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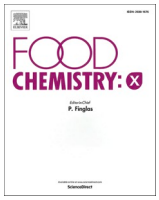

Promoting effects of NaCl and KCl stresses on astaxanthin yield in *Microcystis flos-aquae*☆

Junjie Ma [a,b], Zhehan Yang [a,b], Zhuxin Jin [a,b], Lexin Huang [a,b], Yinggang Wei [a,b], Wangbo Chen [a,b], Zhaojiang Zuo [a,b,*]

[a] National Key Laboratory for Development and Utilization of Forest Food Resources, Zhejiang A&F University, Hangzhou 311300, China
[b] College of Forestry and Biotechnology, Zhejiang A&F University, Hangzhou 311300, China

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ABSTRACT

This study was to uncover the astaxanthin accumulation mechanism of *Microcystis flos-aquae* stressed by NaCl and KCl and select the optimal condition for astaxanthin production. Both of NaCl and KCl stresses showed inhibiting effects on *M. flos-aquae* growth by reducing photosynthetic abilities and causing reactive oxygen species accumulation. With raising the two salt concentrations, astaxanthin content and yield gradually increased, and the highest accumulation was under 300 mM for each salt, which should result from the up-regulation of 6 related genes promoting the precursor (β-carotene and zeaxanthin) transformation. KCl stress was more effective for improving astaxanthin yield than NaCl stress, which was strongly related with the salt concentration and astaxanthin content. Compared with other potential suitable conditions (35°C and purple light), 300 mM KCl also exhibited maximum effect on astaxanthin accumulation. Therefore, *M. flos-aquae* is first identified to synthesize astaxanthin, and KCl stress is more favorable to the compound production.

1. Introduction

Astaxanthin is the strongest natural antioxidant discovered by human up to now, and has been widely used in multiple areas, such as functional foods, healthcare products, cosmetics and pharmaceuticals (Ahirwar et al., 2021; Diao et al., 2024). In the nature, only several algae, bacteria and yeast can synthesize astaxanthin, and natural astaxanthin is extracted from limited species of them, leading to the extreme high price (about $7000 per kg) (Huang et al., 2023; Oslan et al., 2021).

Algae are photosynthetic organisms with fast growth rate and high CO_2 assimilation efficiency, and are considered as the primary sources of natural astaxanthin. At present, several green algae with astaxanthin production potential have been identified, such as *Haematococcus pluvialis*, *Chloromonas zofingiensis*, *Chlorella sorokiniana*, *Scenedesmus acutus*, *Dunaliella viridis*, and *D. salina* (Ahirwar et al., 2021; Chen et al., 2022; Lin et al., 2019), but the studies about improving astaxanthin yield are mainly focused on *H. pluvialis* (Oslan et al., 2021). For cyanobacteria, to the best of our knowledge, only *Microcystis aeruginosa* and *Geitlerinema amphibium* have been identified to have the potential for astaxanthin production (D'Alessandro et al., 2019; Zhou et al., 2022), while no other cyanobacterium has been identified to accumulate the high-value compound. Then, the efforts are still needed to screen out more cyanobacterial species with astaxanthin production potential, as cyanobacteria exhibit huge advantages in photosynthetic abilities and growth rate, with 2 folds in the biomass accumulation rate in contrast to green algae (Farrokh et al., 2019; Noreña-Caro & Benton, 2018).

In algae, β-carotene generated from phytoene is the precursor for astaxanthin formation, and is transformed to astaxanthin in catalysis with two enzymes, such as β-carotene ketolase and hydroxylase (Debnath et al., 2024; Zhou et al., 2022). The catalytic order of the two enzymes leads to two pathways for astaxanthin formation (Huang et al., 2024). Astaxanthin biosynthesis is sensitive to environmental factors, and stress conditions can promote its generation by raising related gene expression and enzyme activities (Oslan et al., 2021; Ramamoorthy et al., 2022; Huang et al., 2023, Huang et al., 2024). Algal growth needs a certain salinity in the medium, and high salt concentration always promotes astaxanthin accumulation. With raising NaCl concentration, the content of astaxanthin in *H. pluvialis* was gradually increasing (Ramamoorthy et al., 2022). Astaxanthin was accumulated to a high

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* Corresponding author at: National Key Laboratory for Development and Utilization of Forest Food Resources, Zhejiang A&F University, Hangzhou 311300, China.
E-mail address: zuozhaojiang@126.com (Z. Zuo).

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level in *C. zofingiensis* treated with NaCl, due to promoting the formation of precursor (β-carotene) and intermediate products (zeaxanthin and canthaxanthin) (Mao et al., 2020). For *D. salina*, NaCl treatment enhanced astaxanthin yield by up-regulating expression of *crtO* that encoded β-carotene ketolase (Chen et al., 2022). Except of NaCl treatment, KCl treatment also showed promoting effect on astaxanthin formation, e.g., astaxanthin and canthaxanthin were accumulated in *Chlorococcum* sp. under KCl stress (Janchot et al., 2019). Recently, a new finding showed that KCl stress is more effective in promoting astaxanthin accumulation in *M. aeruginosa* than NaCl stress, due to the higher expression levels of 8 genes involved in the compound formation (Huang et al., 2024). However, the efforts are still needed to clarify whether the finding can be applicated to other astaxanthin-production algae, especially cyanobacterial species, as they grow fast and can be easily cultured.

Microcystis flos-aquae is one of typical cyanobacterial species, without any utilization potential being proposed up to now. In the present study, this species was first reported to synthesize astaxanthin. Then, the impacts of NaCl and KCl treatments on the algal growth and astaxanthin accumulation were investigated to elucidate the promoting effects of the two salt stresses on astaxanthin synthesis, and the correlation of astaxanthin yield with other indexes was analyzed to reveal the key factors for the compound production. Meanwhile, the astaxanthin content and yield in the algal cells under the optimal salt and other potential conditions (35 °C high temperature and purple light) that were appropriate for *M. aeruginosa* astaxanthin accumulation were compared (Huang et al., 2023; Zhou et al., 2022). The findings are not only beneficial to producing astaxanthin by using *M. flos-aquae*, but also provide reference for improving astaxanthin yield in other cyanobacterial species by using an optimal method.

2. Material and methods

2.1. M. Flos-aquae cell culture and treatment

M. flos-aquae was kept in BG11 medium under 30 μmol·m^{-2}·s^{-1} (16-h light/ 8-h dark regime at 25 °C), and collected by centrifugation at 6000*g* during the mid-logarithmic phase. Then, the harvested cells were transferred into a conical flask with 150 mL fresh media (1×10^7 cells·mL^{-1}) under sterile condition. For NaCl and KCl treatments, a certain amount of each salt was added into the medium, with the concentration of 100, 200 and 300 mM, respectively. Four replicates were set in each treatment, with each conical flask as a replicate. During the treatment within 6 days, the cell density of *M. flos-aquae* was determined with a hemocytometer, and the reactive oxygen species (ROS) levels, chlorophyll content, photosynthetic abilities, astaxanthin and the precursor content, as well as astaxanthin yield were investigated. The gene expression levels in astaxanthin formation under 300 mM NaCl and KCl were determined.

