full Paper

The effects of abscisic acid, salicylic acid and jasmonic acid on lipid accumulation in two freshwater *Chlorella* strains

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Guanxun Wu,^{1,#} Zhengquan Gao,^{1,#,*} Huanmin Du,¹ Bin Lin,¹ Yuchen Yan,³ Guoqiang Li,¹ Yanyun Guo,¹ Shenggui Fu,² Gongxiang Wei,² Miaomiao Wang,¹ Meng Cui,¹ and Chunxiao Meng^{1,*}

¹ School of Life Sciences, Shandong University of Technology, Zibo 255049, China

² School of Sciences, Shandong University of Technology, Zibo 255049, China

³ The Undergraduate Brigade, Second Military Medical University, Shanghai 200433, China

Sustainable renewable energy is being hotly debated globally because the continued use of finite fossil fuels is now widely recognized as being unsustainable. Microalgae potentially offer great opportunities for resolving this challenge. Abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) are involved in regulating many physiological properties and have been widely used in higher plants. To test if phytohormones have an impact on accumulating lipid for microalgae, ABA, JA and SA were used to induce two *Chlorella* strains in the present study. The results showed 1.0 mg/L ABA, 10 mg/L SA, and 0.5 mg/L JA, led strain *C. vulgaris* ZF strain to produce a 45%, 42% and 49% lipid content that was 1.8-, 1.7- and 2.0-fold that of controls, respectively. For FACHB 31 (number 31 of the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences), the addition of 1.0 mg/L ABA, 10 mg/L SA, and 0.5 mg/L JA produced 33%, 30% and 38% lipid content, which was 1.8-, 1.6- and 2.1-fold that of controls, respectively. As for lipid productivity, 1.0 mg/L ABA increased the lipid productivity of *C. vulgaris* ZF strain and FACHB-31 by 123% and 44%; 10 mg/L SA enhanced lipid productivity by 100% and 33%; the best elicitor, 0.5 mg/L JA, augmented lipid productivity by 127% and 75% compared to that of controls, respectively. The results above suggest that the three phytohormones at physiological concentrations play crucial roles in inducing lipid accumulation in *Chlorella*.

Key Words: biodiesel; *Chlorella*; lipid; phytohormones; renewable energy

Introduction

Due to diminishing reserves, the global petroleum supply is shrinking and rising in cost at a fast pace, and further climate change will be enhanced by the continued use of fossil fuels. These factors have increased the demand for alternate fuels, thereby making the production of fuels from alternate sources more feasible. Microalgae, the largest biomass producers, have a high neutral lipid content, outcompeting terrestrial plants for biofuel production, and also have the ability to grow rapidly and synthesize and accumulate large amounts of neutral lipid stored in cytosolic lipid bodies (Mutanda et al., 2011). Biodiesel is produced from microalgal oil, thus crude fossil petroleum could be replaced by mass cultured biomass microalgal oil for eco-sustainable biodiesel production in the near future (Rawat et al., 2013). However, the high cost of algae production is the biggest obstacle to the adoption of an industrial and commercial process. A major conclusion of the cost analyses for large-scale microalgae production, conducted at the end of the last century, is that, even with aggressive assumptions, project costs for biodiesel are two times higher than current petroleum diesel fuel costs (Sheehan et al., 1998). Therefore, the need to search for low-cost and highly efficient ways to induce microalgae to produce lipids is urgent.

*Corresponding authors: Zhengquan Gao and Chunxiao Meng, School of Life Sciences, Shandong University of Technology, Zibo 255049, China.
Tel: +86 533 2762265 Fax: +86 533 2781832 E-mail: zq7723@126.com
Tel: +86 533 2781329 Fax: +86 533 2781832 E-mail: mengchunxiao@126.com

#These authors contributed equally to this work.

