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The influences of phytohormones on triacylglycerol accumulation in an oleaginous marine diatom *Phaeodactylum tricornutum*

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Abstract

Environmental stresses such as nitrate deprivation and high light are effective at increasing lipid content in microalgae, but they can also slow down and even stop growth. In this study, the phytohormones methyl jasmonate, salicylic acid, gibberellin, abscisic acid, and ethephon were introduced to cultures of the oleaginous marine diatom *Phaeodactylum tricornutum* in an attempt to increase growth and lipid production. Single-factor experiments showed that the influences of some of the phytohormones were closely related to their concentrations. Methyl jasmonate, abscisic acid, and salicylic acid promoted *P. tricornutum* growth and lipid accumulation at certain concentrations. The differing effects of the three phytohormones on *P. tricornutum* may be related to the respective phytohormone's responsive *cis*-regulatory elements in the upstream regions of the triacylglycerol (TAG) synthesis genes. Methyl jasmonate, abscisic acid, and salicylic acid were further studied in response surface experiments, through which a 141% increase in TAG production was attained for 10-L cultures of *P. tricornutum* grown under optimal conditions. This study suggests that some phytohormones can promote *P. tricornutum* lipid accumulation without hindering growth. It also provides another strategy for improving the production of microalgae for use as biodiesel.

Keywords *Phaeodactylum tricornutum* · Triacylglycerol · Phytohormones · Single-factor experiments · Response surface experiments

Introduction

Microalgae are the optimal raw material for biodiesel, but are far from being widely used for industrial applications (Liu et al. 2017a, b). To date, there have been many studies and methods on promoting microalgae growth rate (Mohan et al. 2015) and increasing triacylglycerol (TAG) accumulation under environmental stresses (Ge et al. 2014; Peng et al. 2014; Barka et al. 2016). Yet it is difficult to simultaneously obtain rapid growth rates and high lipid production. Therefore, a new method for sustainable TAG production in microalgae has become the focus of attention.

The oleaginous *Phaeodactylum tricornutum* with more than 20% lipid of the dry weight (DW) is a model diatom for studying the lipid synthesis mechanism. The genome of *P. tricornutum* has been reported as having about 27.4 Mbp (Bowler et al. 2008). Many researchers have characterized the TAG synthesis mechanism (Yang et al. 2013; Levitan et al. 2015) and key enzymes (Guihéneuf et al. 2011; Gong et al. 2013; Niu et al. 2013; Cui et al. 2013, 2018) in *P. tricornutum*. The accumulation of lipids in *P. tricornutum* is a consequence of intermediate metabolism remodeling, especially reactions in the tricarboxylic acid and the urea cycles (Levitan et al. 2015). As with other algae, for example, *Chlorella sorokiniana* (Hunt et al. 2010), the synthesis and accumulation of TAG in *P. tricornutum* are always affected by environmental factors, especially nitrate deprivation, and biomass always declines under conditions of stress (Peng et al. 2014; Yang et al. 2014; Yu et al. 2016). Thus, the resulting two-step culture of *P. tricornutum*, which requires a growth phase and TAG accumulation phase, hinders its use for industrial

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applications because of high production costs and long culture cycles.

Several phytohormones such as salicylate and gibberellic acid have been found to affect microalgal growth and metabolism (Xu et al. 2013; Bajguz and Piotrowska-Niczyporuk 2014; Kim et al. 2016; Le Henry et al. 2017; Lin et al. 2018; Parsaeimehr et al. 2017). Naphthylacetic acid and gibberellin could promote *Arthrospira platensis* and *Arthrospira maxima* growth, boosting the growth rate of the normal culture to 150% while increasing the amount of metabolic products such as the total extracellular sugar and total intracellular protein (Chen et al. 2009). Lu et al. (2010) found that jasmonic acid methyl ester and gibberellin promoted the synthesis of the microalga *Haematococcus pluvialis* and accumulation of astaxanthin by regulating the key enzyme BKT in the astaxanthin synthesis pathway. Naphthylacetic acid was reported to improve the fatty acid composition of the microalga *Chlorella pyrenoidosa* (Liu et al. 2017a, b). Recently, Xu et al. (2017) found that 40 μM salicylic acid could stimulate TAG accumulation in *P. tricornutum* at the stationary phase.

Based on previous researches, it can be hypothesized that phytohormones may simultaneously increase the growth rate and TAG production of *P. tricornutum* and, as a result, save on both the time and costs involved in its production for biodiesel. Thus, single-factor and Box-Behnken tests were conducted to detect the influence of single phytohormones on *P. tricornutum* and the optimal proportions needed for the highest lipid yield. Furthermore, the expression patterns of key enzymes in the lipid synthesis pathway were analyzed to investigate the mechanism by which the phytohormones influence *P. tricornutum*.

Materials and methods

Culture of *P. tricornutum*

The *P. tricornutum* strain was kindly donated by Prof. Mingyan Yin of the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences. *P. tricornutum* was cultured in f/2 medium (Guillard and Ryther 1962) at 23 ± 2 °C and illuminated with cool-white fluorescent light at 35 μmol photons m^{-2} s^{-1} on a 12-h:12-h light:dark cycle. The artificial seawater for the f/2 was made using Reef Salt™ (Seachem Laboratories, USA) diluted in distilled water to a concentration of 34 g L^{-1}, and the pH level was adjusted to 7.2 with 10% hydrochloric acid.

Single-factor experiments with phytohormones

The phytohormones methyl jasmonate, salicylic acid, gibberellin, abscisic acid, and ethephon (Sigma, USA) were dissolved in ethyl alcohol before being added separately to *P. tricornutum* cultures at the logarithmic phase of growth. The algae were then cultured under the same conditions described above for 6 days. The final concentration gradients of methyl jasmonate, salicylic acid, and abscisic acid in the 200-mL *P. tricornutum* cultures were 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. The concentrations of gibberellin were 0, 0.15, 1.50, 2.00, and 20.0 mg L^{-1}, while those of ethephon were 0, 0.06, 0.60, 2.00, and 20.0 mg L^{-1}. Absorption was measured at 730 nm every 24 h.

