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Metabolic switching: synergistic induction of carotenogenesis in the aerial microalga, *Vischeria helvetica,* under environmental stress conditions by inhibitors of fatty acid biosynthesis

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Abstract
Objectives To investigate the roles of fatty acid biosynthesis in carotenogenesis in the high-lipid accumulating aerial microalga *Vischeria helvetica* KGU-Y001, we cultured algal cells with fatty acid biosynthesis inhibitors.
Results Under nitrogen-deficient, high-light (200 μmol photons m^{-2} s^{-1}) conditions, the alga accumulated 6.2 mg carotenoids g^{-1} dry weight cells (DWC) after 1 week of culture. The total fatty acid content increased gradually, and reached 290 mg g^{-1} DWC after 9 weeks. When algal cells were cultured with a fatty acid biosynthesis inhibitor (molinate) under nitrogen-deficient, high-light conditions for 1 week, carotenoid accumulation was synergistically increased to 2.4 times that in algal cells cultured without the inhibitor in nitrogen-deficient, low-light conditions (40 μmol photons m^{-2} s^{-1}). The synergistic induction of carotenogenesis was suppressed by an inhibitor of c-jun *N*-terminal kinase, a mitogen-activated protein kinase-like protein.
Conclusion In a commercial context, carotenoid production could be increased by using fatty acid biosynthesis inhibitors to redirect metabolic flux to carotenoid biosynthesis instead of fatty acid synthesis.

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Introduction

Carotenoids are efficient antioxidants for quenching singlet oxygen. These compounds play an important role in protection against oxidative damage in humans and photosynthetic organisms (Demmig-Adams and Adams 2002). Some microorganisms accumulate carotenoids as a part of their response to various environmental stresses. Environmental factors, such as light and temperature, and cultural factors, such as the concentrations of nutrients, metal ions, and salts, can induce carotenoid accumulation in algae, fungi, and bacteria (Bhosale 2004). Photosynthetic organisms use solar energy to fix CO_2 into complex organic substances. Microalgae are good sources of commercially-valuable carotenoids including β-carotene and astaxanthin. Aerial microalgae can produce large amounts and many types of carotenoids during their adaptation to environmental stress conditions, including nitrogen deficiency and high light (Abe et al. 2007; Aburai et al. 2013). The aerial microalga, *Vischeria helvetica* KGU-Y001, in the class Eustigmatophyceae, accumulates large amounts of β-carotene under high-light stress (Aburai et al. 2013).

In microalgae, carotenogenesis increases in response to environmental stress (e.g. high light, nitrogen

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deficiency, and high salt). Intracellular stimulants and chemical promoters, for example oxygen radicals, induce massive β-carotene accumulation in the green microalga *Dunaliella bardawil* (Shaish et al. 1993). Sodium orthovanadate promoted astaxanthin accumulation under low-light conditions (40 μmol photons m^{-2} s^{-1}) by inhibiting protein tyrosine phosphatases, which affected signal transduction related to carotenogenesis in the green microalga *Haematococcus lacustris* (Tran et al. 2009a). Inhibitors of fatty acid biosynthesis affected the interdependence between the formation of triacylglycerol droplets and β-carotene accumulation in *D. bardawil* cultured under nitrogen-sufficient, high-light (690 μmol photons m^{-2} s^{-1}) conditions (Rabbani et al. 1998). Therefore, carotenogenesis may be promoted by bioactive compounds that regulate triacylglycerol metabolism in lipid globules.

In microalgae, bioactive compounds can induce carotenoid accumulation triggered by nitrogen deficiency and high light. We analyzed the synergistic effects of fatty acid biosynthesis inhibitors and environmental stress factors (nitrogen deficiency and high light) on carotenogenesis in the high-lipid accumulating aerial microalga *V. helvetica* KGU-Y001. We discuss the effects of the inhibitors on the responses of this alga to environmental stresses.

