Two DNA methyltransferase inhibitors (5-azacytidine and zebularine) affected the carotenoid accumulation in two green algae, *Chlamydomonas reinhardtii* and *Dunaliella bardawil*

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Background

DNA methylation especially cytosine methylation catalyzed by cytosine-5 DNA methyltransferases (DNMT) plays an important role in cell growth and secondary metabolism [1, 2]. Much research has been done about treatment with DNA methyltransferase inhibitors in plants [3-7], whereas the effects of DNA methyltransferase inhibitors especially zebularine as a novel and more stable DNMT inhibitor on microalgae are less studied [8]. In this study, we made a comparative study on the effects of two DNA methyltransferase inhibitors (5-azacytidine and zebularine) on cell growth, chlorophyll content, carotenoid accumulation, global 5-methylcytosine content and gene expression pattern in two microalgae, the freshwater *Chlamydomonas reinhardtii* and the salt-tolerant *Dunaliella bardawil.*

Methods

Different concentrations of two DNMT inhibitors (5-azacytidine [5AzaC] and zebularine [Zeb]) were added to the algal cultures at inoculation, and the microalgal growth was monitored. DNA methylation level (5mC %) was determined using the MethylFlash™ Global DNA Methylation (5mC) ELISA Easy Kit. The contents of chlorophylls and carotenoids from *C. reinhardtii* and *D. bardawil* can be detected by colorimetric method. Transcriptome sequencing and quantitative Real-Time PCR (qRT-PCR) were used to analyze the gene expression in *C. reinhardtii* and *D. bardawil* in response to 5AzaC and Zeb [9]. The function of carotenoid cleavage dioxygenase 1 from *C. reinhardtii* (*CrCCD1*) was identified by gene complementation assay in *E. coli*. The volatile compounds of *E. coli* were analyzed by solid phase microextraction - gas chromatography mass spectrometry (SPME-GC-MS) [9].

Results

5-Azacytidine and zebularine affected cell growth of *C. reinhardtii* and *D. bardawil*

As shown in Figure 1A and 1C, the effect of 800 μM 5AzaC on cell growth of *C. reinhardtii* and *D. bardawil* was quite different. 800 μM 5AzaC accelerated the growth of *C. reinhardtii*, but suppressed the cell growth of *D. bardawil* at the late log phase. *C. reinhardtii* exposed to Zeb also showed delayed growth (Figure 1B), while inhibition of cell growth of *D. bardawil* was only observed at high concentration of Zeb (Figure 1D). Interestingly, the 5-AzaC- and Zeb-treated algal samples showed unexpected hypermethylation.

5-Azacytidine and zebularine affected pigment accumulation of *C. reinhardtii* and *D. bardawil*

5AzaC at 100 and 800 μM could enhance the synthesis of photosynthetic pigments in *C. reinhardtii* (Figure 2A), in contrast, *D. bardawil* treated with 100~800 μM 5AzaC had an adverse effect on the accumulation of pigment contents (Figure 2C), which were decreased with the increasing concentrations of 5AzaC. Addition with 10~800 μM Zeb to the *C. reinhardtii* cultures led to a significant reduction in

chlorophyll and carotenoid contents (Figure 2B), whereas high concentration of Zeb (up to 1600 μM) could result in a decrease in pigment contents of *D. bardawil* (Figure 2D).