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Algae can modify their lipid metabolism efficiently and display tremendous diversity and sometimes unusual patterns of cellular lipids in response to changes in environmental conditions, and this is one of the most important reasons why algae can survive and proliferate over a wide range of environmental conditions (Guschina and Harwood, 2006; Hu et al., 2008). Lipid production, like the production of other carbohydrate-based storage compounds, is often dependent on environmental conditions, some of which await elucidation and development (US DOE, 2010). As stated in previous studies, most microalgae can alter their lipid accumulation and other secondary metabolic products under unfavorable cultivation conditions as a protective mechanism to cope with stresses. In recent years, considerable attention has been focused on lowering biofuel costs, greenhouse gas emissions, land and water resource needs, and on improving compatibility with fuel distribution systems and vehicle engines (Demirbas, 2011). Growth conditions and the genetics of lipid production have been explored with the goal of enhancing the economic production of microalgae for bioproducts or lipids for biofuels recently (Tate et al., 2013). Various studies have focused on enhancing growth and metabolite production by varying culture conditions of the algae, including pH, temperature, irradiation level, carbon source, aeration and concentrations of specific nutrients (Bhola et al., 2011; Hsieh and Wu, 2009). Other studies have focused on the genes involved in the biosynthesis of fatty acids in microalgae (Merchant et al., 2011). However, thus far no reports have investigated using phytohormones to induce microalgae to accumulate lipids to the best of our knowledge, although they have been widely used in higher plants to enhance production or quality.

In most cases, these phytohormones act as signal molecules to promote and drive many physiological properties in the cells. It has been reported that phytohormones are necessary for manipulating some physiological and biochemical processes in algal cells, including growth and aging performance, metabolic product biosynthesis and resistance to stresses (Tarakhovskaya et al., 2007). However, knowledge of the algal hormonal system and information about hormone metabolism and mechanisms of action in algae are still rather fragmentary, since this information is extremely scarce (Bajguz, 2009; Tarakhovskaya et al., 2007). Currently, the presence of a full-value hormonal system in algae and the correspondence of its biological activities with those of higher-plant hormones are debated; the metabolism and mechanisms of action of phytohormones in microalgae are still unclear.

Chlorella is a well-studied genus of green algae and some species are regarded as potential candidates for microalgae biodiesel production since they can make certain amounts of lipids that are proposed for use in biofuel production (Bajguz, 2010; Hu et al., 2008). *Chlorella* has also been used as a model for plants and a useful tool for studying the influences of phytohormones on growth and metabolite accumulation (Czerpak et al., 2006; Hu et al., 2008). There are reports that some phytohormones, including auxins, cytokinins, ABA, polyamines, brassinosteroids, JA and SA, can improve the adaptability and tolerance of

Fig. 1. Fluorescence (A, C, E, G, I, K, M, O, Q and S) and light microscopy (B, D, F, H, J, L, N, P, R and T) images of *C. vulgaris* ZF strain and FACHB 31 in different statuses of the induction course.

A and B, K and L indicate the beginning status of *C. vulgaris* ZF strain and FACHB 31 (on day 1 of cultivation) with dyeing of Nile red, respectively. C and D, M and N indicate the final status (on day 18 of cultivation and the 3rd day of starvation) of the blanks of *C. vulgaris* ZF strain and FACHB 31, respectively. E and F, O and P indicate the final status of 10 mg/L ABA treatments; G and H, Q and R indicate the final status of 1.0 mg/L SA; I and J, S and T indicate the final status of 0.5 mg/L JA treatments of *C. vulgaris* ZF strain and FACHB 31, respectively.

Chlorella to heavy metal pollution and other abiotic stressors, which suggests that they might play important roles in responding to abiotic stressors and algal adaptability to stresses (Bhola et al., 2011; Piotrowska-Niczyporuk et al., 2012).

These phytohormones should possess a good potential to facilitate microalgal lipid production, since they can promote metabolite production in *C. vulgaris* and *C. pyrenoidosa* (Tate et al., 2013). However, little attention has been paid to the effects of plant hormones on microalgal lipid production. ABA, SA and JA, three typical stress hormones, are involved in the regulation of many physiological properties by acting as signal molecules. Previous studies have also indicated that ABA, SA and JA are used widely in higher plants (Bajguz, 2009). However, the relationship between lipid accumulation and hormone stimulation in microalgae is still unknown. In this regard, the purpose of the present study was to investigate the effects of ABA, SA, and JA, on lipid accumulation in two *Chlorella* strains (*C. vulgaris* ZF strain and FACHB 31, *C. pyrenoidosa*). It was anticipated that the application of ABA, SA, and JA, could lead to improved lipid accumulation in the two *Chlorella* strains, so that a higher lipid production could be achieved.

Fig. 2. Lipid production of *C. vulgaris* ZF strain treated with different ABA, SA, JA concentrations.