High temperature at 35 °C (Huang et al., 2023) and purple light (30 μmol·m^{-2}·s^{-1}) (Zhou et al., 2022) were two optimal conditions for *M. aeruginosa* accumulating astaxanthin. To uncover the effects of the two conditions on astaxanthin accumulation in *M. flos-aquae*, 150 mL cell cultures in a conical flask prepared following the above protocol were treated with purple light at 30 μmol·m^{-2}·s^{-1} (16-h light/ 8-h dark regime at 25 °C) and high temperature (16-h light (35 °C)/ 8-h dark (30 °C) regime, light intensity at 30 μmol·m^{-2}·s^{-1}), respectively. There were also four replicates. The astaxanthin content and yield were investigated after 6 days, and the effects of optimal salt stress (300 mM KCl), purple light and high temperature on astaxanthin content and yield were compared.

2.2. ROS level determination

M. flos-aquae cell cultures were centrifugated at 6000 g, of which the collected cells were added in 10 μM 2′,7′-dichlorodihydrofluorescein diacetate and incubated in the dark. The fluorescent probe entered the cells, and was converted into 2′,7′-dichlorodihydrofluorescein via hydrolysis. After oxidation by ROS, the formed 2′,7′-dichlorofluorescein showed green fluorescence with the wavelength about 530 nm, and the fluorescence intensity was measured with a flow cytometry.

2.3. Assay of chlorophyll content

Algal cell pellets were obtained by centrifugation at 8000*g*, and used to extract chlorophylls by resuspending in 80 % acetone of 3 mL. After extracting for 24 h in darkness, the solution was centrifugated, and the supernatant was used to measure the chlorophyll content by recording the absorption at 649 and 665 nm, according to the previous procedure (Zheng et al., 2020).

2.4. Chlorophyll fluorescence measurement

After centrifugation, the obtained *M. flos-aquae* about 1×10^7 cells were resuspended in 10 μL BG11 medium. Then, they were used to prepare a test spot by dropping onto a small piece of filter paper, with the area about 1 cm^2. After dark-adaptation for 15 min, the chlorophyll fluorescence of the algal cells was measured by using a chlorophyll fluorescence analyzer, and the related parameters about photosystem II (PSII) efficiency were evaluated according to the previous methods (Peng et al., 2020), including maximum quantum yield for photochemistry (φPo), quantum yield from electron transport ($t = 0$) (φEo), photosynthetic performance index (PIabs) and maximum quantum yield for non-photochemical deexcitation (φD_O).

2.5. Determination of astaxanthin and the two precursor levels

According to the protocol of chlorophyll extraction, 80 % acetone was used to extract astaxanthin, β-carotene and zeaxanthin from *M. flos-aquae*. Their levels were determined with a high-performance liquid chromatography. The mixed solution of methanol, dichloromethane and acetonitrile (10: 20: 70) was used as the mobile phase. The extracting solution of 10 μL was used for the analysis, and the compounds were separated in a C18 column and determined with a detector at 450 nm. The standards of the three compounds were used to draw the standard curves for calculating the corresponding compound content (μg·10^{-8} cells) (Zhou et al., 2022). The astaxanthin yield (μg·L^{-1}) after 6 days was evaluated by astaxanthin content × cell density × 1000.

2.6. Analysis of astaxanthin synthesis-associated gene expression

For astaxanthin synthesis-associated genes (*Z-ISO*, *crtP*, *crtQ*, *cruP*, *crtH*, *crtR*, and *crtO*), their expression levels after 6 days were determined with the quantitative real-time PCR (qRT-PCR) method as described in previous study (Huang et al., 2024). A RNA extraction kit was applicated to extract the total RNA from *M. flos-aquae* cells. After reverse transcription into cDNA, the gene expression levels were evaluated through qRT-PCR with referring to *16 s rRNA* gene expression. The primer sequences were supplied in Supplementary Table 1. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the gene expression levels.

2.7. Statistical analysis

The differences among the treatments were analyzed with Origin 8.5 through one-way ANOVA. To assess the correlation of astaxanthin content with other indexes under salt stress, the Pearson's correlation analysis was performed to evaluate the correlation coefficient of each two indexes after 6 days. The relationship of astaxanthin yield with other indexes after 6 days was analyzed through a Mantel test (Zuo et al., 2024), and the ChiPlot (https://www.chiplot.online/) was used to draw the correlation heat map.

3. Results and discussion

3.1. Influence of the two salt stresses on M. Flos-aquae growth

In 100 mM NaCl treatment, *M. flos-aquae* cell growth was not significantly influenced. However, the cell density was significantly ($P < 0.05$) declined in the treatments with 200 and 300 mM NaCl. After 6 days, the cell density was declined by 12.4 % and 19.3 %, respectively (Fig. 1A). KCl stress (except 100 mM at the 2nd day) significantly ($P < 0.05$) inhibited *M. flos-aquae* growth, and the cell density was decreased by 5.5 %, 14.7 % and 23.7 %, respectively, under 100, 200 and 300 mM after 6 days (Fig. 1B).

The impacts of salt stress on cell growth were also found in other algae. For NaCl stress, it can inhibit the growth of *H. pluvialis*, *H. lacustris*, *C. zofingiensis* and *Dunaliella* sp. (Bamary & Einali, 2023; Li et al., 2021; Mao et al., 2020; Zharova et al., 2022), while KCl stress was able to inhibit the growth of *C. sorokiniana*, *C. vulgaris*, *Chlamydomonas reinhardtii* and *Chlorococcum* sp. (Atikij et al., 2019; Church et al., 2017; Janchot et al., 2019; Srivastava et al., 2017). Meanwhile, the inhibitory effects gradually enhanced with raising the two salt concentrations. For *M. aeruginosa*, its growth was inhibited by NaCl ≥100 mM and KCl ≥ 50 mM (Huang et al., 2024). However, 100 mM NaCl did not markedly impact *M. flos-aquae* cell growth, and 100 mM KCl showed inhibitory effect after 4 days (Fig. 1A, B), suggesting that *M. flos-aquae* may have stronger tolerance to salt stress in contrast to *M. aeruginosa*.

