Postprint of Genomic MSAP Analysis of Astaxanthin Accumulation Process in Haematococcus pluvialis under Nitrogen Deficiency Stress

Authors: Keya Zhang, Qingren Wei, Liu Kehuan, Liu Qianqian, Hou Xingguo, Lan Liqiong, Lan Liqiong

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Abstract

Astaxanthin possesses multiple biological activities, and *Haematococcus pluvialis* represents the optimal natural source of astaxanthin. Nitrogen deficiency stress can induce astaxanthin accumulation in *Haematococcus pluvialis*. This study found that during the 0–72 h period of nitrogen deficiency stress, the growth rate of *Haematococcus pluvialis* decreased; however, astaxanthin accumulation primarily occurred during the 12–24 h period of nitrogen deficiency treatment, after which the accumulation rate slowed. Simultaneously, Methylation Sensitive Amplification Polymorphism (MSAP) analysis was performed on the genomic DNA of *Haematococcus pluvialis* under nitrogen deficiency stress at 0 h, 24 h, and 72 h, yielding a total of 291 methylation polymorphic loci. Among these, loci exhibiting methylation changes accounted for 29.90% and 53.95% of the total loci during the 0–24 h and 24–72 h periods, respectively. At the 24 h time point of nitrogen deficiency stress, the DNA hemimethylation rate reached its maximum (12.71%), while the full methylation rate was at its minimum (26.80%). Conversely, at the 72 h time point of nitrogen deficiency stress, the DNA full methylation rate reached its maximum (28.52%), while the hemimethylation rate was at its minimum (1.72%). These results indicate that alterations in DNA methylation regulatory patterns constitute an important regulatory mechanism in the process of astaxanthin accumulation. This study provides a novel research direction for exploring the molecular mechanisms underlying astaxanthin accumulation in *Haematococcus pluvialis* under nitrogen deficiency conditions.

Full Text

Preamble

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Genome MSAP Analysis of Astaxanthin Accumulation in Haematococcus pluvialis Under Nitrogen Depletion Stress

ZHANG Ke-ya[1], QING Ren-wei[1], LIU Ke-huan[1], LIU Qian-qian[1], HOU Xing-guo[1], LAN Li-qiong[1]*

[1]College of Life Sciences, Sichuan University, Key Laboratory of Bio-Resource and Eco-Environment of Ministry of Education, Chengdu 610065, Sichuan Province, China

Abstract

Astaxanthin possesses multiple biological activities, and *Haematococcus pluvialis* represents the optimal natural source of this compound. Nitrogen depletion stress induces astaxanthin accumulation in *H. pluvialis*. This study found that during 0–72 h of nitrogen depletion, the growth rate of *H. pluvialis* decreased, while astaxanthin accumulation occurred primarily between 12–24 h, after which the accumulation rate slowed. Concurrently, methylation-sensitive amplification polymorphism (MSAP) analysis of genomic DNA from *H. pluvialis* under nitrogen depletion at 0 h, 24 h, and 72 h revealed 291 methylation polymorphic loci. Among these, loci exhibiting methylation changes accounted for 29.90% and 53.95% of total loci during 0–24 h and 24–72 h, respectively. The semi-methylation rate peaked at 12.71% after 24 h of nitrogen depletion, while the full-methylation rate reached its minimum (26.80%) at this time point. Conversely, after 72 h of stress, the full-methylation rate attained its maximum (28.52%) and the semi-methylation rate its minimum (1.72%). These findings demonstrate that altered DNA methylation patterns represent an important regulatory mechanism during astaxanthin accumulation. This study provides a novel research direction for exploring the molecular mechanisms underlying astaxanthin accumulation in *H. pluvialis* under nitrogen deficiency.

Keywords: phycology, nitrogen depletion stress, *Haematococcus pluvialis*, astaxanthin, methylation change

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Author Bio: ZHANG Ke-ya (1991–), female, from Neijiang City, Sichuan Province, Master' s degree candidate, primarily engaged in phycology research, (E-mail) keya_2016@163.com.