A shows the lipid production of *C. vulgaris* ZF strain treated with different ABA concentrations; B shows the lipid production of *C. vulgaris* ZF strain treated with different SA concentrations; C shows the lipid production of *C. vulgaris* ZF strain treated with different JA concentrations.

Materials and Methods

Algal strains and culture medium. The *C. vulgaris* ZF strain was obtained from the Institute of Oceanology, Chinese Academy of Sciences and FACHB 31 was purchased from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The two *Chlorella* strains in the exponential growth phase were cultured in 1000 ml Erlenmeyer flasks with a modified BBM medium (Ebrahimian et al., 2014) containing the following components (per liter): 0.25 g NaNO_3 , 0.075 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.175 g KH_2PO_4 , 0.075 g K_2HPO_4 , 0.025 g NaCl , 0.00498 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g Na_2EDTA , 8.05 mg H_3BO_3 , 1.81 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.222 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.079 mg

Fig. 3. Lipid production of FACHB 31 treated with different ABA, SA, JA concentrations.

A shows the lipid production of FACHB 31 treated with different ABA concentrations; B shows the lipid production of *C. vulgaris* ZF strain treated with different SA concentrations; C shows the lipid production of FACHB 31 treated with different JA concentrations.

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.390 mg $\text{NaMoO}_4 \cdot 5\text{H}_2\text{O}$ and 0.0494 mg $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ at 23°C , under $40 \mu\text{mol photons/m}^2 \text{ s}$ light illumination (12h:12h), and the medium was added once a week. The cultures were hand-shaken three to five times daily to avoid clumping.

Phytohormones induction. ABA (Shanghai Yuanye Bio-Technology), SA (Tianjin Fuchen) and JA (Sigma) were used in this study. The three phytohormones were added to an equal mass of ethanol to increase their solubility and then diluted with deionized water which were mother liquors. Briefly, the whole process of the experiment was conducted as “a two-stage culture system”. Firstly, the microalgal cells were cultured in $1 \times \text{BBM}$ medium until the logarithmic growth phase was achieved with about a 1.0×10^8 cell density. Secondly, the microalgal solutions

Table 1. Biomass concentration, total lipid content and lipid productivity of *C. vulgaris* ZF strain and FACHB 31 treated by ABA gradients at the end of the culture.

Treatments of ABA	<i>C. vulgaris</i> ZF strain			FACHB31		
	Biomass (g/L)	Total lipid (g/L)	Lipid productivity (mg/L/d)	Biomass (g/L)	Total lipid (g/L)	Lipid productivity (mg/L/d)
Control	1.81 ± 0.17	0.44 ± 0.07	24.42 ± 0.36	3.42 ± 0.80	0.64 ± 0.02	35.03 ± 1.31
0.1 mg/L	2.50 ± 0.09 ^b	0.60 ± 0.06 ^a	33.34 ± 0.31 ^a	3.01 ± 0.70	0.49 ± 0.01	27.18 ± 0.67
0.5 mg/L	2.26 ± 0.09 ^a	0.64 ± 0.05 ^a	35.61 ± 0.28 ^a	2.94 ± 0.65	0.71 ± 0.15 ^a	16.12 ± 0.59
1 mg/L	2.22 ± 0.13 ^a	0.98 ± 0.10 ^b	54.41 ± 0.56 ^b	2.78 ± 0.8	0.92 ± 0.13 ^b	51.13 ± 0.72 ^a
10 mg/L	2.04 ± 0.21	0.27 ± 0.07	15.01 ± 0.38	3.02 ± 0.27	0.33 ± 0.07	18.27 ± 0.39
20 mg/L	1.78 ± 0.31	0.15 ± 0.02	8.32 ± 0.11	2.61 ± 0.17	0.25 ± 0.02	13.93 ± 1.42

Table 2. Biomass concentration, total lipid content and lipid productivity of *C. vulgaris* ZF strain and FACHB 31 treated by SA gradients at the end of the culture.