A Water-PAM fluorometer (Walz GmbH, Germany) was used to monitor F_v/F_m in *P. tricornutum* every 72 h. *Phaeodactylum tricornutum* cultures were first dark adapted for 5 min, then exposed to a saturating pulse (0.8 s; 5640 μmol photons m^{-2} s^{-1}) and a set of actinic irradiances at 322 μmol photons m^{-2} s^{-1} for 0.8 s every 20 s over a 5-min interval. There was a 40-s delay between the saturating pulse and the actinic light. The experiments were performed in triplicate.

Box-Behnken experiment and response surface methodology

The Box-Behnken test design in the MyDesign-Design-Expert 8.0.6 software (www.statease.com/soft_ftp.html) was used to find the optimal amounts of additives, with the TAG content as the response value (Kansedo and Keatteong 2013). Based on the results of the single-factor experiments, methyl jasmonate at concentrations of 10 to 14 mg L^{-1} and salicylic acid and abscisic acid at ranges of 2 to 6 mg L^{-1} were studied further. Ten-liter *P. tricornutum* cultures were exposed to the phytohormones at these concentrations for 6 days, and the results were analyzed using analysis of variance (ANOVA). The growing conditions for the 10-L cultures were as described above with continuous aeration at 1.3 m^3 min^{-1}.

Lipid extraction and analysis

The *P. tricornutum* cultures from the single-factor experiments and the cultures exposed to multiple phytohormones were harvested after 6 days by centrifugation at 1500×*g* at 18 °C and then freeze-dried for 48 h. The resulting powders were broken down and digested with 4 mol L^{-1} hydrochloric acid at room temperature for 1 h, and the total lipids were extracted from the powder using the chloroform-methanol method (Yoon et al. 2012). The weight and concentration of the total lipids were measured with an electronic balance. The TAG was separated by thin-layer chromatography (silica gel plate, HSGF254, Yellow Sea, China) with a mixture of normal hexane/diethyl ether/acetic acid (70:30:1 by volume) as the mobile phase. The lipid and TAG quantification was performed by gas chromatography-mass spectrometry (GC-MS), as previously described (Yoon et al. 2012).

Expression pattern of TAG synthesis enzymes in *P. tricornutum*

To investigate the influences of the three phytohormones methyl jasmonate, salicylic acid, and abscisic acid on the lipid synthesis pathway, the expression patterns of the key enzymes in *P. tricornutum* for TAG synthesis were examined by quantitative PCR (Q-PCR). The key enzymes included acetyl-CoA carboxylase (ACC, NC_011686.1 and NC_011698.1), long-chain acyl-coenzyme A synthetases (LACS, KF359938.1, KF359939.1, KF359940.1, KF359941.1, and KF359942.1), lysophosphatidic acid acyltransferase (LPAAT, JQ837824.1), and diacylglycerol acyltransferase (DGAT, HQ589265.1, JQ837823.1, JX469837.1, XP_002184474.1). The Q-PCR primers for these enzymes are listed in Table 1. The *cis*-regulatory elements in the 5′ upstream region of the key enzymes' genes were analyzed through the PLANTCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Phaeodactylum tricornutum induced by the three phytohormones for 0, 3, and 6 days were harvested and frozen in liquid N_2. RNA was isolated from algal samples using RNAiso extraction reagent (TAKARA, China) according to the manufacturer's instructions. cDNA synthesis and relative quantitative real-time PCR were performed as described by Cui et al. (2018) using the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA) with SYBR Green PCR Master Mix (TAKARA, China). The mRNA expression level was normalized using the *18S* cDNA gene as the internal control.

Table 1 The primers for Q-PCR in the research

Number	Name	Sequence (5′-3′)
1	18S rDNA-for	CCAGGTCCAGACATAGTAAG
	18S rDNA-rev	GTACAAAGGGCAGGGACGTA
2	ACC1-for	GGCCACCGAGTTTGCGGATTT
	ACC1-rev	CACCCTGGCGCATTTGACCC
3	ACC2-for	TGTTGGTATGGTGGCGTGGCT
	ACC2-rev	TTCTCGGGTTCCAAAGCTCCC
4	LAPPT-for	GGCAACTATTTGGCCGGGTAG
	LAPPT-rev	AGATGGCGACGATGATGAGG CA
5	LACS1-for	CGCTGTTCAAGGCGCTCGTC
	LACS1-rev	TCCCTCCACATCCCGGCGAT
6	LACS2-for	CGACGCACCGCTGGACGAAT
	LACS2-rev	TCGTCTGTCACCGGCTGCAC
7	LACS3-for	CCAGCCCACCGTGCTCTTT
	LACS3-rev	TAGTGCATCCCGCGTCCCT
8	LACS4-for	GTGGCGGATGTCGCTTGGA
	LACS4-rev	AGACACCGGCAGGGATTCCTCC
9	LACS5-for	CCGTCGCTCTTGGAACCCTG
	LACS5-rev	TGCAGGGACCGAGCGGGTTCA
10	DGAT1-for	TTATGCACGAGGTGCTTG
	DGAT1-rev	CCGGGAATTTGCGATAGAG
11	DGAT2A-for	CGCTAGTATGGGTTCCATTGA
	DGAT2A-rev	ATAACGAGAACTGCCAGAATC
12	DGAT2D-for	CAATTTGTGTTCGCCGTTAG
	DGAT2D-rev	ATCTTGCTTGCAGTCTGT
13	WS/DGAT-for	AGCTCCCACAACAATCATC
	WS/DGAT-rev	CGTGAAAGCAAGCATAGGT

Statistical analysis

All experiments were performed using biological triplicates to ensure reproducibility. Values are presented as means ± SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., USA). Paired sample *t* tests were applied. Differences were considered statistically significant when *p*-values < 0.05.