Corresponding Author: LAN Li-qiong, Ph.D., Professor, primarily engaged in phycology research, (E-mail) lanlq@scu.edu.cn.

Introduction

Haematococcus pluvialis is a freshwater unicellular green alga belonging to Chlorophyta, Chlorophyceae, Volvocales, Haematococcaceae, and *Haematococcus*. Under adverse environmental conditions such as intense light, high salinity, elevated temperature, and nutrient deficiency, it forms large, thick-walled cysts that accumulate astaxanthin and lose their flagella to become non-motile cells (Duan et al., 2017). Astaxanthin is a xanthophyll carotenoid that appears deep pink and shares a chemical structure similar to -carotene (Martinez-Delgado et al., 2017). It is widely found in shrimp, crabs, fish, and the feathers of certain birds. Beyond its pigmentation function, astaxanthin exhibits strong antioxidant and free radical scavenging capacities, protecting cells from oxidative damage. Astaxanthin exists as three stereoisomers: 3S,3' S; 3R,3' R; and 3R,3' S (corresponding to levorotatory, dextrorotatory, and meso forms, respectively) (Ambati et al., 2014). In *H. pluvialis*, the 3S,3' S isomer predominates (Wan et al., 2015). Liu et al. (2016) demonstrated through in vitro and in vivo experiments that the antioxidant activity of these three stereoisomers follows the order: (3S,3' S) > (3R,3' R) > (3R,3' S). Currently, natural astaxanthin in nature exists primarily in certain algae, yeasts, and bacteria, with *H. pluvialis* representing the optimal source of natural astaxanthin (Zhao et al., 2016). Due to its high biological activity, astaxanthin has become a research focus for investigators and industries including aquaculture, cosmetics, pharmaceuticals, and food.

DNA methylation typically refers to the process by which DNA methyltransferases transfer methyl groups from S-adenosylmethionine (SAM) to the 5-carbon position of cytosine residues in DNA molecules, thereby modifying the genome. Genomic DNA methylation can maintain plant genome stability to help resist environmental stress (Ma et al., 2013) and regulate normal plant growth and development, alter vernalization, promote flowering, and induce transgene silencing (Huang et al., 2009). Methylation-sensitive amplification polymorphism (MSAP) was developed based on amplified fragment length polymorphism (AFLP) (Vos et al., 1995). It utilizes the differential methylation sensitivity of isoschizomers HpaII/MspI to the recognition sequence CCGG, generating different DNA cleavage fragments to reveal methylation sites (Li et al., 2012). HpaII recognizes externally hemimethylated cytosine sites, while MspI recognizes internally fully methylated sites. External cytosine methylation and internal hemimethylation or full methylation cannot be cleaved by either enzyme, whereas unmethylated CCGG sequences can be cleaved by both (Schulz et al., 2013). Based on the different banding patterns produced, the methylation status of genomic DNA can be effectively distinguished. Due to its low cost, high sensitivity, independence from genome sequence, ability to detect numerous loci, and capacity for simultaneous analysis of multiple samples (Xiong et al., 2017), MSAP has been increasingly applied to detect plant genomic DNA methylation levels and shows promising applications in plant epigenetic research.

Current research on *H. pluvialis* focuses on its efficient cultivation (Jaime Fábregas et al., 2001; Zhang et al., 2014; Chen et al., 2005), investigation of astaxanthin accumulation and extraction conditions (Duan et al., 2017; Li et al., 2015), and observation of astaxanthin accumulation structures—plastoglobuli—and cloning of plastoglobule structural protein genes (Fan et al., 2012). However, no reports have documented genome-wide MSAP analysis of *H. pluvialis* during astaxanthin accumulation under nitrogen deficiency, which is crucial for revealing the specific mechanisms of astaxanthin accumulation under such conditions. This study investigated changes in genomic methylation levels during astaxanthin accumulation in *H. pluvialis* under nitrogen depletion using MSAP, providing preliminary insights into the adaptive mechanisms of *H. pluvialis* to nitrogen deficiency. This work offers a theoretical foundation for in-depth study of the mechanisms of astaxanthin accumulation in *H. pluvialis* under nitrogen stress and enriches epigenetic research in algae.