Materials and methods

Strain and culture conditions

Vischeria helvetica KGU-Y001 was precultured in Bold's basal (BB) medium (Aburai et al. 2013) and maintained at 25 ± 2 °C, pH 8.0, under low-light conditions (40 μmol photons m^{-2} s^{-1}) in a 500 ml Erlenmeyer flask with aeration for 3 weeks. The green vegetative cells were then transferred to 50 ml Erlenmeyer flasks containing nitrogen-deficient medium. The cells were incubated under low-light and high-light conditions (200 μmol photons m^{-2} s^{-1}) at 25 ± 2 °C for 1 week on a reciprocal shaker at 120 rpm, in the presence or absence of fatty acid biosynthesis inhibitors (cerulenin, sethoxydim, or molinate; Wako Pure Chemicals Industries Co., Ltd., Osaka, Japan). The compounds SB203580, SP600125, and PD98059, which inhibit mitogen-activated protein kinases (MAPKs), were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). All inhibitors were dissolved in 0.1 % (w/v) saponin aqueous solution. This surfactant was used to improve the permeability of the compounds without organic solvents (Augustin et al. 2011).

Analyses

The growth of algal cells was monitored by measuring the dry weight. cells (DWC) were collected on a 0.45 μm membrane filter, washed with distilled water, and then dried under reduced pressure before weighing.

The nitrate concentration in the culture medium was measured by ion chromatography. Samples were deionized with Amberlite IR120B and then analyzed using an IA-300 ion analyzer (TOA-DKK Co., Tokyo, Japan) equipped with an anion-exchange column (PCI-211, 4.6 × 100 mm; TOA-DKK Co., Tokyo, Japan). The mobile phase was 2.3 mM phthalic acid/2.8 mM 6-aminohexanoic acid/200 mM boric acid.

Total carotenoids were estimated according to Aburai et al. (2013). Pigments were extracted with methanol/dichloromethane (3:1, v/v). Extracted carotenoids were analyzed by HPLC with a reversed-phase column (Inertsil 5 μm ODS-2, 4.6 × 150 mm; GL Sciences Inc., Tokyo, Japan).

Fatty acids were analyzed by a method described previously by Abe et al. (2007). Fatty acid methyl esters were prepared by acid transesterification. Lyophilized cells were incubated with 5 % (v/v) HCl/methanol at 100 °C for 3 h. Fatty acid methyl esters were extracted with hexane and analyzed by GC equipped with a capillary column (InertCap 17, 30 m × 0.25 mm × 0.25 μm; GL Sciences Inc., Tokyo, Japan). Heptadecanoic acid was used as an internal standard for the quantitative analysis.

Statistical analyses

Means and standard deviations were calculated for each treatment. One-way analysis of variance (ANOVA) was used for statistical analysis, and tests were considered statistically significant at $P < 0.05$.

Results and discussion

Accumulation of carotenoids and fatty acids under nitrogen-deficient and high-light conditions

Nitrogen deprivation and high light increase carotenoid accumulation in *Haematococcus* sp. (Tran et al. 2009b). We measured the growth and nitrate

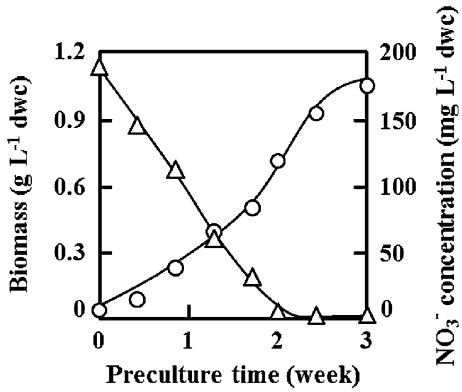

Fig. 1 Cell growth (*open circle*) and nitrate concentration (*open triangle*) in a preculture of *V. helvetica* KGU-Y001. Algal cells were cultured in a flat glass bottle for 3 weeks in BB medium at 40 µmol photons m^{-2} s^{-1}. Values shown are the average of two independent experiments

concentration in a preculture of *V. helvetica* KGU-Y001. Algal cells were cultured in a flat glass-bottle for 3 weeks in BB medium at 40 µmol photons m^{-2} s^{-1}. Under these conditions, the cells reached the stationary growth phase after 17 days, when the nitrate removal rate reached 0.5 mg l^{-1} h^{-1} (Fig. 1). The algal cells precultured for 3 weeks were then grown in nitrogen-deficient medium to promote carotenoid accumulation.