3.2. Impacts of the two salt stresses on M. Flos-aquae photosynthetic abilities

Chlorophylls are crucial photosynthetic pigments in algae, of which levels are sensitive to stress conditions, e.g., the chlorophyll levels in *C. sorokiniana*, *D. tertiolecta*, *D. salina*, *Tetraselmis tetrathele* and *M. aeruginosa* gradually declined with enhancing NaCl stress (Tammam et al., 2011; El-Kassas and El-Sheekh, 2016; Srivastava et al., 2017; Huang et al., 2024). When *M. flos-aquae* cells were treated with 200 and 300 mM NaCl, the significant ($P < 0.05$) reduction was also detected in the chlorophyll levels (Fig. 2A). After 6 days, it was reduced by 6.0 % and 8.7 %, respectively. The similar significant ($P < 0.05$) decrease was also detected in the treatments with 100, 200 and 300 mM KCl, and the chlorophyll content was declined by 5.2 %, 8.4 % and 12.2 %, respectively, at the 6th day (Fig. 2B). This was similar with previous findings in *Scenedesmus* sp., *Ulva lactuca*, *Chlorococcum* sp. and *M. aeruginosa* under KCl stress (Aburai et al., 2015; El-Adl et al., 2021; Huang et al., 2024; Janchot et al., 2019). With respect to NaCl stress, KCl stress exhibited stronger lowering effect on chlorophyll content in *C. sorokiniana*, *U. lactuca* and *M. aeruginosa* (El-Adl et al., 2021; Huang et al., 2024; Srivastava et al., 2017). In this study, the similar stronger lowering effect was also found under KCl stress (Fig. 2A, B).

φPo reflects PSII maximum photochemical quantum yield, and is always used as a typical indicator of photosynthetic ability (Xu et al., 2022). In the treatments with NaCl at 200 and 300 mM for 6 days, the φPo was significantly ($P < 0.05$) decreased by 6.1 % and 8.9 %, respectively (Fig. 2C), while it was significantly ($P < 0.05$) decreased by 6.9 %, 9.2 % and 11.9 %, respectively, in the treatments with KCl at 100, 200 and 300 mM for 6 days (Fig. 2D). φEo indicates the quantum yield from electron transport during light reaction, and PIabs reflects the ability of solar energy absorbing, excitation energy trapping and electron transporting (Peng et al., 2020). In the treatments with NaCl and KCl, the variations of the two parameters were consistent with φPo (Fig. 2E-H). Similarly, NaCl stress showed declining effect on the photochemical quantum production and transfer in *C. zofingiensis*, *H. lacustris* and *M. aeruginosa* (Huang et al., 2024; Mao et al., 2020; Zharova et al., 2022), and KCl stress also showed the decreasing effect in *Micrasterias denticulate* and *M. aeruginosa* (Affenzeller et al., 2009; Huang et al., 2024). Meanwhile, the declining effect gradually enhanced with raising the two salt concentrations.

In photosynthesis, chlorophylls absorb light energy and transform it to electric energy in the reaction centers. Both the two stresses caused the decrease of chlorophyll content in *M. flos-aquae*, which was not beneficial to the light energy absorption and electric energy formation, leading to the decline of φPo, φEo and PIabs (Fig. 2C-H). For the absorbed light energy, salt stress exhibits inhibitory effect on its transport and transformation to electric energy, of which the unutilized part is dissipated as heat (Sun et al., 2021). The heat dissipation (φDo) of *M. flos-aquae* also increased in the treatments with NaCl (except 100 mM) and KCl (Fig. 2I, J), which may also lead to the decrease of PSII efficiency and photochemical activity. The decrease of chlorophyll levels and PSII efficiency demonstrated that the photosynthetic abilities in *M. flos-aquae* were declined under the two salt stresses, which should lead to the inhibitory effects on the algal growth.

3.3. ROS accumulation under the two salt stresses

In algae, ROS are mainly generated from the photosynthetic and respiratory electron transport chains as the by-products, and abiotic and biotic stresses can promote their production by affecting the normal electron transport, resulting in electron leakage to form ROS (Li et al., 2021; Zheng et al., 2020). In NaCl treatment, ROS accumulation was detected in *M. denticulate*, *H. pluvialis* and *M. aeruginosa* (Affenzeller et al., 2009; Huang et al., 2024; Li et al., 2021), and the accumulation levels exhibited positive correlation with NaCl concentration (Huang et al., 2024; Tammam et al., 2011). The treatments with 200 and 300 mM NaCl significantly ($P < 0.05$) increased *M. flos-aquae* ROS levels, and raised the ROS to the highest level at the 2nd day, with the increase of 2.2 and 5.0 folds, respectively (Fig. 3A). To lower oxidative damage, the cells can increase antioxidant enzyme activity to quench the ROS (Ma et al., 2019). Under 200 and 300 mM NaCl stresses for 2 days, the significant ($P < 0.05$) increase was found in the activity of superoxide

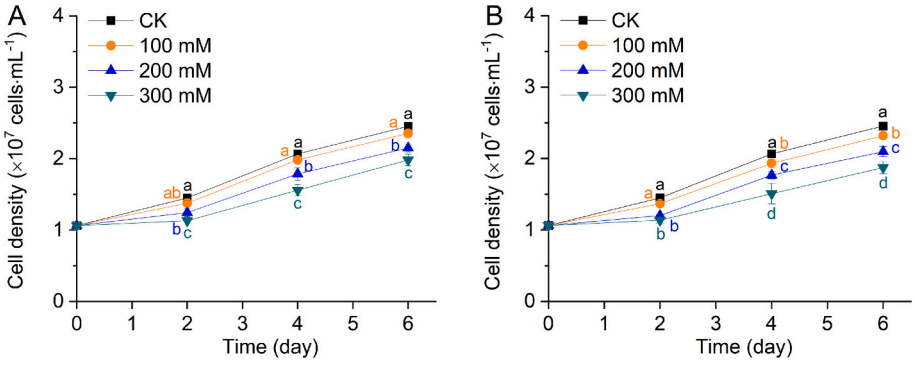

Fig. 1. Effects of NaCl (A) and KCl (B) stresses on *M. flos-aquae* cell growth. CK: The control, without NaCl or KCl treatment. Different lowercase letters indicate the differences at $P < 0.05$. Means ± SE ($n = 4$).