Results

Effects of phytohormones on growth and TAG accumulation in *P. tricornutum*

In the single-factor experiments, methyl jasmonate, salicylic acid, and abscisic acid were found to stimulate the growth rate of *P. tricornutum* to 0.7 to 0.9 mg L^{-1} day^{-1} at several concentrations. These include methyl jasmonate at 10 and 14 mg L^{-1}; salicylic acid at 2, 6, and 30 mg L^{-1}; and abscisic acid at 2 mg L^{-1}. Meanwhile, gibberellin and ethephon did not promote the growth of *P. tricornutum* at any of the tested concentrations (Fig. 1).

The DW, TAG productivity, and TAG content (TAG/DW%) of *P. tricornutum* following 6 days of induction with the phytohormones are shown in Table 2. The TAG productivity of *P. tricornutum* cultures induced by methyl jasmonate, salicylic acid, and abscisic acid at some concentrations was higher than that of the control. For methyl jasmonate, the highest biomass and TAG productivity of *P. tricornutum* were achieved at 10 mg L^{-1}, which is 1.51-fold the control value. Meanwhile, for abscisic acid, the highest TAG production was 1.97-fold of the control at 10 mg L^{-1}. In contrast, gibberellin and ethephon caused the TAG productivity of *P. tricornutum* to decline by a large margin.

The TAG content in *P. tricornutum* induced by these phytohormones in most tested concentrations—except 10 mg L^{-1} methyl jasmonate, 0.15 and 1.5 mg L^{-1} gibberellin, and 0.06 mg L^{-1} ethephon—was lower than that of the control. This suggests that salicylic acid and abscisic acid could promote the growth of *P. tricornutum* to achieve high TAG production, but not strengthen TAG synthesis. The phytohormones

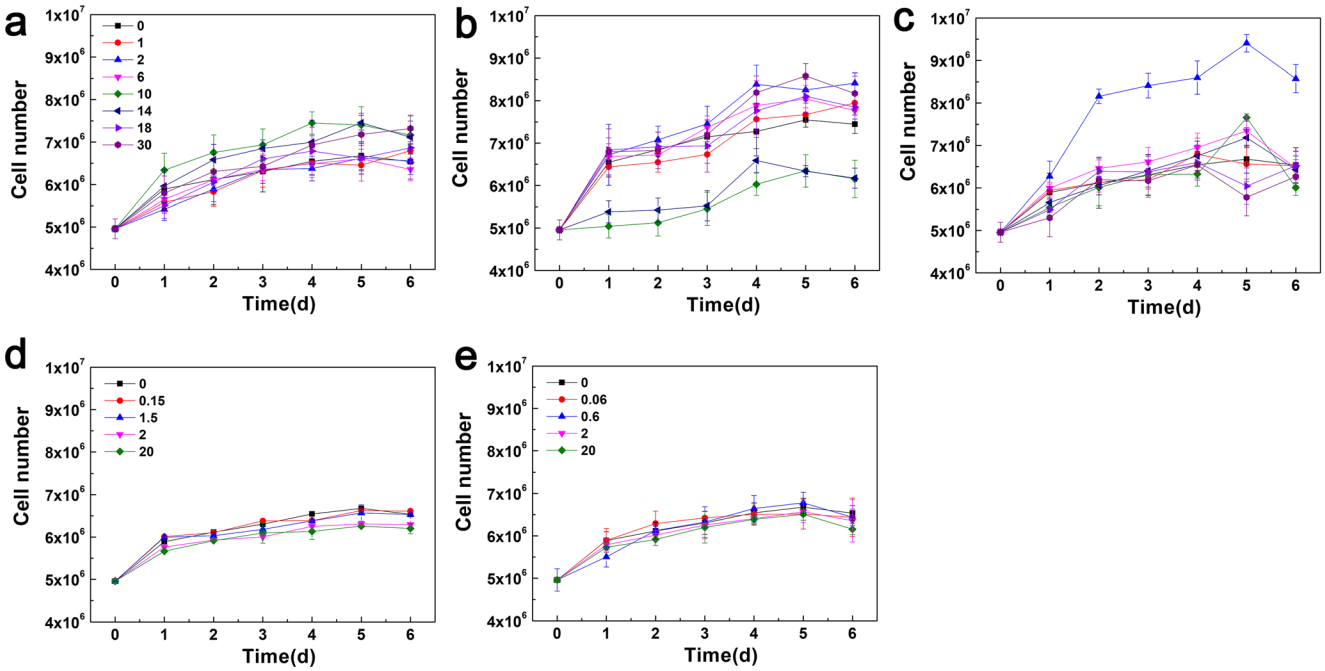

Fig. 1 Growth curves of *P. tricornutum* cultures containing the phytohormones. **a** *P. tricornutum* containing methyl jasmonate. The numbers in the figure are the concentrations of methyl jasmonate: 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. **b** *P. tricornutum* containing salicylic acid. The numbers in the figure are the concentrations of salicylic acid: 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. **c** *P. tricornutum* containing abscisic acid. The numbers in the figure are the concentrations of abscisic acid: 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. **d** *P. tricornutum* containing gibberellin with. The numbers in the figure are the concentrations of gibberellin: 0, 0.15, 1.50, 2.00, and 20.0 mg L^{-1}. **e** *P. tricornutum* containing ethephon. The numbers in the figure mean the concentrations of ethephon: 0, 0.06, 0.60, 2.00, and 20.0 mg L^{-1}. (Data are mean ± SD, $n = 3$)

gibberellin and ethephon may promote TAG synthesis at several concentrations, but inhibit growth at the same time.

The F_v/F_m of *P. tricornutum* showed a slight decline during the late logarithmic phase of growth (Fig. 2). Methyl jasmonate, salicylic acid, and abscisic acid did not influence photosynthetic activity ($p > 0.05$, two-way repeated measures ANOVA), while gibberellin and ethephon limited photosynthetic activity after 3 days of induction.

Based on these results, the phytohormones methyl jasmonate, salicylic acid, and abscisic acid could improve the TAG production as well as the growth of *P. tricornutum*; thus, they were further analyzed for optimization. Gibberellin and ethephon did not increase growth or TAG production, and thus were not evaluated further.