When KGU-Y001 cells were cultured in the nitrogen-deficient medium at 200 µmol photons m^{-2} s^{-1}, the cells turned yellow-orange because of a massive increase in β-carotene accumulation and a decrease in the total chlorophyll content (Aburai et al. 2013). We monitored carotenoids and fatty acids accumulation in cultures of *V. helvetica* KGU-Y001 for 12 weeks (Fig. 2). Carotenoids reached a transient maximum of 6.2 mg g^{-1} DWC after 1 week at 200 µmol photons m^{-2} s^{-1} in nitrogen-deficient medium, and then gradually decreased during the rest of the culture (Fig. 2a).

In the nitrogen-deficient medium, the total fatty acids (TFA) content of the algal cells cultured at 200 µmol photons m^{-2} s^{-1} increased gradually to reach approx. threefold its initial level after 9 weeks (290 mg g^{-1} DWC; Fig. 2b). The rates of carotenoids and TFAs accumulation were faster under high-light conditions than under low-light conditions (at 40 µmol photons m^{-2} s^{-1}, the maximum carotenoids and TFA concentrations were 5.8 and 272 mg g^{-1}

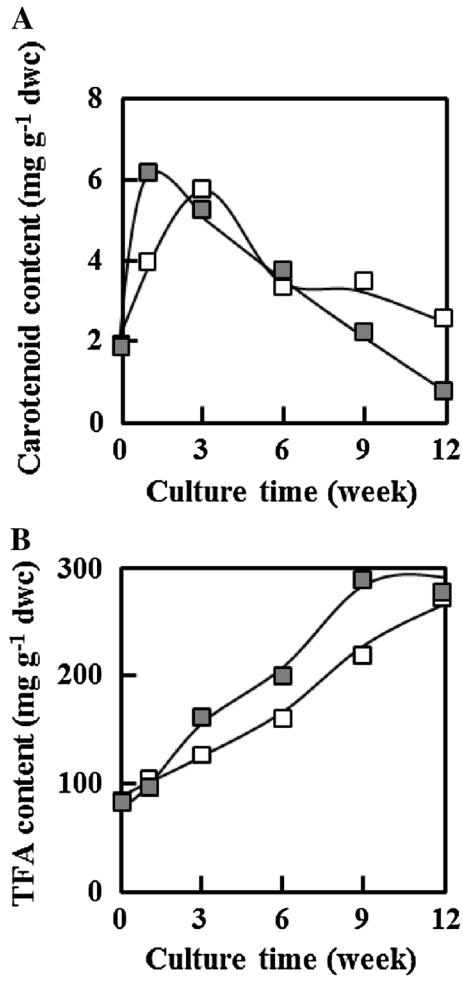

Fig. 2 Accumulation of **a** carotenoids and **b** fatty acids in a culture of *V. helvetica* KGU-Y001. Algal cells were cultured for 12 weeks in nitrogen-deficient medium at 40 (*open square*) and 200 (*filled square*) µmol photons m^{-2} s^{-1}. Values shown are the average of two independent experiments

DWC, respectively). GC analysis showed that palmitic acid (C16:0) and palmitoleic acid (C16:1) were the most abundant fatty acids, each accounting for approx. 40 % of the TFAs (data not shown). Under nitrogen-deficient and high-light conditions, *D. salina* accumulated oleic acid (18:1) as the main fatty acid, and there was a significant correlation between oleic acid accumulation and β-carotene accumulation (Mendoza et al. 1999). The modes of carotenoids and fatty acids accumulation in KGU-Y001 clearly differed from those in the green microalga *D. salina* (Rabbani et al. 1998).