Treatments of SA	<i>C. vulgaris</i> ZF strain			FACHB31		
	Biomass (g/L)	Total lipid (g/L)	Lipid productivity (mg/L/d)	Biomass (g/L)	Total lipid (g/L)	Lipid productivity (mg/L/d)
Control	1.81 ± 0.17	0.44 ± 0.07	24.42 ± 0.36	3.42 ± 0.80	0.64 ± 0.02	35.03 ± 1.30
0.1 mg/L	1.82 ± 0.20	0.22 ± 0.08	12.21 ± 0.45	3.24 ± 0.55	0.50 ± 0.10	27.75 ± 0.55
0.5 mg/L	2.04 ± 0.11	0.69 ± 0.12 ^a	38.32 ± 5.33 ^a	2.94 ± 0.23	0.49 ± 0.10	27.21 ± 0.55
1 mg/L	1.74 ± 0.50	0.60 ± 0.12 ^a	33.27 ± 6.71 ^a	2.61 ± 0.42	0.57 ± 0.12	31.73 ± 6.70
10 mg/L	2.13 ± 0.65	0.88 ± 0.27 ^b	48.89 ± 15.10 ^b	2.81 ± 0.28	0.84 ± 0.11 ^a	46.72 ± 6.1 ^a
20 mg/L	1.64 ± 0.41	0.33 ± 0.12	8.91 ± 0.67	2.84 ± 0.47	0.51 ± 0.17	28.33 ± 9.41

Table 3. Biomass concentration, total lipid content and lipid productivity of *C. vulgaris* ZF strain and FACHB 31 treated by JA gradients at the end of the culture.

Treatments of JA	<i>C. vulgaris</i> ZF strain			FACHB31		
	Biomass (g/L)	Total lipid (g/L)	Lipid productivity (mg/L/d)	Biomass (g/L)	Total lipid (g/L)	Lipid productivity (mg/L/d)
Control	1.81 ± 0.17	0.44 ± 0.07	24.42 ± 0.36	3.42 ± 0.80	0.64 ± 0.02	35.03 ± 1.30
0.1 mg/L	2.04 ± 0.12	0.53 ± 0.03	29.42 ± 0.14	2.83 ± 0.07	0.84 ± 0.15 ^a	46.66 ± 0.83 ^a
0.5 mg/L	2.14 ± 0.26	1.03 ± 0.02	56.12 ± 0.01 ^b	2.81 ± 0.11	1.11 ± 0.06	61.10 ± 3.59 ^b
1 mg/L	2.34 ± 0.41 ^a	0.59 ± 0.14 ^a	32.80 ± 0.78 ^a	2.64 ± 0.10	0.92 ± 0.25 ^b	50.57 ± 1.44 ^a
10 mg/L	2.60 ± 0.12 ^a	0.45 ± 0.04	5.04 ± 0.21	2.93 ± 0.44	0.78 ± 0.03 ^a	43.32 ± 1.80
20 mg/L	2.74 ± 0.19 ^a	0.43 ± 0.03	23.90 ± 0.16	2.52 ± 0.32	0.58 ± 0.21	32.16 ± 0.99

a, b: Significant difference between the exposure and control groups (a: p -value < 0.05, b: p -value < 0.01).

were divided into six treatments with three replicates (each 500 ml), and different volume gradients of three kinds of phytohormone mother liquors were added into six different treatments, respectively. The final concentrations of ABA, SA, and JA, were 0, 0.1, 0.5, 1.0, 10.0 and 20.0 mg/L, respectively. An equal amount of deionized water was added to the controls.

Microscopic analyses. Microalgae cells were stained with 2 mg/mL Nile red (dissolved in dimethyl actone; Sigma, USA) for 15 minutes (Lim et al., 2012). Observations of lipid bodies were conducted using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and photographs were taken with a Nikon CCD DS- DP10 file digital camera (excitation: 510–550 nm, emission: 590 nm).

Lipid quantification. Algal cells were harvested by centrifugation at 18 days. The supernatant after being centrifugate with $8000 \times g$, at 4°C for 10 min was decanted and cell pellets were heat dried in the oven at 100°C for 24 hours, and then the biomass was ground into a final powder. The total lipid content was then extracted with

chloroform/methanol (1/2, v/v) and quantified gravimetrically based on the method of Bligh and Dyer (1959) with some modifications (Bajguz, 2009). A 100 mg sample of dried microalgal powder was added to 4.0 mL methanol and 2.0 mL chloroform in a 50-mL test tube and mixed for 1 min. Thereafter, the samples were ultrasonically disrupted (15/5S, with 15 second treatments and 5 second intervals) and centrifuged at $8000 \times g$ for 10 min. The upper layer was withdrawn using a pipette, and then 18.0 mL of chloroform and 18.0 mL of water were added and mixed for 1 min in a 50-mL test tube. The lower chloroform phase containing the extracted lipids was transferred into a new tube, and algal pellets were re-extracted two more times until no color was visible in the extract. Finally, all of the chloroform phases were combined and evaporated in a nitrogen evaporator until dry lipid was obtained. The lipid content was calculated as the percentage of algal dry weight.