Effects of methyl jasmonate, salicylic acid, and abscisic acid on the TAG synthesis pathway

The TAG content (TAG product per 1 mg DW) in *P. tricornutum* induced by methyl jasmonate, salicylic acid, and abscisic acid did not rise obviously or even declined in most cases as listed in Table 2, which may indicate the TAG synthesis pathway in *P. tricornutum* was not strengthened by these phytohormones. To survey the influences of methyl jasmonate, salicylic acid, and abscisic acid on the TAG synthetic pathway, the key enzymes related to ACC1, ACC2, LAPPT, and so on were further monitored by Q-PCR every 72 h (Fig. S1). The R^2 values for all the primer pairs were all higher than 0.90. The results showed that these key enzymes' expression were related to the phytohormone concentrations and the inducing time (Figs. 3, 4, and 5), and were suppressed in most sets except 6-day inducing of 10 mg L^{-1} methyl jasmonate (Fig. 3).

The *cis*-regulatory elements responsive to the three phytohormones in these gene upstream regions were also analyzed and are listed in Table 3. The methyl jasmonate-responsive elements (CGTCA-motif and TGACG-motif) were found in the upstream regions of most genes except DGAT1, while the abscisic acid- and salicylic acid-responsive elements were present in just some of the gene upstream regions. Considering the Q-PCR results (Fig. 3) and the TAG content measurements (Table 2), methyl jasmonate appears to influence TAG content through the expression of the key enzymes in the TAG synthesis pathway. Its most effective concentration range for increasing TAG synthesis in *P. tricornutum* was 10 to 14 mg L^{-1} for 6 days. Salicylic acid and abscisic acid suppressed genes' expression at all the concentrations, including DGAT2A which contained the regulatory elements responsive to the phytohormones, and the TAG content of *P. tricornutum* induced by the two phytohormones decreased. Based on these results, salicylic acid and abscisic acid were predicted to increase TAG production through biomass but not the TAG content.

Table 2 TAG product of *P. tricornutum* induced by PGRs

PGRs	Concentration (mg L^{-1})	DW (mg L^{-1} day^{-1})	TAG productivity (mg L^{-1} day^{-1})	TAG content (TAG/DW%)
Methyl jasmonate	0	67.67 ± 2.01	2.61 ± 0.14	3.85 ± 0.21
	1	63.37 ± 1.01	2.04 ± 0. 15	3.21 ± 0.19
	2	66.00 ± 3.01	2.20 ± 0.13	3.34 ± 0.23
	6	68.67 ± 1.01	2.20 ± 0.13	3.20 ± 0.21
	10	100.33 ± 1.01	3.93 ± 0.12	3.92 ± 0.15
	14	97.00 ± 2.01	3.59 ± 0.13	3.70 ± 0.17
	18	87.67 ± 1.01	3.04 ± 0.09	3.47 ± 0.18
	30	95.33 ± 0.01	3.20 ± 0.13	3.36 ± 0.18
Salicylic acid	0	71.00 ± 1.01	2.61 ± 0.14	3.67 ± 0.21
	1	67.10 ± 2.21	2.49 ± 0.06	3.71 ± 0.19
	2	82.33 ± 1.01	2.95 ± 0.07	3.58 ± 0.15
	6	74.63 ± 0.98	2.56 ± 0.08	3.43 ± 0.24
	10	58.20 ± 0.82	1.48 ± 0.04	2.55 ± 0.25
	14	61.47 ± 2.01	1.66 ± 0.07	2.69 ± 0.18
	18	71.19 ± 1.02	2.42 ± 0.05	3.40 ± 0.24
	30	79.67 ± 2.01	2.32 ± 0.08	2.91 ± 0.19
Abscisic acid	0	67.67 ± 1.21	2.61 ± 0.04	3.85 ± 0.22
	1	67.00 ± 1.28	2.32 ± 0.03	3.47 ± 0.20
	2	133.67 ± 0.71	5.13 ± 0.04	3.84 ± 0.25
	6	80.33 ± 1.00	2.54 ± 0.02	3.17 ± 0.18
	10	66.00 ± 0.81	1.43 ± 0.02	2.17 ± 0.08
	14	74.53 ± 1.31	2.03 ± 0.04	2.72 ± 0.35
	18	63.67 ± 0.61	2.15 ± 0.12	3.38 ± 0.17
	30	62.33 ± 0.31	2.02 ± 0.03	3.24 ± 0.38
Gibberellin	0	67.67 ± 2.01	2.61 ± 0.14	3.85 ± 0.22
	0.15	64.83 ± 2.01	2.53 ± 0.04	3.90 ± 0.21
	1.5	64.50 ± 0.91	2.56 ± 0.13	3.97 ± 0.21
	2	61.33 ± 1.01	1.48 ± 0.04	2.41 ± 0.35
	20	60.25 ± 2.01	2.14 ± 0.03	3.56 ± 0.16
Ethephon	0	67.67 ± 2.01	2.61 ± 0.14	3.85 ± 0.21
	0.06	66.05 ± 0.81	2.60 ± 0.04	3.94 ± 0.18
	0.6	66.65 ± 0.91	2.57 ± 0.03	3.85 ± 0.17
	2	65.65 ± 0.41	1.92 ± 0.03	2.93 ± 0.15
	20	62.33 ± 2.00	0.35 ± 0.13	2.17 ± 0.17

$$\text{TAG productivity} = \frac{\text{TAG product in the induced cultures (mg)} - \text{TAG product in the cultures before phytohormones exposure(mg)}}{\text{the volume (L)} \times \text{the inducing days}}$$

Combination of methyl jasmonate, abscisic acid, and salicylic acid for inducing TAG accumulation in *P. tricornutum*

Based on the single-factor experiments, the effects of methyl jasmonate, abscisic acid, and salicylic acid were further examined using the response surface test. Ten-liter cultures of *P. tricornutum* with three phytohormones inducing were established according to the design of the software, and the resulting TAG products (Table 4) were used as the response values to find the optimal conditions for growth. These results were then analyzed using ANOVA. The resulting parameters calculated based on TAG yield as the response are presented in Table 5 and the response surface model was established with $p < 0.05$. The combination of methyl jasmonate and salicylic acid (group AB in Table 5) and of methyl jasmonate and abscisic acid (group AC in Table 5) may increase TAG production in *P. tricornutum* cultures, much more than just one of these phytohormones will do (Table 5). And salicylic acid (group C in Table 5) may be more effective to increase TAG production in *P. tricornutum* than the other two phytohormones (group A and group B) during inter-group analysis.