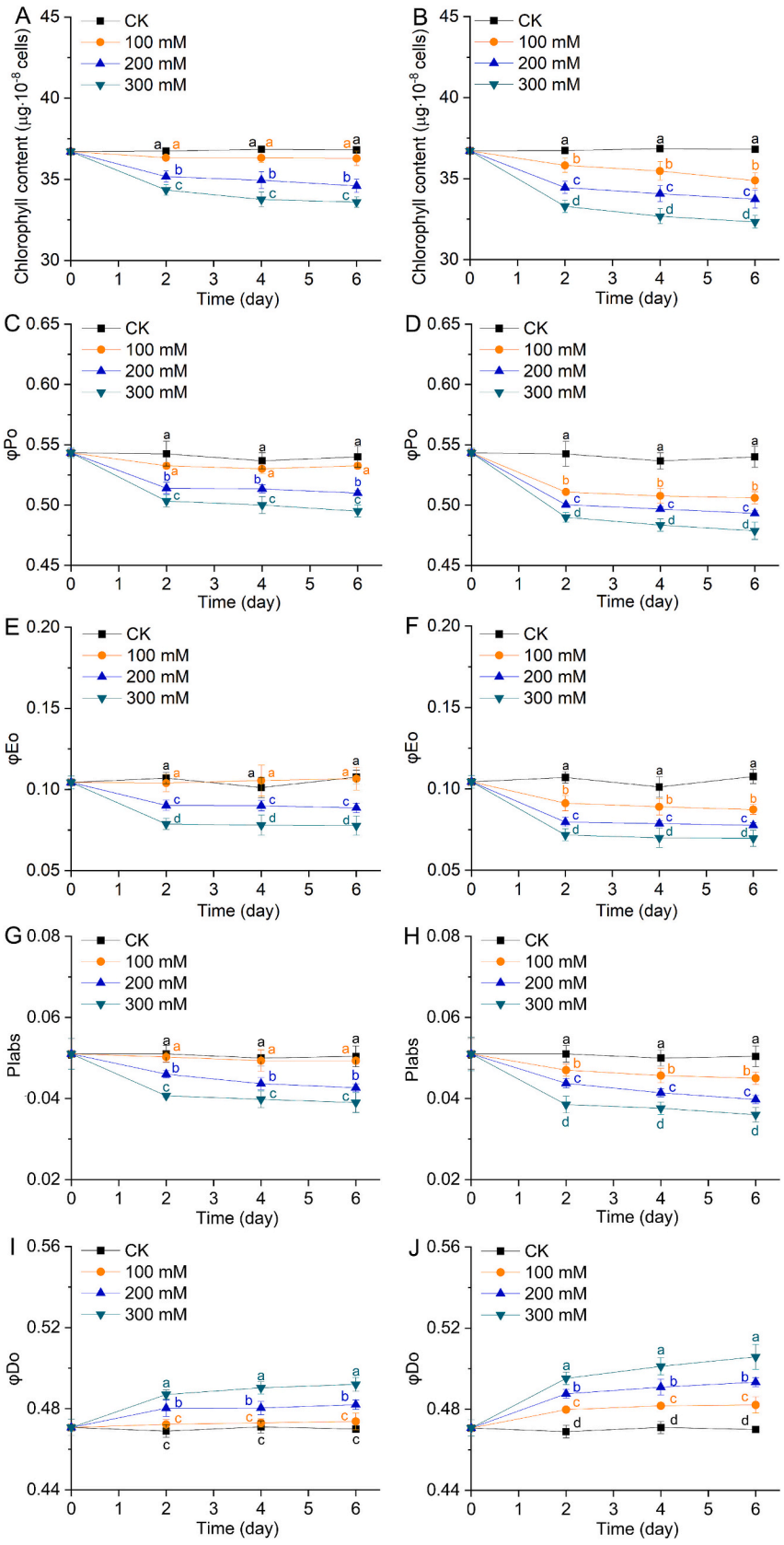

Fig. 2. Effects of NaCl (A, C, E, G and I) and KCl (B, D, F, H and J) stresses on *M. flos-aquae* photosynthetic abilities. CK: The control, without NaCl or KCl treatment. A and B: Chlorophyll content; C and D: Maximum quantum yield for photochemistry (φPo); E and F: Quantum yield from electron transport (φEo); G and H: Photosynthetic performance index (PIabs); I and J: Maximum quantum yield for non-photochemical deexcitation (φD_O). Different lowercase letters indicate the differences at $P < 0.05$. Means ± SE (n = 4).

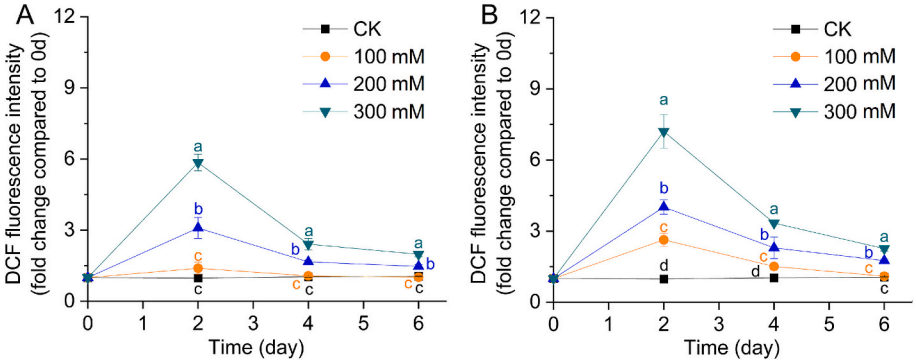

Fig. 3. Effects of NaCl (A) and KCl (B) stresses on the reactive oxygen species levels in *M. flos-aquae*. DCF: 2′,7′-Dichlorofluorescein. CK: The control, without NaCl or KCl treatment. Different lowercase letters indicate the differences at $P < 0.05$. Means ± SE (n = 4).

dismutase (SOD) (Supplementary Fig. 1 A).

Under KCl stress, the ROS was also significantly ($P < 0.05$) accumulated in *M. flos-aquae* cells, with the increase of 1.7, 3.1 and 6.3 folds, respectively, at 100, 200 and 300 mM after 2 days (Fig. 3B). This was consistent with previous findings in H_2O_2 accumulation in *C. reinhardtii* and *M. denticulate* treated with KCl (Darehshouri and Lütz-Meindl, 2010; Vavilala et al., 2016), as well as ROS accumulation in *M. aeruginosa* stressed by KCl (Huang et al., 2024). Meanwhile, the ROS levels were positively related with KCl stress intensity (Huang et al., 2024; Yao et al., 2010). In addition, the SOD activity was also markedly increased under KCl stress for 2 days (Supplementary Fig. 1B).

For *M. aeruginosa* and *Chenopodium album*, NaCl and KCl stresses showed remarkable differences in inducing ROS generation, with higher levels under KCl stress (Huang et al., 2024; Yao et al., 2010). KCl stress also exhibited stronger inducing effect on ROS generation compared with NaCl stress (Fig. 3), which might be caused by the impact differences of K^+ and Na^+ salt stresses on the physiological activities, especially associated with ROS production (Huang et al., 2024; Sun et al., 2021; Yao et al., 2010). The massively accumulated ROS can cause oxidative degradation of photosynthetic pigments (Petrov et al., 2015; Zheng et al., 2020), which may cause the reduction of chlorophyll content in *M. flos-aquae* stressed with the two salts. The oxidative damages of ROS on membrane lipids, photosynthetic systems, proteins and DNA (Petrov et al., 2015; Zheng et al., 2020) may also contribute to the low photosynthetic abilities and cell growth of *M. flos-aquae* stressed by the two salts. With respect to NaCl stress, KCl stress exhibited stronger impacts on the cell growth and photosynthetic abilities at the same concentration, which might result from the higher ROS levels under this stress.