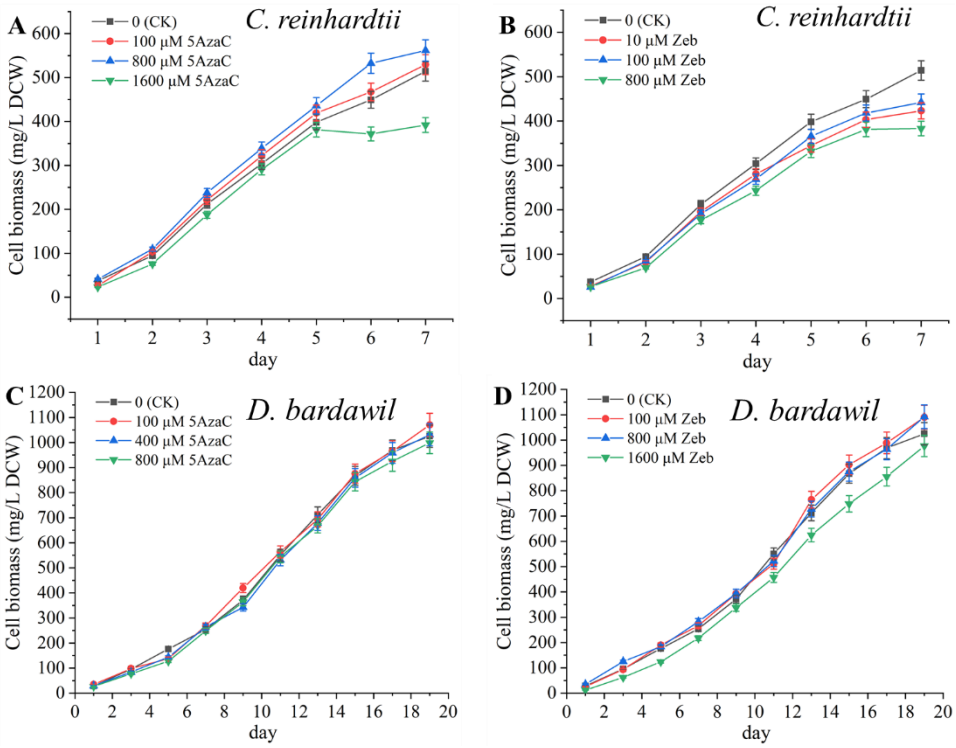


Figure 1 Effects of different concentrations of 5AzaC and Zeb on cell biomass of *C. reinhardtii* and *D. bardawil*. A, treatment with 5AzaC on *C. reinhardtii*; B, treatment with Zeb on *C. reinhardtii*; C, treatment with 5AzaC on *D. bardawil*; D, treatment with Zeb on *D. bardawil.*

Expression of specific genes in response to 5-azacytidine and zebularine in *C. reinhardtii* and *D. bardawil*

800 μM 5-azacytidine enhanced the carotenoid accumulation of *C. reinhardtii*, which may be due to the up-regulation of quite a lot of carotenogenic genes, such as *CrPDS*, *CrCRTISO*, *CrLcyB*, *CrBCH*, and *CrCYP97A*. Notablely, 800 μM zebularine repressed carotenoid accumulation of *C. reinhardtii*, which may be associated with the prominent up-regulation of carotenoid cleavage dioxygenase 1 (*CrCCD1*). In *D. bardawil*, 800 μM 5-azacytidine repressed multiple genes including *DbGGPS*, *DbPSY*, *DbPDS*, and *DbLcyB* responsible for carotenoid metabolism, thus inhibiting the carotenoid accumulation.

Functional identification of CrCCD1

Carotenoid cleavage dioxygenase 1 (*CrCCD1*) from *C. reinhardtii* CC-503 cw92 mt+ (*CrCCD1*) and *Arabidopsis thaliana* (*AtCCD1*) were cloned into the expression vector pET32a to constructed pET-CrCCD1 and pET-AtCCD1. These two expression vectors and pET32a were introduced into *E. coli* BL21 (DE3) strains accumulating β-carotene (carrying pACCAR16Δ*crtX* [10]), and these recombinant strains were named as Ec1-CrCCD1, Ec2-AtCCD1, and Ec0, respectively. The existence of small amounts of β-ionone in the control meant non-enzymatic degradation of β-carotene even occurred at room temperature. But much more β-ionone can be detected in *E. coli* cells overexpressing CrCCD1 and AtCCD1. Obviously, CrCCD1 encoded a functional CCD1 enzyme like AtCCD1, which can cleave the 9,10 (9',10') double bonds of β-carotene into β-ionone (Figure 3). The up-regulation of *CrCCD1* expression may act as a strong contributor to the carotenoid degradation induced by Zeb in *C. reinhardtii*.