Promotion of carotenogenesis by inhibition of fatty acid biosynthesis

For the control culture without inhibitors of fatty acid biosynthesis, cells of KGU-Y001 were cultivated in nitrogen-deficient medium at 40 or 200 μmol photons m^{-2} s^{-1} for 1 week. The carotenoid content of cells cultured under high-light conditions was 160 % of that in cells cultured under low-light conditions (Fig. 3a). The algal cells were cultured with each of three differently acting inhibitors of fatty acid biosynthesis: cerulenin, which inhibits β-ketoacyl-(acyl carrier protein) synthase (Ōmura 2011), sethoxydim, an inhibitor of acetyl-CoA carboxylase (Casida 2009), and molinate, a probable inhibitor of acetyl-CoA elongase (Galhano et al. 2010). When cells cultured in nitrogen-deficient, high-light conditions were supplemented with 10 μM molinate (dissolved in saponin solution) and then further cultured for 1 week, the carotenoids content of the cells synergistically increased to 150 and 240 % of the that (11.2 ± 1.6 mg g^{-1} DWC) in the controls (with no inhibitor) at 200 and 40 μmol photons m^{-2} s^{-1}, respectively. Under the same culture conditions, molinate significantly reduced the TFA content from 118 to 92 mg g^{-1} DWC (for comparison, see control culture at 200 μmol photons m^{-2} s^{-1} in Fig. 3b). The decrease (26 mg g^{-1} DWC) was almost same as the concentration of TFAs (25 mg g^{-1} DWC) biosynthesized by cells cultured under nitrogen-deficient, high-light conditions for 1 week (Fig. 2b). However, addition of cerulenin and sethoxydim did not affect the cells cultured under nitrogen-deficient, high-light conditions (Fig. 3). Compared with molinate, cerulenin and sethoxydim have a lower solubility in water and possibly a lower permeability into algal cells. In these experiments, the bioactive compounds were dissolved in 0.1 % saponin as a surfactant, instead of using organic solvents to increase permeability (Augustin et al. 2011). The use of other surfactants to increase the solubility and permeability of cerulenin and sethoxydim would be helpful to study carotenogenesis and fatty acid biosynthesis in these algal cells.

In general, molinate functions as a selective pre-emergence herbicide to control broad-leaved and grassy plants in rice and other crops. This herbicide was shown to inhibit the growth of a cyanobacterium (Galhano et al. 2010). In KGU-Y001, carotenogenesis was promoted by molinate in a dose-dependent manner from 0.1 to 10 μM. This was accompanied by the suppression of fatty acid biosynthesis (Fig. 4).

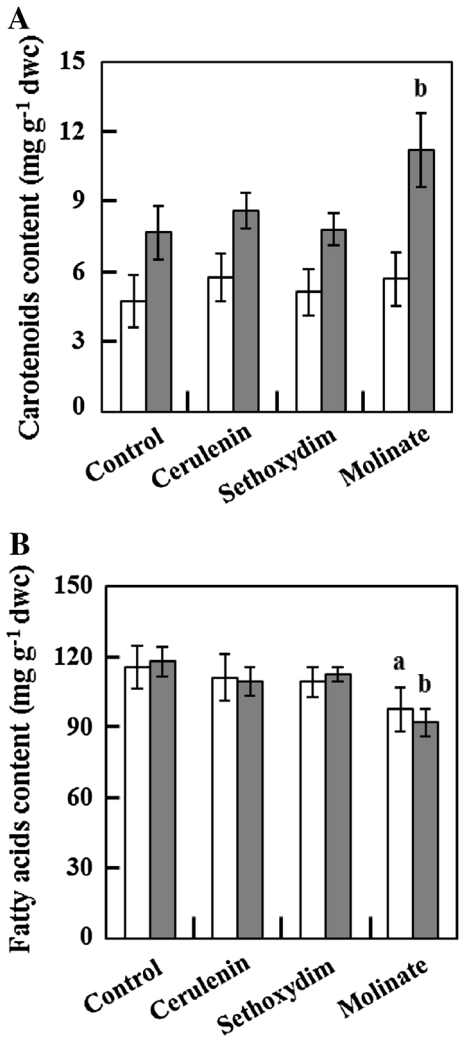

Fig. 3 Effects of inhibitors of fatty acid biosynthesis on accumulation of **a** carotenoids and **b** fatty acids in cells of *V. helvetica* KGU-Y001. Algal cells were cultured for 1 week in nitrogen-deficient medium without (control) or with 10 μM cerulenin, sethoxydim, or molinate at 40 (*open square*) and 200 (*filled square*) μmol photons m^{-2} s^{-1}. Values shown are mean ± SD of four replicate cultures (*a* and *b*, $P < 0.05$, as compared with untreated control at 40 and 200 μmol photons m^{-2} s^{-1}, respectively)