3.4. Astaxanthin accumulation under the two salt stresses

In algae, β-carotene is considered as the direct precursor for astaxanthin biosynthesis, while zeaxanthin is an intermediate product in the process (Zhou et al., 2022). In the treatments with NaCl (except 100 mM) and KCl, the content of the two compounds was significantly ($P < 0.05$) declined in *M. flos-aquae* cells, and the two salts at 300 mM showed the maximum declining effect. At the 6th day, β-carotene and zeaxanthin content was declined by 16.9 % and 13.4 % in 300 mM NaCl treatment, respectively, and declined by 22.9 % and 19.9 % in 300 mM KCl treatment, respectively (Fig. 4A-D). For *M. aeruginosa*, the content of the two compounds gradually decreased with elevating the temperature and light intensity (Huang et al., 2023), but gradually raised with increasing NaCl and KCl concentration (Huang et al., 2024). Moreover, purple light also showed promoting effect on the formation of the two compounds in *M. aeruginosa* (Zhou et al., 2022). These results demonstrate that the changes of β-carotene and zeaxanthin levels depend on the algal species and stress condition.

In NaCl and KCl treatments, the astaxanthin content in *M. flos-aquae* gradually increased with raising the two salt concentrations. When *M. flos-aquae* was treated with 300 mM NaCl and 300 mM KCl for 6 days, the astaxanthin content was significantly ($P < 0.05$) increased by 37.4 % and 62.0 %, respectively, (Fig. 4E, F), and the astaxanthin yield was significantly ($P < 0.05$) increased by 6.2 % and 9.9 %, respectively (Fig. 4G, H). Similarly, NaCl stress also raised astaxanthin content in *H. pluvialis*, *C. zofingiensis*, *D. salina* and *M. aeruginosa* (Chen et al., 2022; Huang et al., 2024; Li et al., 2022; Mao et al., 2020), and there was a positive relationship between the salt concentration and astaxanthin content (Huang et al., 2024; Ramamoorthy et al., 2022). For KCl stress, it improved astaxanthin levels in *Chlorococcum* sp. and *M. aeruginosa* cells (Huang et al., 2024; Janchot et al., 2019).

Under normal condition, the astaxanthin content in *M. aeruginosa* was about 0.52 $\mu g \cdot 10^{-8}$ cells, and it reached to the high levels of 0.85 and 0.96 $\mu g \cdot 10^{-8}$ cells, respectively, in 200 mM NaCl and KCl treatments for 6 days, due to 300 mM salt stress causing the cell death (Huang et al., 2024). For *M. flos-aquae*, the astaxanthin content was about 1.04 $\mu g \cdot 10^{-8}$ cells under normal condition, and it can reach to 1.42 and 1.68 $\mu g \cdot 10^{-8}$ cells, respectively, in 300 mM NaCl and KCl treatments for 6 days (Fig. 4E, F). These indicate that *M. flos-aquae* can accumulate more astaxanthin in the cells with strong salt tolerance, and has stronger potential for astaxanthin production.

Compared with NaCl stress, KCl stress exhibited stronger promoting effects on astaxanthin content and yield in *M. flos-aquae* at the same concentration (Fig. 4E-H). This was consistent with the higher astaxanthin levels in *M. aeruginosa* stressed by KCl than by NaCl (Huang et al., 2024). In high light and temperature treatments, *M. aeruginosa* synthesized massive astaxanthin that was transformed from the precursor and intermediate products, leading to the reduction of β-carotene and zeaxanthin levels (Huang et al., 2023). In the present study, the two salt treatments also promoted β-carotene and zeaxanthin transforming into astaxanthin, resulting in the low precursor content (Fig. 4A-D) and high product content (Fig. 4E, F). Compared with NaCl stress, KCl stress may be more effective for promoting the transformation, which led to the lower β-carotene and zeaxanthin content and higher astaxanthin content.

3.5. Up-regulation of related genes under the two salt stresses

In algae, phytoene desaturase, ζ-carotene isomerase and desaturase, as well as prolycopene isomerase are four crucial enzymes in catalyzing the formation of all-trans-lycopene from phytoene, and their encoded genes are *crtP*, *Z-ISO*, *crtQ* and *crtH*, respectively (Fig. 5A) (Huang et al., 2023; Zhou et al., 2022). In the treatment with NaCl, the up-regulation of *crtP* was found in *U. prolifera*, and there was a positive correlation between NaCl stress intensity and the gene expression level, due to up-regulation of the transcription factor (He et al., 2022). Similarly, *crtP* up-regulation was also detected in NaCl and KCl mixed-treatment (Liu et al., 2022), and 200 mM NaCl promoted the astaxanthin formation in