Materials and Methods

1.1 Materials

The *Haematococcus pluvialis* strain was maintained by the Algae Laboratory, College of Life Sciences, Sichuan University.

1.2.1 Culture Method

Cultures were grown in BBM medium with aeration (V_air:V_CO = 98:2) at (22±1)°C under a 12 h:12 h light-dark cycle at 900–1100 lx illumination. For preculture, algae in logarithmic growth phase were inoculated at a 1:10 ratio and aerated until reaching logarithmic phase. For the formal experiment, cultures were transferred at a 1:6 ratio into 1 L Erlenmeyer flasks. When cultures reached logarithmic phase, algae were harvested by centrifugation and transferred to an equal volume of freshly prepared nitrogen-deficient BBM medium for stress treatment. The control group was cultured normally in complete BBM medium.

1.2.2 Cell Counting and Microscopic Observation

Samples were collected from both control and nitrogen-depleted groups at 0 h, 12 h, 24 h, 48 h, and 72 h. Algal cell morphology was observed under microscope and cell numbers were determined using a hemocytometer.

1.2.3 Astaxanthin Content Determination

Samples were collected at the same time points for astaxanthin content measurement using a modified method from Cyanotech Corporation (Zhang & J, 1995). Algal pellets were dried in 15 mL centrifuge tubes, then 1 g quartz sand and 5 mL DMSO were added and incubated at 45–50°C for 30 min, with vortexing for 30 s every 5 min (6 times total). After centrifugation at 3500 rpm for 5 min to pellet cellular material, the supernatant was transferred to a 10 mL volumetric

flask. One milliliter of acetone was added to the pellet, vortexed for 30 s, and centrifuged again at 3500 rpm for 5 min. The supernatant was transferred to the volumetric flask, and acetone extraction was repeated at least 3 times until the supernatant was essentially colorless (absorbance < 0.05). The volume was adjusted to 10 mL with acetone, mixed thoroughly, and 5–10 mL was transferred to a centrifuge tube and centrifuged at 3500 rpm for 5 min to remove particulates carried over from previous steps. Maximum absorbance was measured at 474 nm using acetone as blank. If absorbance exceeded 1.25, samples were diluted with acetone (typically 1:5–1:10) before measurement. Three replicates were performed for each group. Calculations were performed as follows:

Carotenoid mass (mg) = × 10 mL (acetone) × dilution factor

Astaxanthin (%) = × 80%

1.2.4 MSAP Analysis

DNA was extracted from *H. pluvialis* samples at 0 h (pre-stress control), 24 h, and 72 h of nitrogen depletion using the Ezup Column Plant Genomic DNA Kit (Sangon Biotech, Shanghai). The extracted DNA was subjected to digestion, ligation, pre-amplification, selective amplification, and electrophoresis following a modified protocol from Xiong et al. (1999). The adapter sequences, pre-amplification primers, and selective amplification primers used for MSAP analysis are listed in Table 1 . Adapters and primers were synthesized by Sangon Biotech (Shanghai). Restriction enzymes were purchased from Thermo Fisher Scientific, T4 DNA Ligase from TaKaRa, and PCR mix from Chengdu Tsingke Biotechnology.

Table 1 Adapters and primers for MSAP

Primer Type	Name and Sequence (5' -3')
Adapter	
EcoRI (E)	EA1: CTCGTAGACTGCGTACC EA2: AATTGGTACGCAGTCTAC
HpaII/MspI (H/M)	H/M1: GATCATGAGTCCTGCT H/M2: CGAGCAGGACTCATGA
Pre-amplification primer	
E0: GACTGCGTAC-CAATTC	H/M0: ATCATGAGTCCTGCTCGG
Selective amplification primer pairs	
No.10	E+AGC: GACTGCGTACCAATTCAGC / H/M+TCCA: ATCATGAGTCCTGCTCGGTCCA