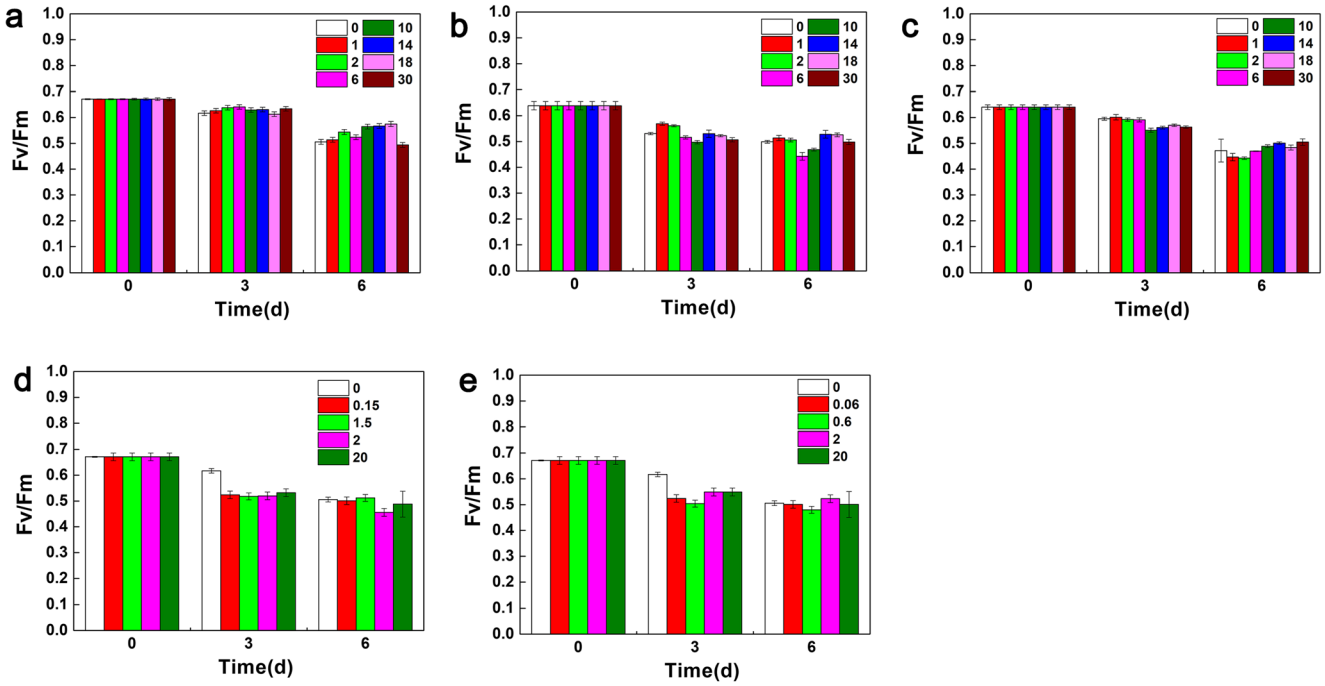

Fig. 2 The F_v/F_m of *P. tricornutum* cultures with phytohormones. **a** Cultures with methyl jasmonate. The 8 different concentrations of methyl jasmonate were 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. **b** Cultures with salicylic acid. The 8 different concentrations of salicylic acid were 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. **c** Cultures with abscisic acid. The 8 different concentrations of abscisic acid were 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}. **d** Cultures with gibberellin. The 5 different concentrations of gibberellin were 0, 0.15, 1.50, 2.00, and 20.0 mg L^{-1}. **e** Cultures with ethephon. The 5 different concentrations of ethephon were 0, 0.15, 1.50, 2.00, and 20.0 mg·L^{-1}. (Data are mean ± SD, $n = 3$)