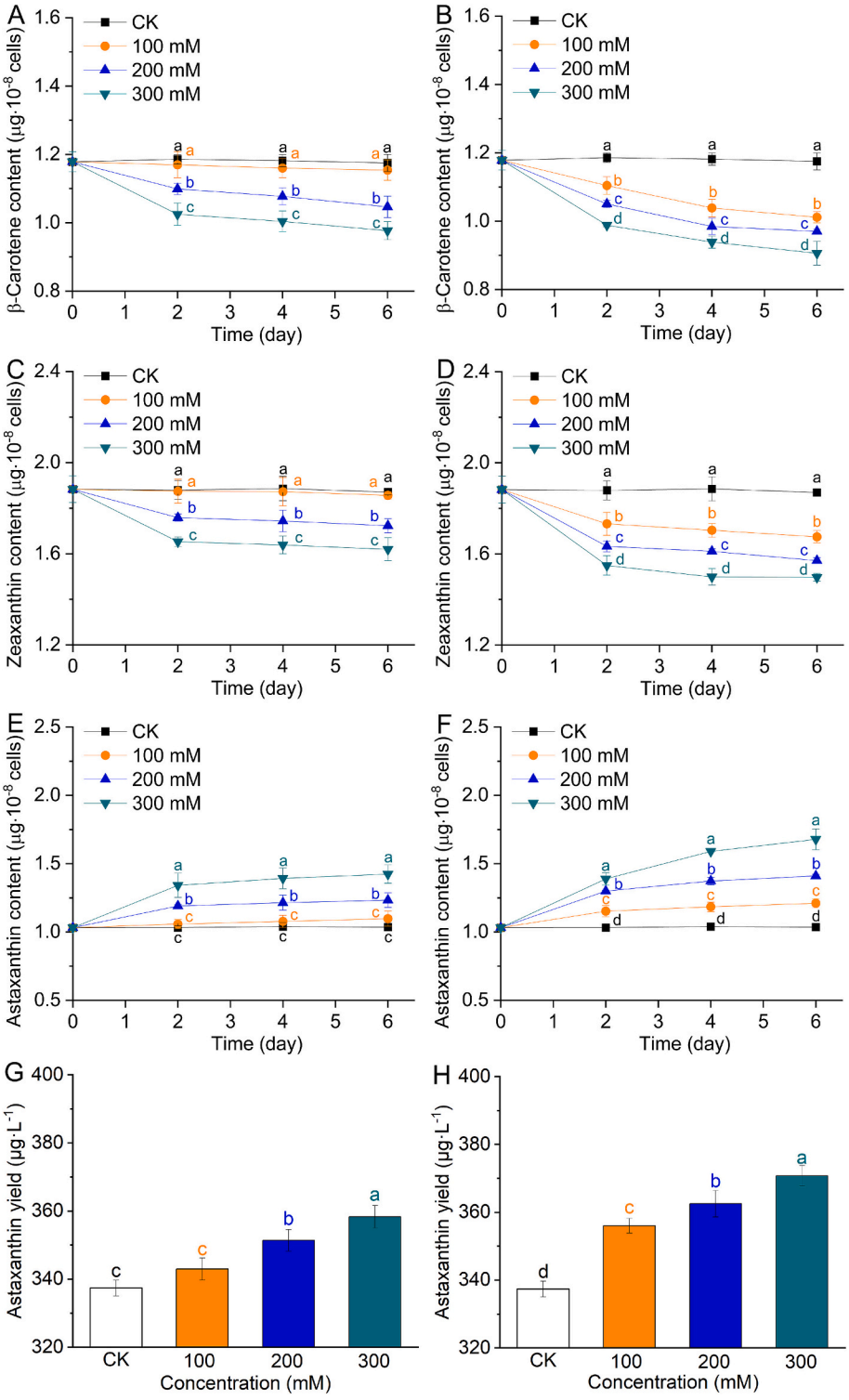

Fig. 4. Effects of NaCl (A, C, E and G) and KCl (B, D, F and H) stresses on astaxanthin and the precursor content in *M. flos-aquae*. G and H: Astaxanthin yield after 6 days. CK: The control, without NaCl or KCl treatment. Different lowercase letters indicate the differences at $P < 0.05$. Means ± SE (n = 4).

C. zofingiensis by raising *Z-ISO*, *crtQ* and *crtH* expression (Mao et al., 2020). When *M. aeruginosa* was stressed by 200 mM NaCl or KCl, the four genes were up-regulated to promote the formation of all-trans-lycopene (Huang et al., 2024). For *M. flos-aquae*, the *crtP*, *Z-ISO*, *crtQ* and *crtH* expression levels were significantly ($P < 0.05$) raised in 300 mM NaCl treatment for 6 days, with the increase of 1.4, 1.0, 1.6 and 1.1 folds, respectively. Similarly, their expression levels were significantly ($P < 0.05$) increased by 2.8, 3.2, 4.7 and 4.9 folds, respectively, in 300 mM KCl treatment (Fig. 5B-E). The raised expression of the four genes under the two salt stresses might be caused by up-regulation of the related transcription factors (He et al., 2022), resulting in the enhancement of the corresponding enzyme activities and promotion of all-trans-lycopene formation.

In catalysis with lycopene cyclase, all-trans-lycopene is converted into β-carotene, the direct precursor of astaxanthin (Fig. 5A) (Huang et al., 2023). *CruP* encoded the enzyme, of which expression levels in

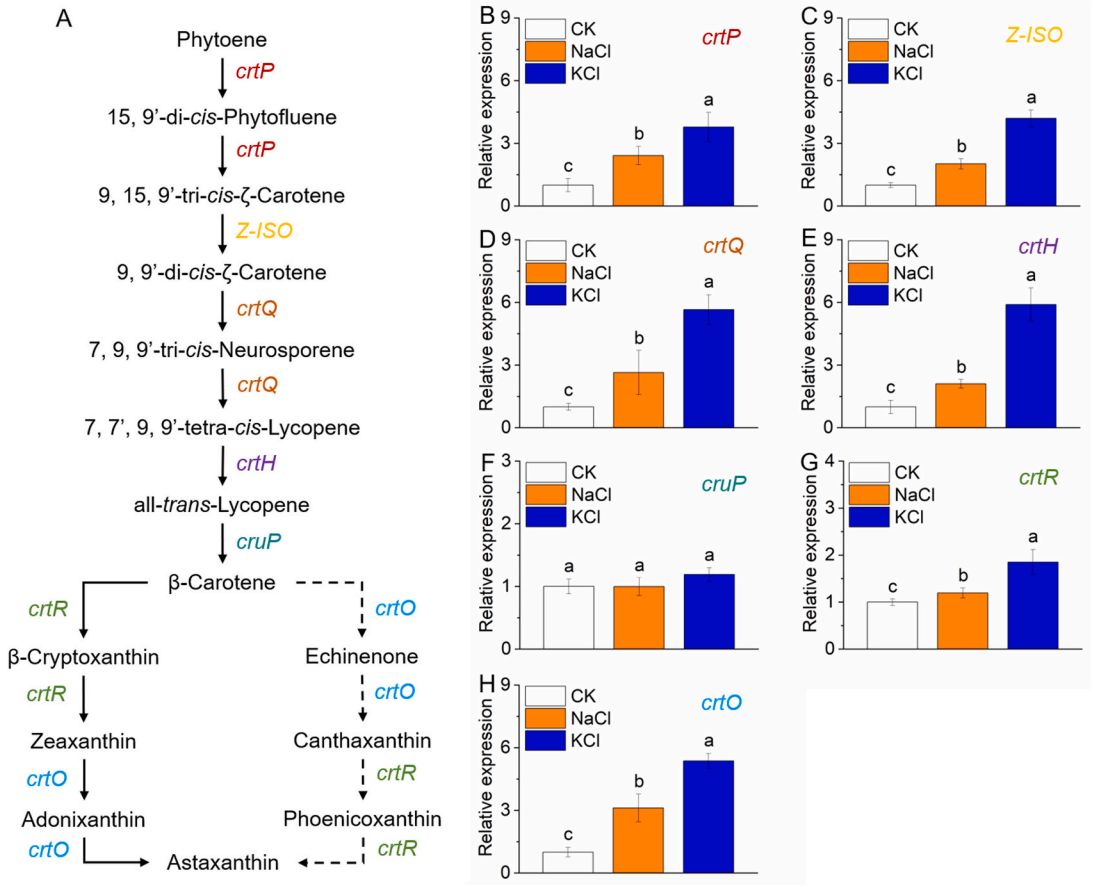

Fig. 5. Gene expression in astaxanthin synthesis in *M. flos-aquae* under 300 mM NaCl and KCl stresses. A: Astaxanthin synthesis pathway; B: *crtP*; C: *Z-ISO*; D: *crtQ*; E: *crtH*; F: *cruP*; G: *crtR*; H: *crtO*. CK: The control, without NaCl or KCl treatment. Different lowercases indicate the significant difference at $P < 0.05$. Means ± SE (n = 4).