Effects of MAPK inhibitors on molinate-induced carotenoid accumulation

The MAPKs expressed in all eukaryotic cells are highly conserved serine/threonine kinases. In plants,

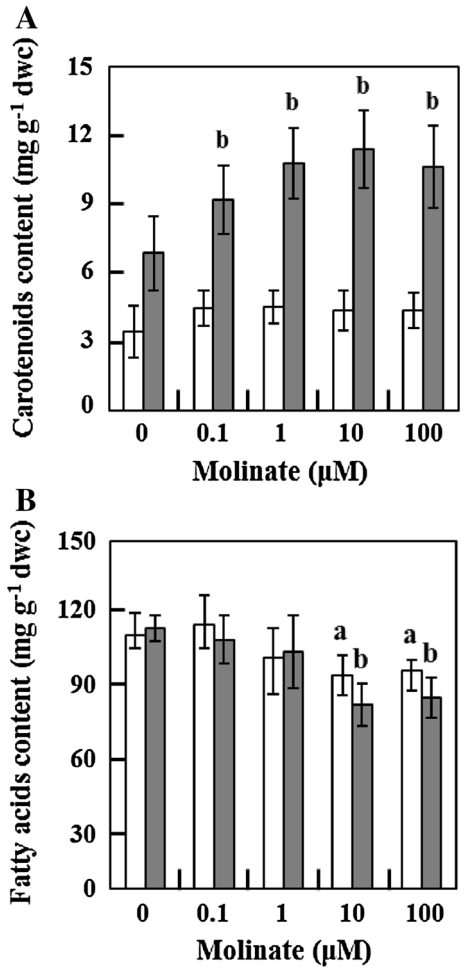

Fig. 4 Synergistic effects of molinate and high light on accumulation of **a** carotenoids and **b** fatty acids in *V. helvetica* KGU-Y001. Algal cells were cultured for 1 week in nitrogen-deficient medium containing 0.1, 1.0, 10, or 100 μM molinate at 40 (*open square*) or 200 (*filled square*) μmol photons m^{-2} s^{-1}. Values shown are mean ± SD of four replicate cultures (*a* and *b*, $P < 0.05$, as compared with untreated control at 40 and 200 μmol photons m^{-2} s^{-1}, respectively)

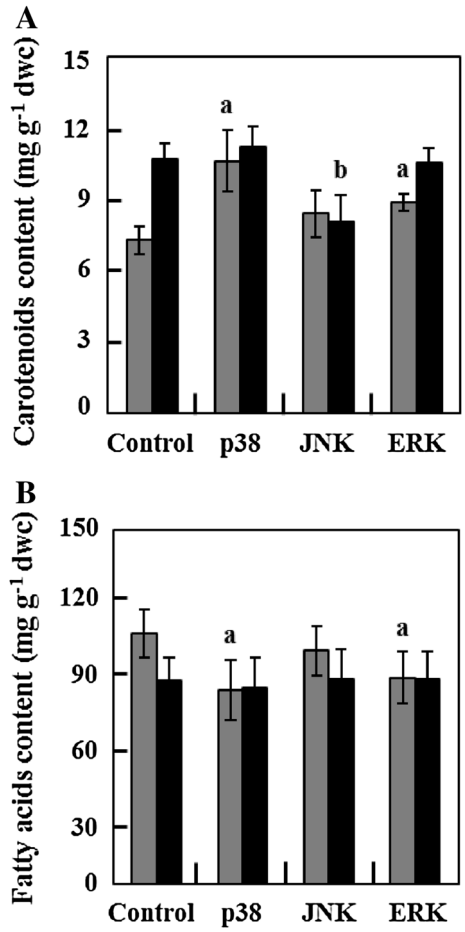

Fig. 5 Effects of MAPK inhibitors on accumulation of **a** carotenoids and **b** fatty acids induced by molinate. Cells were incubated for 1 week in nitrogen-deficient medium without (control) or with 20 μM SB203580 (p38), SP600125 (JNK), or PD98059 (ERK) at 200 μmol photons m^{-2} s^{-1}. Saponin aqueous solution (*grey filled square*) or 10 μM molinate in saponin aqueous solution (*black filled square*) were added to the medium. Values shown are mean ± SD of four replicate cultures (*a* and *b*, $P < 0.05$, as compared with molinate untreated and treated control, respectively)