Statistical analysis. All the data were statistically analyzed with one-way ANOVA (SPSS 17.0). LSD multiple com-

parison tests were used to test the differences among different concentrations in each treatment.

Results

Microscopic analyses

Figure 1 showed the fluorescence (A, C, E, G, I, K, M, O, Q and S) and light microscopy images (B, D, F, H, J, L, N, P, R and T), respectively, of the two strains of *Chlorella* at the beginning and end of the induction course. A and B, and K and L, indicate the status (on day 1 of cultivation) of *C. vulgaris* ZF strain and FACHB 31, respectively, at the beginning with the addition of Nile red. C and D, and M and N, indicate the final status (on day 18 of cultivation) of the blanks of the two strains dyed with Nile red, respectively. E and F, and O and P, indicate the final status of the two strains treated with 10 mg/L SA and dyed with Nile red, respectively. G and H, and Q and R, indicate the final status of the two strains treated with 1.0 mg/L ABA and dyed with Nile red, respectively. I and J, and S and T, indicate the final status of the two strains treated with 0.5 mg/L JA and dyed with Nile red, respectively.

No orange fluorescence, due to lipid, was found in the beginning for the two *Chlorella* strain blanks dyed with Nile red (A and B, K and L), but fluorescence was distinct in the final status of the two *Chlorella* strains blanks (C and D, and M and N). However, the samples treated with the three plant hormones displayed much stronger fluorescence than the blanks. Moreover, in the two *Chlorella* strain treatments, the order of fluorescence intensity was: 10.0 mg/L SA samples < 1.0 mg/L ABA samples < 0.5 mg/L JA samples (Fig. 1). These results indicated the three plant hormones had an obvious effect on the induction of lipid accumulation in the two *Chlorella* strains: JA had the greatest effect since it could induce the strongest fluorescence strength with the minimum concentration; ABA had the second best induction effect; and SA had the weakest effect.

Determination of lipid content and lipid productivity

Lipid content. Figures 2A–C show the changes in total lipid contents (% w/w) after 18 days of cultivation with ABA, SA, and JA, induction in *C. vulgaris* ZF strain. 1.0 mg/L ABA, 10 mg/L SA and 0.5 mg/L JA, with 45%, 42% and 45%, which were 1.8-, 1.7- and 2.0-fold higher compared with the controls (% w/w), respectively. On day 18, the 0.1 mg/L ABA treatment had little effect on lipid accumulation (24%), whereas an initial increase in lipid content (28%) occurred at 0.5 mg/L ABA induction, and it reached its highest level (45%) with 1.0 mg/L ABA treatment, then decreased sharply with 10.0 and 20.0 mg/L ABA treatment to 13% and 8%, respectively (Fig. 2A). With SA treatments, on day 18, 0.1 mg/L SA induction led to an obvious decrease in lipid content (12%), whereas 0.5 and 1.0 mg/L SA stimulated lipid content significantly (35% and 35%), resulting in a maximum (42%) at 10.0 mg/L that was sharply reduced for 20.0 mg/L SA treatment (21%) (Fig. 2B). With JA treatments on day 18, an initial increase in lipid content (26%) was obtained for 0.1 mg/L JA treatment, and a maximum yield appeared for 0.5 mg/L JA treatment (49%), which gradually de-

creased for 1.0, 10.0 and 20.0 mg/L JA treatments to 26%, 17% and 16%, respectively (Fig. 2C).