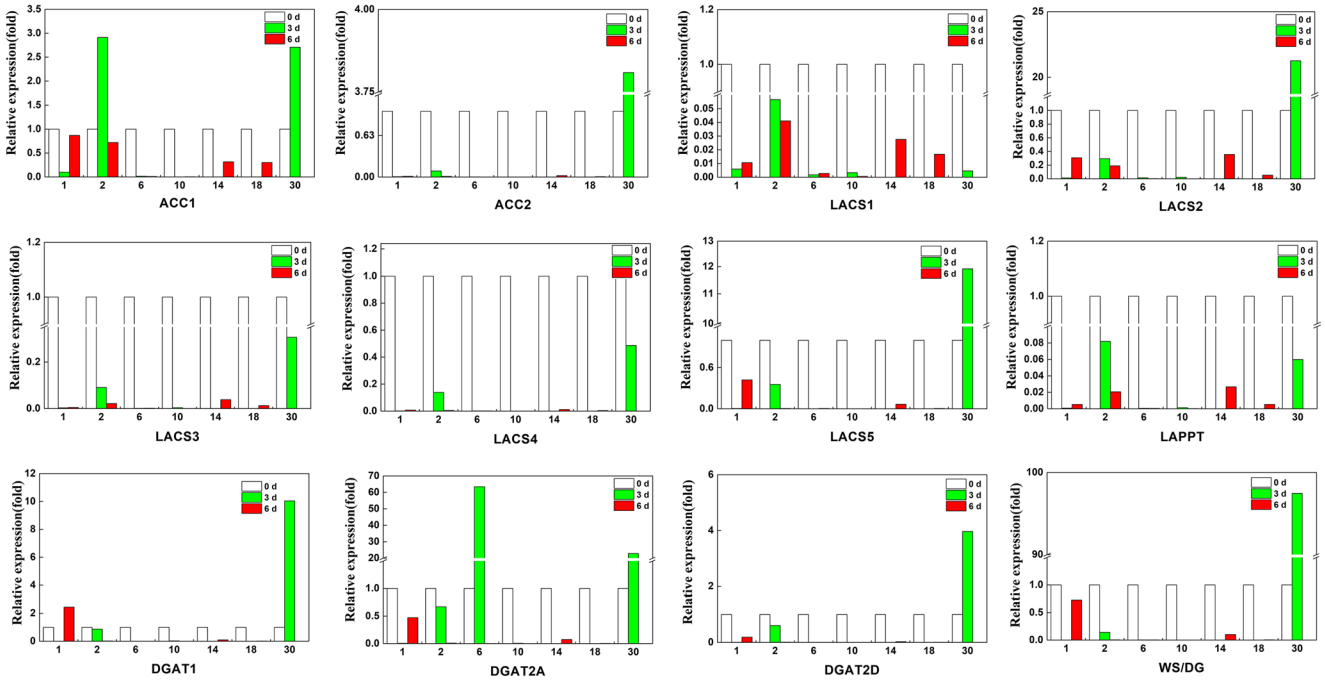

Fig. 3 The expression pattern of the key enzyme genes in TAG synthetic pathway in *P. tricornutum* with methyl jasmonate induction. The *P. tricornutum* cultures were conducted with Q-PCR for every 3 days and each sample has three biological replicates, while each biological triplicate sample has three analytical triplicates in Q-PCR. The numbers on the *X*-axis are the concentrations of methyl jasmonate: 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}

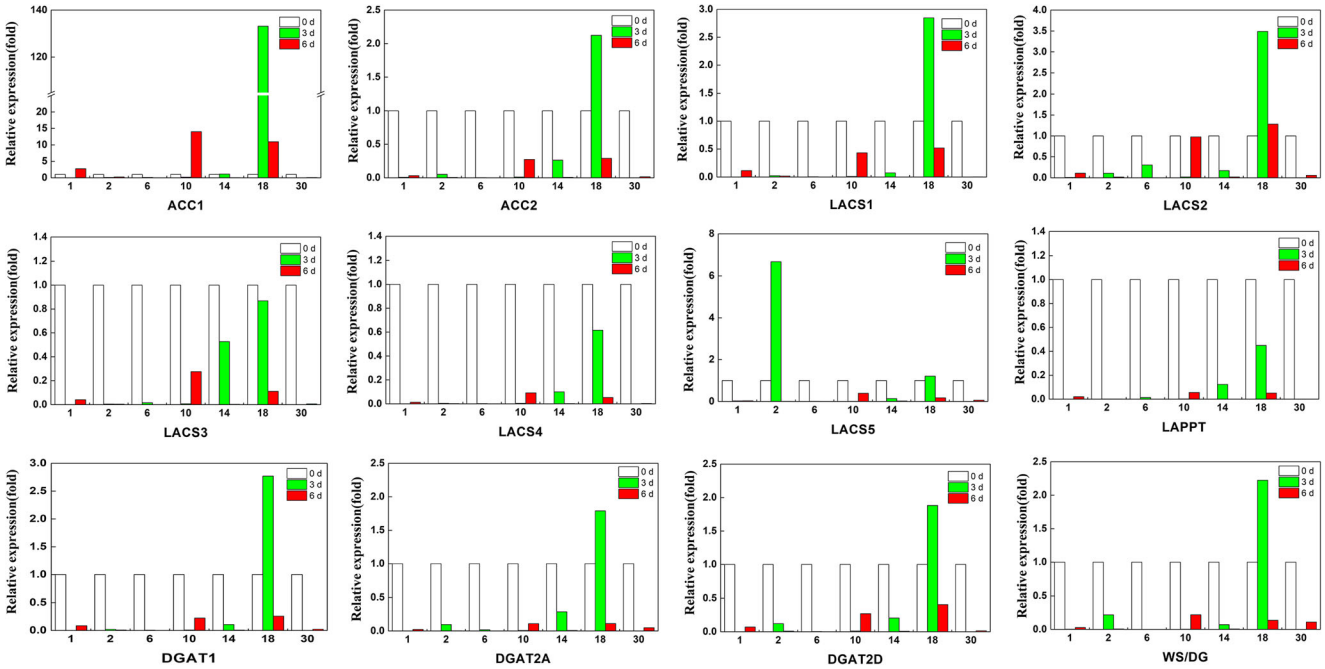

Fig. 4 The expression pattern of the key enzyme genes in TAG synthetic pathway in *P. tricornutum* with salicylic acid induction. The *P. tricornutum* cultures were conducted with Q-PCR for every 3 days and each sample has three biological replicates, while each biological triplicate sample has three analytical triplicates in Q-PCR. The numbers on the *X*-axis are the concentrations of salicylic acid: 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}