these kinases transduce extracellular signals during a variety of cytoplasmic and nuclear events (Moustafa et al. 2014). In the green microalga *D. tertiolecta*, two kinds of MAPK-like proteins, p38 and c-jun *N*-terminal kinase (JNK), are phosphorylated during environmental stresses including osmotic and thermal shock, ultraviolet irradiation, and nitrogen starvation (Jiménez et al. 2004). The phosphorylation levels of extracellular signal-regulated kinase (ERK)-like proteins in *D. viridis* decrease markedly after exposure to stresses such as hyperosmotic shock, sublethal ultraviolet irradiation, and nitrogen deficiency that impaired cell division (Jiménez et al. 2007).

We used such inhibitors to explore the interdependence between carotenogenesis and MAPK cascades in KGU-Y001. Algal cells were cultured in nitrogen-deficient medium at 200 μmol photons m^{-2} s^{-1} for 1 week. As shown in Fig. 5, treatment with 10 μM molinate synergistically increased the carotenoids content from 7.3 ± 0.6 to 10.7 ± 0.7 mg g^{-1} DWC and decreased the fatty acids content from 106 ± 10.1

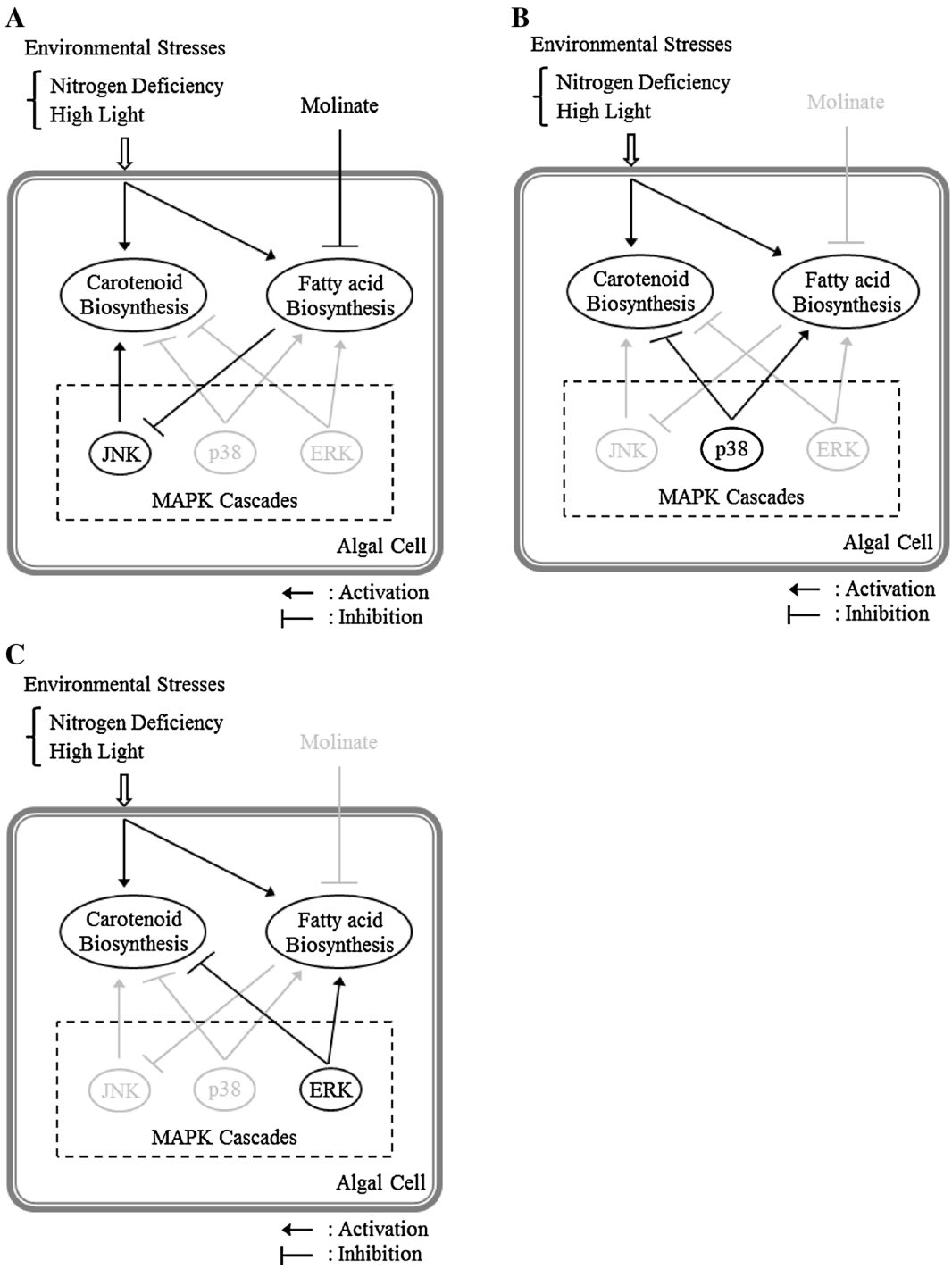

Fig. 6 Proposed model of regulation of carotenoid and fatty acid biosynthesis affected by **a** JNK, **b** p38, and **c** ERK MAPK cascades in the aerial microalga, *V. helvetica* KGU-Y001

to 86 ± 9.7 mg g^{-1} DWC. On the other hand, in the algal cells cultured under nitrogen-deficient, high-light conditions and treated with molinate, further addition of 20 μM SP600125 (JNK inhibitor) to the medium decreased the carotenoids content from 10.7 ± 0.7 to 8.1 ± 1.1 mg g^{-1} DWC, but did not

affect the TFAs content (87 ± 12.8 mg g^{-1} DWC). These results suggested that algal carotenogenesis could be regulated by fatty acid biosynthesis via the JNK pathway under nitrogen-deficient, high-light conditions.