Figures 3A–C show the changes in total lipid content (% w/w) after 18 days cultivation after ABA, SA and JA induction in FACHB 31. The three maximum lipid contents occurred for 1.0, 10.0 and 0.5 mg/L with 33%, 30% and 38%, which were 1.8-, 1.6- and 2.1-fold higher compared with the controls (19%), respectively. On day 18, 0.1 mg/L ABA treatment produced only an insignificant decrease in lipid accumulation (17%), whereas an initial increase in lipid content (24%) occurred with the 0.5 mg/L ABA treatment, and this reached its highest level (33%) with the 1.0 mg/L ABA treatment, then decreased sharply with the 10.0 and 20.0 mg/L ABA treatments to 11% and 10%, respectively (Fig. 3A). With SA treatments on day 18, 0.1 and 0.5 mg/L SA induction led to an obvious decrease in lipid production (16% and 17%), whereas 1.0 mg/L SA enhanced lipid production to 22%, and a maximum (30%) then resulted with 10 mg/L SA treatment, which was reduced sharply with 20.0 mg/L SA treatment (18%) (Fig. 3B). With JA treatments on day 18, an initial increase in lipid content (30%) was obtained with the 0.1 mg/L JA treatment and the maximum (38%) occurred with the 0.5 mg/L JA treatment, which then gradually decreased with the 1.0, 10.0 and 20.0 mg/L JA treatments to 35%, 27% and 23%, respectively (Fig. 3C). In conclusion, our results showed that ABA, SA and JA, at physiological concentrations can induce lipid accumulation in *Chlorella C. vulgaris* ZF strain and FACHB 31. Moreover, among the three kinds of plant hormones, the 0.5 mg/L JA treatment appeared to have the greatest stimulatory effect on the lipid content (49% and 38%).

Lipid productivity. Tables 1–3 display the total biomass dry weight and total lipid contents of the controls and different phytohormone treatments after 18 days induction. As is shown in Tables 1–3, with the same inoculum size, and the same cultivation length and using different kinds and concentrations of phytohormones, different biomasses and total lipid productivities were obtained. In the *C. vulgaris* ZF strain treatments, all ABA concentrations induced a biomass increase, and for the 0.1, 0.5, and 1.0 mg/L treatments the biomass increased significantly compared to that of the controls. Moreover, all 0.1, 0.5, and 1.0 mg/L treatments could induce a significant increase in total lipids compared to the controls. However, 10.0 mg/L and 20.0 mg/L ABA treatments were associated with a decreased lipid production. As for SA, all five concentrations had no evident effect on the biomass production. 0.5, 1.0, and 10.0 mg/L SA treatments increased the total lipid production and the increases for 0.5 and 10.0 mg/L SA treatments were significant. 0.1 and 20.0 mg/L SA reduced the lipid production significantly compared to that of the controls. For the JA treatments, 1.0, 10.0, and 20.0 mg/L JA augmented biomass significantly, and 0.5 mg/L JA increased the total lipid production very significantly to 2.3-fold that of the controls.

In the FACHB 31 treatments, all five ABA concentrations reduced biomass by an insignificant extent; 0.5 and 1.0 mg/L ABA increased total lipid production significantly but the remaining three concentrations decreased total production significantly. For the SA treatments, all

five SA concentrations reduced biomass insignificantly; only 10.0 mg/L SA increased the total lipid production very significantly; the remaining four concentrations decreased total lipid production insignificantly. All five JA concentrations reduced biomass insignificantly; but 0.1, 0.5 and 1.0 mg/L JA enhanced the total lipid production significantly. The optimum JA treatment was 0.5 mg/L, which could induce 1.1 g/L lipid production, which was 1.8-fold that of the controls. All lipid productivities display the same trends as total lipids in the three tables. The *C. vulgaris* ZF strain control had 2.44 ± 0.036 mg/L/d lipid productivity and *C. vulgaris* ZF strain, treated with the three kinds of phytohormones, displayed 0.5 ± 0.02 – 5.6 ± 0.01 mg/L/d lipid productivity. As for FACHB31, the controls showed 3.5 ± 1.3 mg/L/d and the treatments with three kinds of phytohormones displayed 1.4 ± 0.1 – 6.1 ± 0.4 mg/L/d lipid productivity.

Discussion

Many microalgae synthesize and sequester lipids into cytosolic lipid bodies as storage lipids, and these appear to play an active role in the stress response by which algal cells resist stress conditions, such as photo-oxidative stress or nutrient starvation, and so on (Breuer et al., 2013; Hu et al., 2008). One of the lipids is carotenoid (e.g. β -carotene, lutein or astaxanthin), which serves as a “sunscreen” to prevent or reduce excess light from irradiating the chloroplast under stress (Zhekisheva et al., 2002). Moreover, lipid synthesis may also utilize phosphatidylcholine, phosphatidyl ethanolamine and galactolipids or toxic fatty acids excluded from the membrane system as acyl donors, thereby serving as a mechanism to detoxify membrane lipids and deposit them in the form of triacylglycerols (Breuer et al., 2013; Hu et al., 2008).