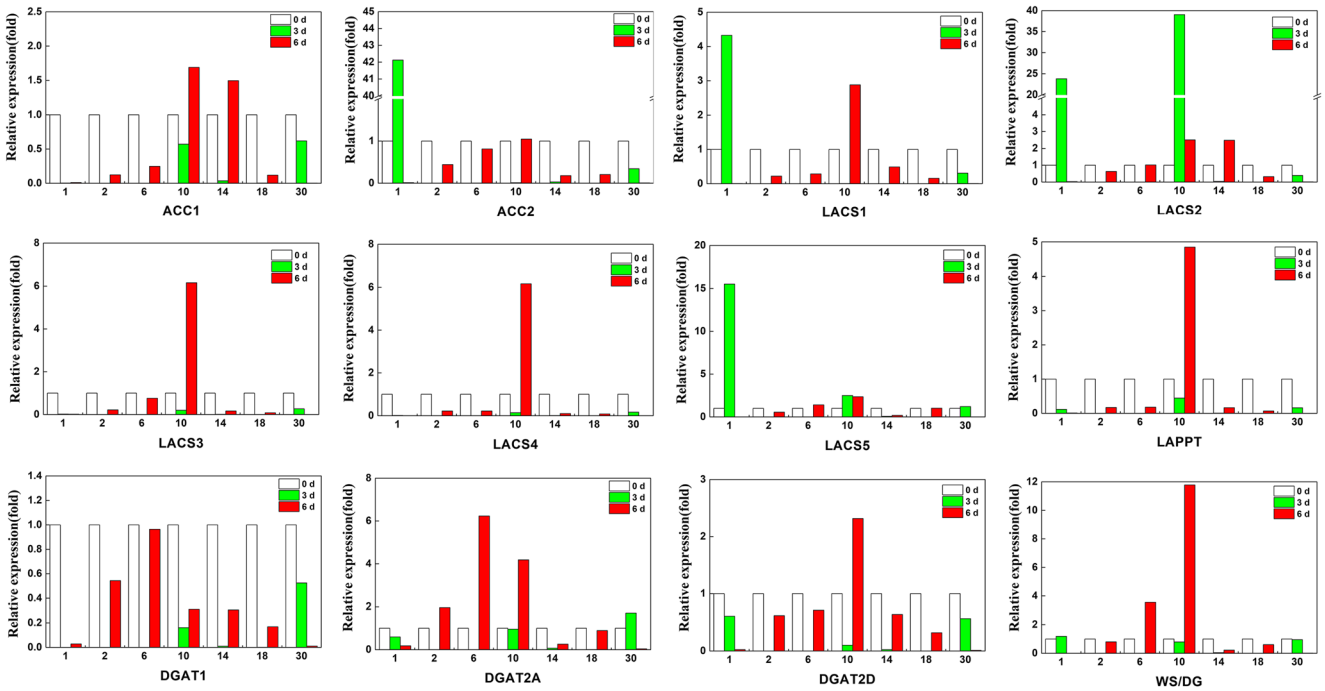

Fig. 5 The expression pattern of the key enzyme genes in TAG synthetic pathway in *P. tricornutum* with abscisic acid induction. The *P. tricornutum* cultures were conducted with Q-PCR for every 3 days and each sample has three biological replicates, while each biological triplicate sample has three analytical triplicates in Q-PCR. The numbers on the *X*-axis ae the concentrations of abscisic acid: 0, 1, 2, 6, 10, 14, 18, and 30 mg L^{-1}

Table 3 The *cis*-regulatory elements related to the PGR responsiveness presented in the upstream of the key enzymes' genes in *P. tricornutum*

Function	Elements	Sequences	Number	Genes
Involved in the methyl jasmonate responsiveness	TGACG-motif	TGACG	2	ACC1
	CGTCA-motif	CGTCA	2	
	TGACG-motif	TGACG	1	ACC2
	CGTCA-motif	CGTCA	1	
	TGACG-motif	TGACG	1	LAPPT
	CGTCA-motif	CGTCA	1	
	TGACG-motif	TGACG	5	LACS1
	CGTCA-motif	CGTCA	5	
	TGACG-motif	TGACG	5	LACS2
	CGTCA-motif	CGTCA	5	
	TGACG-motif	TGACG	2	LACS3
	CGTCA-motif	CGTCA	2	
	TGACG-motif	TGACG	1	LACS4
	CGTCA-motif	CGTCA	2	
	CGTCA-motif	CGTCA	1	LACS5
	TGACG-motif	TGACG	1	DGAT2A
	CGTCA-motif	CGTCA	1	
	TGACG-motif	TGACG	1	DGAT2D
	CGTCA-motif	CGTCA	1	
	TGACG-motif	TGACG	2	WS/DGAT
	CGTCA-motif	CGTCA	2	
Involved in the abscisic acid responsiveness	ABRE	TACGTGTC/TACGTG	2	LACS1
			2	LACS2
			1	LACS5
			1	DGAT1
			1	DGAT2A
Involved in salicylic acid responsiveness	TCA-element	CCATCTTTTT	1	LACS4
			1	DGAT2A

Finally, according to the model, 6.83 mg L^{-1} day^{-1} TAG product, which was 2.41-fold the control value, was obtained from 10-L cultures of *P. tricornutum* with 14 mg L^{-1} methyl jasmonate, 6 mg L^{-1} abscisic acid, and 2 mg L^{-1} salicylic acid. The combined influence of the three phytohormones was stronger than just one of them for increasing TAG accumulation in *P. tricornutum*.

Discussion

In the photoautotrophic culture of *P. tricornutum*, some environmental conditions such as nitrate deprivation and high light could increase TAG content 2- to 3-fold (Peng et al. 2014; Yang et al. 2014; Yu et al. 2016). But these environmental conditions, especially nitrate deprivation, inhibit algal growth when *P. tricornutum* is exposed to a nitrate-deprived medium at the logarithmic growth phase. In this study, the effects of five commonly used phytohormones—methyl jasmonate, salicylic acid, gibberellin, abscisic acid, and ethephon—on TAG accumulation in this diatom were investigated. All of these five phytohormones play important roles in the growth and development of the higher plants (Hara et al. 2012; Vankova 2012; Muhammad et al. 2013; Ozturk et al. 2018; Thongkum et al. 2018). They also participate in the signaling of biotic and abiotic stress responses in the higher plants (Hara et al. 2012; Vankova 2012; Jiang et al. 2018). Salicylic acid could increase the heat stress tolerance of bread wheat seeds (Kousar et al. 2018) and hinder the biotrophic pathogen, for example, *Pseudomonas syringae* (Vlot et al. 2009). The phytohormones were also used to increase the secondary metabolites in higher plants and algae. The methyl jasmonate promoted astaxanthin synthesis in *H. pluvialis* (Lu et al. 2010) and TAG synthesis in *P. tricornutum* (Table 2).