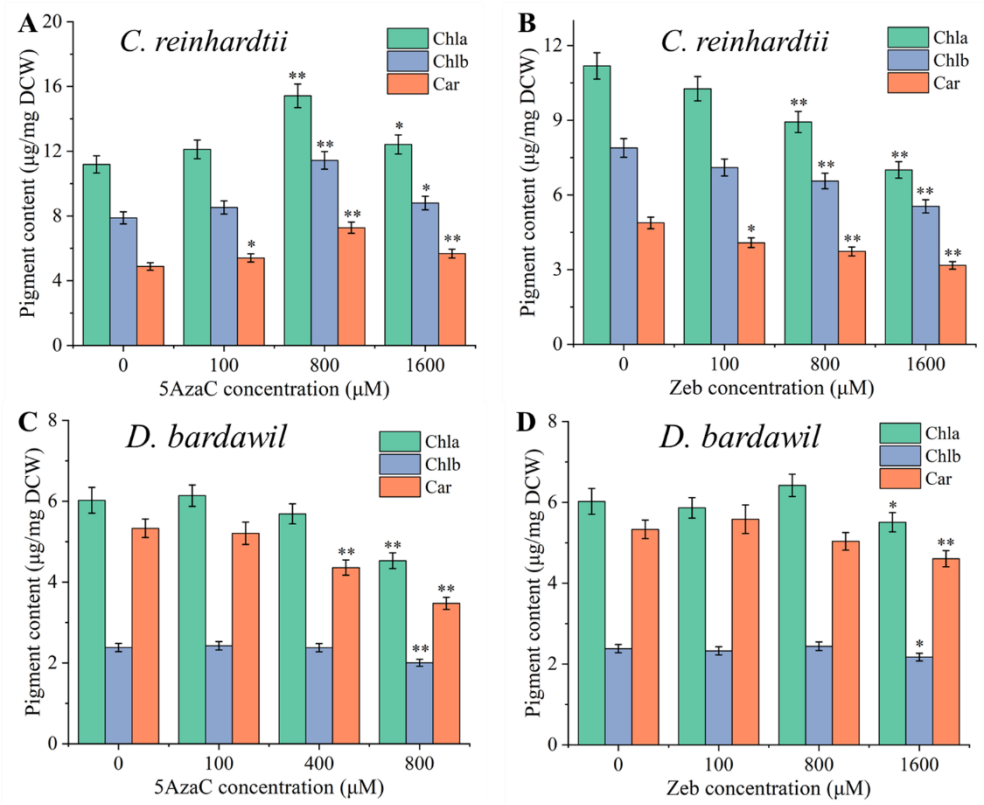
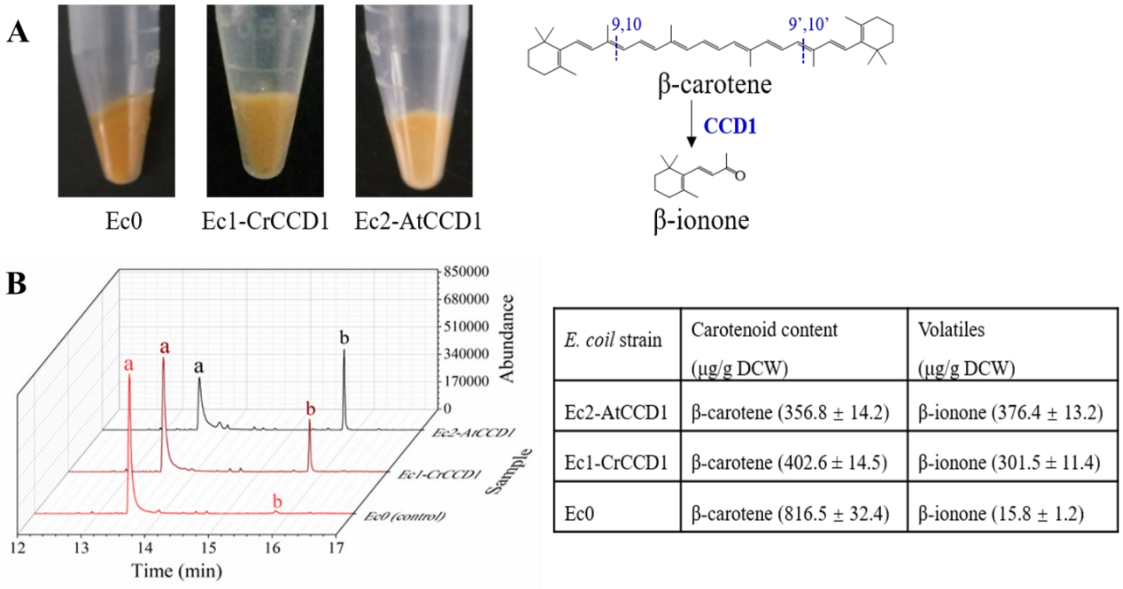


Figure 2 Effects of different concentrations of 5AzaC and Zeb on pigment contents of *C. reinhardtii* and *D. bardawil*. A-B, effects of different concentrations of 5AzaC and Zeb on Chla, Chlb, and carotenoid contents of *C. reinhardtii*; C-D, effects of different concentrations of 5AzaC and Zeb on Chla, Chlb, and carotenoid contents of *D. bardawi*.



E. coil strain	Carotenoid content (μg/g DCW)	Volatiles (μg/g DCW)
Ec2-AtCCD1	β-carotene (356.8 ± 14.2)	β-ionone (376.4 ± 13.2)
Ec1-CrCCD1	β-carotene (402.6 ± 14.5)	β-ionone (301.5 ± 11.4)
Ec0	β-carotene (816.5 ± 32.4)	β-ionone (15.8 ± 1.2)

Figure 3 Functional identification of CCD1s in *E. coli* cells accumulating β-carotene. A, *E. coli* recombinant strains for functional identification of CCD1s; B, SPME-GC-MS analysis of CCD1 activity after transformation in *E. coli* cells accumulating β-carotene. Peak a, indole; peak b, β-ionone.

Conclusion

In conclusion, both 5-Azacytidine and zebularine were found to influence the cell growth, carotenoid accumulation, global 5mC content, and gene expression pattern in *C. reinhardtii* and *D. bardawil*. Moreover, the effect of DNMT inhibitors on cell growth and the biosynthesis of secondary metabolites varied from the dose of DNMT inhibitors and the treated species. All in all, DNA methylation plays an important role in the regulation of carotenoid biosynthesis in microalgae. Furthermore, genome-wide methylome analysis can be used to reveal the correlation between the changes in methylation status and expression levels.

Funding

This work was supported by the National Natural Science Foundation of China (31801468), Guangdong Basic and Applied Basic Research Foundation (2019A1515010656), China Postdoctoral Science Foundation (2020M682717), Guangzhou Basic Research Program (202102020109), and the Fundamental Research Funds for the Central Universities (2018MS89).

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