Though microalgae possess many advantages compared to other biodiesel-producing organisms, there is a long way to go before commercial production is realised, as it is inferior to fossil-derived energy at present in terms of cost. The two best ways to decrease cost are to enhance the lipid concentration within algae cells and to accelerate the accumulation of algae biomass. After detailed and careful research and analyses, Neenan et al. (1986) concluded that only if the lipid concentration achieved 50–60% of algae dry biomass would algal biodiesel be competitive compared with fossil energy. Therefore, increasing lipid production is an efficient method for lowering the cost and accelerating the process of commercial algae biodiesel production.

Phytohormones are small signaling molecules essential for the regulation of almost every aspect of the plant life cycle, including plant growth, development, reproduction, and survival. The key role of phytohormones, such as ABA, JA, and SA, as critical components of complex signaling networks and primary signals involved in the defensive responses against various stresses has been well established, and compelling evidence demonstrates that these hormones are the primary signals inducing defense responses through recognized defense hormone signaling pathways (Bari and Jones, 2009; Raman and Ravi, 2011). These hormones generate a signal transduction network

that leads to a cascade of events responsible for the physiological adaptation of the plant to stresses (Aimar et al., 2011).

The plant hormone ABA regulates many key processes in plants including the response to abiotic stress and regulation of the water balance (Boneh et al., 2012). ABA could trigger stress and defense responses to protect plants from oxidative damage (Wang et al., 2010). In our previous studies, results showed that the addition of 2.0 mg/L ABA efficiently promoted astaxanthin production by 13.5 mg/L on day 28, whereas high concentrations of ABA (>5 mg/L) might be toxic for the growth of *H. pluvialis* (Meng and Gao, 2007). Our present data also indicate that application of 1.0 mg/L ABA led to an increasing lipid content, whereas the application of 10.00–20.0 mg/L ABA caused decreasing lipid production in *Chlorella C. vulgaris* ZF strain and FACHB 31.

SA, as a signal molecule, is involved in plant responses to several environmental stress factors and has been reported to affect the growth and development of plants (Gao et al., 2012b). SA has been found to play a key role in the regulation of plant growth, development, interaction with other organisms, and in responses to environmental stresses (Hayata et al., 2010) and SA triggered the accumulation of ABA (Szepesi et al., 2009). Our previous work has also shown that the addition of 5.0 mg/L (or 10.0 mg/L) SA promoted astaxanthin content, increasing it by 16.86 mg/L, whereas high concentrations of SA (>10.0 mg/L) might be inhibitory for algae growth (Gao et al., 2007), which is also in agreement with our present data regarding enhanced lipid production with 10 mg/L SA and reduced lipid production with 20 mg/L SA in the present *Chlorella* strains. Moreover, Czerpak et al. (2002) investigated the influence of SA on the growth and photosynthetic pigment levels in the green alga *C. vulgaris*. Their results showed that 10^{-4} M SA increased the cell numbers 1.4-fold, the chlorophyll a and b contents 1.5–1.7-fold, and the total carotenoids 1.3–1.6-fold, between 8 and 12 days of cultivation.

JA and methyl jasmonate (MJ) are known to be active in higher plants. Many plant secondary metabolites may be induced by JAs via up-regulation of the expression of a series of key enzyme genes when plants are under stress or treated by exogenous JA (Endt et al., 2002). For example, the caffeoylputrescine content of tomato seedlings increased when treated with JA (Chen et al., 2006). JA also promoted nicotine biosynthesis in transgenic tobacco by causing over-expression of allene oxide cyclase from *Hyoscyamus niger* (Jiang et al., 2009). Recently, attempts were made to use JA on algae to enhance metabolite production. JA enhanced heavy metal toxicity leading to a decrease in cell number, chlorophylls, and carotenoids, as well as antioxidant enzyme activity in *C. vulgaris* (Piotrowska-Niczyporuk et al., 2012). Moreover, Czerpak et al. (2006) discussed the influence of exogenous JA upon the growth and changes in some metabolite levels in the cells of green alga *C. vulgaris*. They found that JA at high concentrations (10^{-5} – 10^{-4} M) resulted in a decrease in cell number and photosynthetic pigments, whereas JA at low concentrations (10^{-8} – 10^{-6} M) induced increases in both of these, which is also in accordance with our present data

regarding increased lipid content under 0.5 mg/L JA induction and decreased lipid production under 10–20.0 mg/L JA induction in *Chlorella* strains. Furthermore, both SA and MJ at 500 μ M concentrations displayed inhibitory effects on the growth of algal cells and carotenoid and chlorophyll contents, whereas higher concentrations of SA and MJ inhibited astaxanthin accumulation in *Haematococcus pluvialis* (Raman and Ravi, 2011). Gao et al. (2012a) also reported that JA was an effective regulator that stimulated astaxanthin production in *H. pluvialis*. However, it appeared to play no role in brown algae for defense induction (Wiesemeier et al., 2008).