N. oceanica, *C. zofingiensis* and *H. pluvialis* were raised under NaCl stress, resulting in promoting β-carotene generation for astaxanthin synthesis (Chen et al., 2022; Li et al., 2022; Liu et al., 2022; Mao et al., 2020). Meanwhile, a positive correlation was found between lycopene cyclase transcription levels and NaCl concentration (Chen et al., 2022). Except of NaCl stress, KCl stress also promoted *cruP* expression in *M. aeruginosa* (Huang et al., 2024). However, 300 mM NaCl and KCl stresses did not impact *cruP* expression in *M. flos-aquae* (Fig. 5F), indicating that *crtP*, *Z-ISO*, *crtQ* and *crtH* may serve main functions in the algal β-carotene synthesis under salt stresses.

In generation of astaxanthin from β-carotene, two enzymes β-carotene ketolase and hydroxylase serve the catalytic functions, and are encoded by *crtO* and *crtR*, respectively. The action order of the two enzymes leads to two pathways of astaxanthin synthesis in algae (Fig. 5A) (Huang et al., 2024; Zhou et al., 2022). In NaCl treatment, *D. salina* (Chen et al., 2022), *C. zofingiensis* (Mao et al., 2020) and *H. pluvialis* (Li et al., 2021) increased expression of the two genes to improve astaxanthin level. For *M. aeruginosa*, both of NaCl and KCl treatments can raise *crtR* and *crtO* expression to increase astaxanthin content (Huang et al., 2024). For *M. flos-aquae*, β-carotene might be successively catalyzed by *crtR* and *crtO* to form astaxanthin, as the intermediate product zeaxanthin was found (Fig. 4C, D), rather than echinenone, canthaxanthin and phoenicoxanthin. Under 300 mM NaCl and KCl stresses, *crtR* and *crtO* were mrakedly ($P < 0.05$) up-regulated by 19.4 % and 2.1 folds, as well as 85.1 % and 4.4 folds, respectively (Fig. 5G, H). The up-regulation of the two genes should increase the corresponding enzyme activities, and then promote massive β-carotene and zeaxanthin to convert into astaxanthin, leading to their low levels in the algal cells (Fig. 4A-D).

Among natural compounds, astaxanthin shows the strongest ability in scavenging ROS, and its biosynthesis can be triggered by the ROS to lower the oxidative damages to the cells (Janchot et al., 2019). In exposure to H_2O_2, the accumulation of astaxanthin was found in *Rhodotorula glutinis* by increasing the related gene expression, such as *cruP* and *crtP* (Zhao & Li, 2023). H_2O_2 also improved astaxanthin levels in *Xanthophyllomyces dendrorhous* by increasing the expression of *crtR* (Torres-Haro et al., 2024). The ROS levels in *M. flos-aquae* cells were massively increased under NaCl and KCl stresses, which may induce astaxanthin synthesis-related gene expression (except *cruP*) to improve the corresponding enzyme activities, resulting in the promotion on the compound biosynthesis and accumulation (Fig. 5B-H).

Compared with NaCl stress, KCl stress caused more ROS accumulation (Fig. 3), which might induce higher expression levels of the astaxanthin-synthesis genes (Fig. 5) to form more astaxanthin (Fig. 4E-H) for quenching ROS and lowering oxidative damage. When *M. aeruginosa* was treated with NaCl and KCl, the higher astaxanthin-synthesis gene expression and the compound accumulation was found under KCl stress (Huang et al., 2024), indicating that there are ionic differences between Na^+ and K^+ in causing astaxanthin accumulation in algae, with stronger effect under K^+ stress. However, the specific difference mechanism of Na^+ and K^+ in inducing astaxanthin accumulation is still needed further investigation from the stress signaling transduction, transcriptional control and compound conversion by using the transgenic lines. Meanwhile, other conditions such as osmotic stress should be also considered, as salt stress always cause osmotic stress. The revelation of the difference mechanism is not only beneficial to uncovering the ionic differences in regulating astaxanthin biosynthesis, but also promotes the application of suitable condition for improving astaxanthin yield.

3.6. Correlation of astaxanthin accumulation with other indexes and environmental conditions

M. flos-aquae cell growth, photosynthesis, ROS levels, astaxanthin accumulation and related gene expression showed similar responses to NaCl and KCl stresses. Then, the correlation of each two indexes (except astaxanthin yield) was analyzed by using the Pearson's correlation analysis without distinguishing the two salts. The salt concentration, astaxanthin content and ROS levels were positively related with *crtP*, *Z-ISO*, *crtQ*, *crtH*, *crtR* and *crtO* expression levels, but negatively related with β-carotene and zeaxanthin content. A positive correlation was found among salt concentration, astaxanthin content and ROS levels. Moreover, the expression levels of *crtp*, *Z-ISO*, *crtQ*, *crtH*, *crtR* and *crtO* were negatively related with β-carotene and zeaxanthin content (Fig. 6A, the correlation coefficients in Supplementary Table 2). With distinguishing the salt types, the similar correlation was also detected under NaCl and KCl stresses (Supplementary Fig. 2). These results suggest that the salt concentration determines the ROS levels, and then the ROS induce the 6 astaxanthin-synthesis gene expression to promote β-carotene and zeaxanthin transforming to astaxanthin, leading to astaxanthin accumulation and the precursor content reduction.

According to the Mantel test results, salt concentration and astaxanthin content showed the strongest correlations with astaxanthin yield under KCl stress, but showed weak correlations under NaCl stress. For *crtP*, *Z-ISO*, *crtQ*, *crtH*, *crtR* and *crtO*, they showed the similar strong correlations with the astaxanthin yield under the two salt stresses. For cell density, ROS levels, β-carotene content and zeaxanthin content, they weakly impacted the astaxanthin yield under the two slat stresses. Under NaCl stress, the effects of cell density and zeaxanthin content on astaxanthin yield were weaker in contrast to KCl stress (Fig. 6A, the Mantel test results in Supplementary Table 3). These results demonstrated that salt concentration and astaxanthin content were two crucial factors for astaxanthin production by using KCl stress.