In this study, salicylic acid increased TAG productivity in *P. tricornutum* by stimulating algal growth but not the TAG

Table 4 The design and resulting TAG productivity in response to surface experiment

Test number	Content of methyl jasmonate (mg L^{-1})	Content of abscisic acid (mg L^{-1})	Content of salicylic acid (mg L^{-1})	TAG productivity (mg L^{-1} day^{-1})
1	12	4	4	4.17 ± 0.11
2	12	2	2	4.83 ± 0.17
3	12	4	4	4.00 ± 0.12
4	12	4	4	4.17 ± 0.14
5	12	2	6	3.33 ± 0.11
6	10	4	2	3.50 ± 0.11
7	10	4	6	5.17 ± 0.09
8	14	4	2	5.00 ± 0.14
9	12	4	4	4.17 ± 0.07
10	12	6	6	4.00 ± 0.06
11	10	2	4	5.33 ± 0.05
12	10	6	4	3.50 ± 0.13
13	14	4	6	3.17 ± 0.11
14	14	6	4	6.00 ± 0.12
15	12	4	4	4.00 ± 0.14
16	14	2	4	3.83 ± 0.12
17	12	6	2	5.17 ± 0.21
Control 1	10	0	0	3.93 ± 0.21
Control 2	0	2	0	5.13 ± 0.12
Control 3	0	0	2	2.95 ± 0.21
Control 4	0	0	0	2.83 ± 0.21

Table 5 Analysis of variance (ANOVA) for TAG from BBD design

Source	df	Sum of squares	Mean square	*F* value	*P* value
Model	9	0.04	3.83E−03	6.27	0.01
A-MJ	1	1.36E−04	1.36E−04	0.22	0.65
B-AC	1	5.03E−04	5.03E−04	0.82	0.39
C-SC	1	3.81E−03	3.81E−03	6.24	0.04
AB	1	0.02	0.02	25.04	0.00
AC	1	0.01	0.01	17.92	0.00
BC	1	9.34E−05	9.34E−05	0.15	0.71
A^2	1	9.31E−04	9.31E−04	1.52	0.26
B^2	1	2.49E−03	2.49E−03	4.07	0.08
C^2	1	2.30E−04	2.30E−04	0.38	0.56

Source meant the names the analysis sets; Model meant the total analysis; A-MJ meant the group A was methyl jasmonate treatment; B-AC meant the group was abscisic acid treatment; C-SC meant the group C was salicylic acid treatment. The inter-group analysis contained A-MJ, B-AC, and C-SC, and the intra-group analysis contained AB (group A to group B), AC (group A to group C), BC (group B to group C), A^2 (group A to group A), B^2 (group B to group B), and C^2 (group C to group C)

content per DW, but interestingly, it was found to stimulate TAG synthesis of *P. tricornutum* at the stationary phase (Xu et al. 2017). The effects of salicylic acid application depend on numerous factors such as the species and the developmental stage of the plant (Hara et al. 2012). The extent of salicylic acid's various influences on *P. tricornutum* may be related to the growth phases: the stationary phase in the research by Xu et al. (2017) and the logarithmic phase in the present study.

F_v/F_m is the maximum photochemical efficiency of open reaction centers in photosystem II, and has been used as a character for reflecting the PSII efficiency of a light-dependent process. Environmental factors and endogenous diel patterns can both impact the F_v/F_m value (Cosgrove and Borowitzka 2011). In the present study, environmental factors such as light, temperature, and nutrient status were set as the parallel parameters during all the experiments, and the added phytohormones in the cultures were the only extracellular factor to influence F_v/F_m. Gibberellin and ethephon limited algae growth and decreased F_v/F_m, whereas methyl jasmonate, salicylic acid, and abscisic acid did not influence F_v/F_m though they stimulated algal growth and promoted TAG synthesis (Table 2 and Figs. 2, 3, 4, and 5) in *P. tricornutum*. These results may be related to the cellular metabolisms influenced by the respective phytohormones and further studies including the enzymatic reaction of PSII, the transcriptomes, and metabolome analysis will be needed.

The relative elements responsive to these phytohormones were found in the 5′ upstream region of the TAG synthesis-related genes in this study. The gene expression patterns in *P. tricornutum* induced by phytohormones were consistent with the TAG content changes compared with those of the control. Methyl jasmonate promoted TAG synthesis and up-regulated the genes related to TAG synthesis, while salicylic acid and abscisic acid declined TAG synthesis and downregulated these genes. Methyl jasmonate at the concentration of 10 mg L^{-1} could promote the TAG synthesis-related gene expression (such as ACC1, LACS1, LACS2, LACS5, LAPPT, DGAT2A in Fig. 3) and increase the TAG content to 3.92% compared with 3.85% TAG content in the control. Meanwhile, salicylic acid and abscisic acid downregulated the TAG synthesis-related gene expression (ACC1, ACC21, LACS1, LACS2, DGAT2A, DGAT2D,WS/DGAT) at the concentration of 2 mg L^{-1} (Figs. 4 and 5) and decline the TAG content to 3.58 and 3.84% respectively (Table 2). TAG synthesis and accumulation in *P. tricornutum* are closely related to endogenous intermediate metabolism (Levitan et al. 2015) and are influenced by many factors such as light and nutrients. The phytohormones methyl jasmonate, salicylic acid, and abscisic acid may act as signals for regulating internal mechanisms such as the tricarboxylic acid cycle and starch synthesis, but not directly induce TAG synthesis. The mechanisms of the phytohormones influencing TAG synthesis and accumulation will be explored in future studies.

The three phytohormones—methyl jasmonate, salicylic acid, and abscisic acid—were found to increase TAG production in *P. tricornutum*, while salicylic acid and abscisic acid just stimulated the growth rate. The combination of these three phytohormones could increase TAG productivity to 6.00 mg L^{-1} day^{-1}, which is higher than that of *P. tricornutum* with N deprivation (5.40 mg L^{-1} day^{-1}; Yang et al. 2013; Cui et al. 2018). The TAG content of *P. tricornutum* induced by phytohormones was 1.7-fold than that of the control, indicating that TAG accumulation is strengthened by the combination of the three phytohormones. These results suggest that phytohormone induction is a promising method for improving TAG accumulation in *P. tricornutum* for industrial use.

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