In our study, the lipid contents of the *C. vulgaris* ZF strain and FACHB 31 controls were 24% and 19%, respectively. They displayed similar results for lipid content as previous reports. Han et al. (2013) found that the lipid contents of *Chlorella* sp. SDEC-10 and *C. ellipsoidea* SDEC-11 diversified from 26% to 28% within 14 days in autoclaved synthetic sewage. Hu et al. (2013) found that locally isolated *Chlorella* sp. achieved a relatively high lipid content (26%) when cultivated in wastewater. Ebrahimian et al. (2014) reported the lipid concentration in *C. vulgaris* was from 21% to 33% when incubated in secondary municipal wastewater. Wang et al. (2014) found the lipid productivity of *C. pyrenoidosa* using a semi-continuous cultivation system was about 18%. Similarly, Han et al. (2014) reported that *C. pyrenoidosa* yielded a 24–31% lipid content using the semi-continuous cultivation system. In our work, three phytohormones, ABA, JA, and SA, enhanced lipid content up to 2.0-fold in the *Chlorella* strains *C. vulgaris* ZF strain, and 2.1-fold in FACHB 31, which suggests that phytohormone supplementation might contribute to large-scale commercial biodiesel production with microalgae in the future. However, elucidation of the molecular regulatory mechanism is needed before commercial application is realised in industrial production.

Many kinds of environmental stresses, such as nutrients stresses (e.g., nitrogen and/or phosphorus starvation), high light, high salinity, high toxic reactive oxygen species, osmotic stress, radiation, acidic or alkaline conditions, cold or heat stresses, heavy metals etc., are known to induce microalgae to accumulate lipid efficiently, mainly with the form of triacylglycerides (Hu et al., 2008; Liu et al., 2011; Mata et al., 2010; Sharma et al., 2012; Shemesh et al., 2016; Yang et al., 2013). Pieterse et al. (2009) proposed that diverse small-molecule hormones could play pivotal roles in the regulation of a plants defense signaling network. They found that changes in hormone concentration or sensitivity, which can be triggered under biotic and abiotic stress conditions, mediated the adaptive host plant responses to a wide range of stresses, such as pathogen invasion, insect herbivory, drought, cold and heat stress, and so on. Utilizing a microcosm that mimics the biotic and abiotic stress to the plants, researchers found that endogenous phytohormones deteriorated biotic and abiotic stress conditions and triggered vigorous kinds of related physical reactions of plants (Galston and Sawhney, 1990; Griffiths and Parry, 2002; Jackson, 1993). The results above suggested that the application of phytohormones in working concentrations might trigger lipid accumulation

as a stress condition in the two *Chlorella* strains. Therefore, we speculated that the three kinds of stress phytohormones at physiological concentrations triggered lipid accumulation as a defensive method for microalgae cells. Possibly, the increased concentrations of phytohormones simulated the stress response and misled microalgae cells to produce an overdose of lipids in the defensive system.

Notwithstanding the fact that analytically pure ABA and JA are too expensive for the large-scale production of biodiesel using microalgae, the study demonstrated the possibility of the application of exogenous ABA, SA and JA to enhance lipid production in microalgae. Considering the reasonable price of SA and the industrial purity of JA and ABA, the application of the above chemicals in biodiesel production via microalgae could be a promising alternative for future biodiesel generation.

Conclusions

In the present paper, these phytohormones in the concentrations of 1.0 mg/L ABA, 10.0 mg/L SA, and 0.5 mg/L JA, were effective elicitors for lipid production in two fresh *Chlorella* strains. However, the relationship between exogenous and endogenous phytohormone concentrations, as well as the genes involved in the synthesis, is not yet clarified. Obviously, further studies regarding the molecular mechanisms for lipid accumulation via phytohormones warrant greater attention from researchers.

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