Temperature and light quality are two important environmental factors for algal growth, which can affect astaxanthin accumulation in algae (Huang et al., 2023; Pereira & Otero, 2020). In previous studies, *M. aeruginosa* increased astaxanthin content under high temperature, with the maximum increasing effect at 35°C (Huang et al., 2023). Among different light qualities, purple light was able to promote *M. aeruginosa* accumulating astaxanthin (Zhou et al., 2022). For *M. flos-aquae*, 300 mM KCl exhibited the maximum effect on astaxanthin accumulation (Fig. 4E-H). To select the optimal condition among 35°C, purple light and 300 mM KCl for astaxanthin production, the compound content and yield were assayed in *M. flos-aquae* cells under the three conditions. High temperature at 35°C and purple light can improve the astaxanthin content in *M. flos-aquae* (Fig. 4E-F, Fig. 6B), which may be caused by up-regulation of the genes involved in the compound biosynthesis, as 35°C (9 genes) and purple light (6 genes) can raise the related gene expression to improve astaxanthin levels in *M. aeruginosa* (Huang et al., 2023; Zhou et al., 2022). Among of the three conditions, the highest astaxanthin content and yield were detected under 300 mM KCl stress. For astaxanthin content, it was 1.5 and 1.5 folds of that under 35°C and purple light, respectively (Fig. 6B), while the astaxanthin yield was 1.2 and 1.4 folds of that under 35°C and purple light, respectively (Fig. 6C). These results suggest that KCl stress is more favorable to improve astaxanthin accumulation than other conditions. Moreover, the astaxanthin content in *M. flos-aquae* stressed by 300 mM KCl under purple light was not significantly higher than that in single 300 mM KCl treatment (Supplementary Fig. 3), indicating that there is no additive effect in inducing astaxanthin accumulation by these conditions.

M. flos-aquae is a typical cyanobacterial species, and can form enormous biomass during water blooms, e.g., the salvaged cyanobacteria with *M. flos-aquae* as the main species from Lake Taihu in China is 1000 tons per day, and the dry biomass of cyanobacteria (*M. flos-aquae* as the main species) is expected to reach to about 500,000 tons in Lake Chaohu in China (Hu et al., 2011; Huang et al., 2023). Up to now, there is still no an effective method to utilize these salvaged cyanobacteria. The content of astaxanthin in *M. flos-aquae* under normal condition was about 1.04 $\mu g \cdot 10^{-8}$ cells (7.9 $mg \cdot g^{-1}$ dry weight (DW)), which can be improved to 1.68 $\mu g \cdot 10^{-8}$ cells (12.8 $mg \cdot g^{-1}$ DW) under 300 mM KCl stress. Although there is no advantage in contrast to the astaxanthin content in *H. pluvialis* (10–50 $mg \cdot g^{-1}$ DW), the cultivation cost can be effectively saved if the salvaged cyanobacteria (mainly *M. flos-aquae*) are used to produce astaxanthin by imposing KCl stress. Seawater contains high concentration of NaCl (about 400 mM) and KCl (about 4.7 mM) (Elahinik et al., 2024), and NaCl and KCl mixed-treatment can

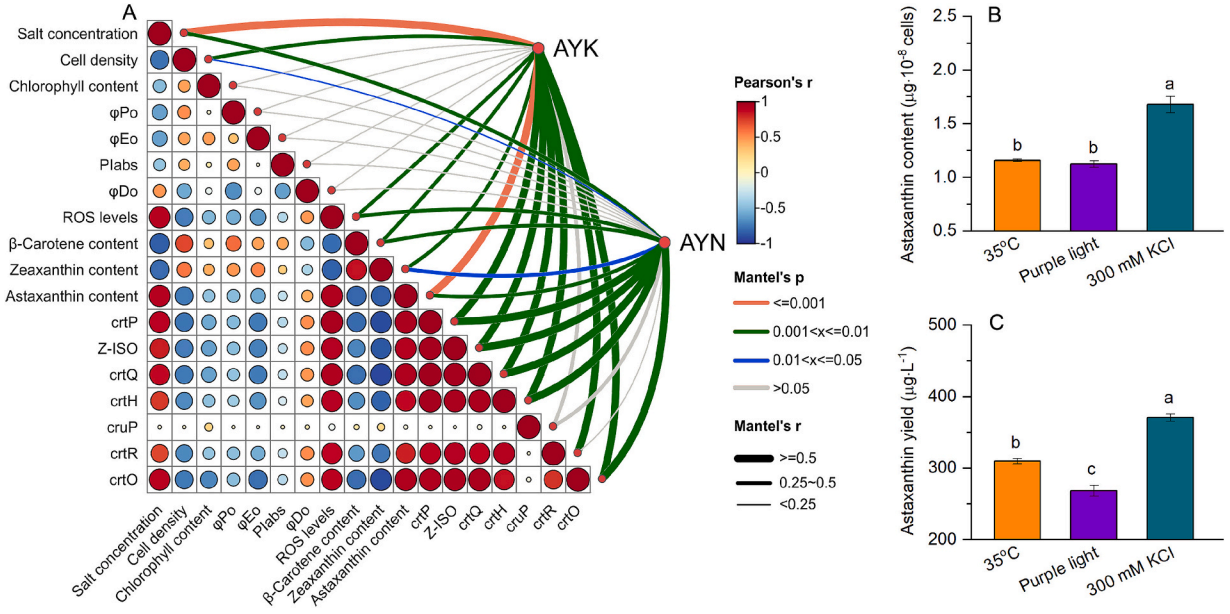

Fig. 6. Correlation of astaxanthin yield with other indexes under the two salt stresses (A) and comparation of astaxanthin content (B) and yield (C) under 35 °C, purple light and 300 mM KCl. AYK: Astaxanthin yield under KCl stress; AYN: Astaxanthin yield under NaCl stress. Different lowercases indicate the significant difference at $P < 0.05$. Means ± SE (n = 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enhance astaxanthin accumulation in *N. oceanica* (Liu et al., 2022), indicating that seawater has a potential for treating the salvaged cyanobacteria to improve the astaxanthin content for further saving cost. In addition, this treatment can be carried out in outdoor small ponds for large-scale application as an economic way, which is not only beneficial to improving astaxanthin yield, but also resolving the utilization problem of salvaged cyanobacteria to avoid environmental pollution.

4. Conclusion

Both of NaCl and KCl stresses can improve astaxanthin content and yield in *M. flos-aquae*, which resulted from the up-regulation of 6 genes in the compound biosynthesis, leading to the reduction of β-carotene and zeaxanthin levels for promoting their transformation. With raising the two salt concentrations, astaxanthin was gradually accumulated and reached to the maximum level at 300 mM. KCl stress was more effective in improving astaxanthin yield than NaCl stress, which was strongly related with the salt concentration and astaxanthin content. Moreover, 300 mM KCl stress was more favorable to astaxanthin production in contrast to other potential conditions, such as 35°C and purple light.

CRediT authorship contribution statement

Junjie Ma: Writing – original draft, Investigation, Data curation. **Zhehan Yang:** Investigation, Data curation. **Zhuxin Jin:** Investigation. **Lexin Huang:** Investigation. **Yinggang Wei:** Investigation. **Wangbo Chen:** Investigation. **Zhaojiang Zuo:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102442.

Data availability

Data will be made available on request.

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