A model of regulation of carotenoid and fatty acid biosynthesis affected by MAPK cascades in the aerial microalga, *V. helvetica* KGU-Y001, is proposed in Fig. 6. JNK-like proteins are associated with cell survival, cell death, DNA repair, and metabolism in *D. tertiolecta* (Jiménez et al. 2007; García-Gómez et al. 2012). Although the influence of the JNK pathway on carotenoid biosynthesis in microalgae is not well understood, it may play an important role in regulating carotenogenesis.

When cells of KGU-Y001 were cultured for 1 week at 200 μmol photons m^{-2} s^{-1} in nitrogen-deficient medium (without molinate) with 20 μM SB203580 (p38 inhibitor) or PD98059 (ERK inhibitor), the carotenoids content synergistically increased to 145 or 120 % of that in the control, respectively, while the TFAs content decreased to 82 ± 12.7 and 87 ± 11.1 mg g^{-1} DWC, respectively. The p38 and ERK pathways could up-regulate fatty acid biosynthesis in these algal cells; therefore, treatment with inhibitors of these pathways promoted carotenogenesis under nitrogen-deficient, high-light culture conditions (Fig. 6).

We explored the roles of the fatty acid biosynthesis and MAPKs pathways in carotenogenesis in KGU-Y001 using various inhibitors. The use of bioactive compounds that modulate biological functions is a powerful strategy to dissect the pathways of carotenoid production. The mechanisms underlying carotenogenesis, including fatty acid biosynthesis and MAPKs signal transduction, were investigated in this alga. In future research, we intend to examine large-scale, rapid carotenoid production by aerial microalgae under environmental stress conditions (e.g. nitrogen-deficient and high-light) and in response to certain bioactive compounds, such as intercellular biosynthesis and signal transduction inhibitors.

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