Primer Type	Name and Sequence (5' -3')
No.11	E+AGA: GACTGCGTACCAATTCAGA / H/M+TAG: ATCATGAGTCCTGCTCGGTAG E+AAA: GACTGCGTACCAATTCAAA / H/M+TAG: ATCATGAGTCCTGCTCGGTAG E+AAC: GACTGCGTACCAATTCAAC / H/M+TAG: ATCATGAGTCCTGCTCGGTAG E+AAG: GACTGCGTACCAATTCAAG / H/M+TAG: ATCATGAGTCCTGCTCGGTAG E+AAT: GACTGCGTACCAATTCAAT / H/M+TAG: ATCATGAGTCCTGCTCGGTAG E+AAC: GACTGCGTACCAATTCAAC / H/M+TAC: ATCATGAGTCCTGCTCGGTAC E+AAG: GACTGCGTACCAATTCAAG / H/M+TAC: ATCATGAGTCCTGCTCGGTAC E+AGC: GACTGCGTACCAATTCAGC / H/M+TAC: ATCATGAGTCCTGCTCGGTAC E+AAC: GACTGCGTACCAATTCAAC / H/M+TTG: ATCATGAGTCCTGCTCGGTTG E+AGG: GACTGCGTACCAATTCAGG / H/M+TTG: ATCATGAGTCCTGCTCGGTTG

Extracted DNA was digested with two enzyme combinations: EcoRI/HpaII and EcoRI/MspI. The digestion reaction (20 L total) contained: EcoRI 10 U, HpaII & MspI 10 U, 10×Tango buffer 4 L, 300 ng DNA sample, and ddH O to 20 L. Digestion was performed at 37°C for 10 h, and efficiency was verified by 1% agarose gel electrophoresis.

Ligation was performed in a 20 L reaction containing: 10 L digestion product, 1 L each of EcoRI adapter (5 M) and HpaII(MspI) adapter (50 M), 350 U T4 DNA Ligase, 2 L 10×T4 DNA Ligase buffer, and ddH O to 20 L. Ligation proceeded at 16°C for 12 h, after which the product was diluted 10-fold for pre-amplification.

The pre-amplification reaction (20 L) included: 1 L each of pre-amplification primers E0 (10 M) and H/M0 (10 M), 5 L of 10× diluted ligation product, 10 L 2×PCR mix, and double-distilled water to 20 L. The amplification program was: 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min; and final extension at 72°C for 10 min. Pre-amplification products were diluted 30-fold for selective amplification.

Selective amplification was performed in a 20 L reaction containing: 1 L each of EcoRI selective primer (10 M) and H/M selective primer (10 M), 5 L of 30× diluted pre-amplification product, 10 L 2×PCR mix, and ddH O to 20 L. The amplification program was: 94°C for 5 min; 12 cycles of 94°C for 30 s, 65°C

(decreasing 0.7°C per cycle) for 30 s, 72°C for 1 min; 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min; and final extension at 72°C for 10 min.

Selective amplification products were denatured, and 2–3 L was loaded onto 6% denaturing polyacrylamide gels. After electrophoresis, gels were silver-stained using a modified method from Gao et al. (2009), then photographed and documented.

For band scoring, HpaII/MspI digestion products were scored as "0" for absence and "1" for presence of bands. The numbers of each band type were tallied and analyzed using Excel 2013.

Results

2.1.1 Growth Curve of Haematococcus pluvialis

The growth of *H. pluvialis* in normal and nitrogen-deficient BBM medium is shown in Figure 1 [Figure 1: see original paper]. Throughout the experiment, algae under normal culture conditions grew vigorously, reaching 13.2×10 cells · mL ^{1} by 72 h. In contrast, the nitrogen-depleted group exhibited reduced growth rate, with cell numbers reaching only 8.7×10 cells · mL ^{1} at 72 h, equivalent to 0.66-fold of the control group.

2.1.2 Microscopic Observation

Microscopic observations are presented in Figure 2 [Figure 2: see original paper]. During 0–24 h of nitrogen depletion, no significant morphological changes were observed in *H. pluvialis*. By 72 h, however, microscopic examination revealed initial astaxanthin accumulation within algal cells.

Figure 2 Microscopy of *Haematococcus pluvialis* under nitrogen depletion stress after 0 h (A), 12 h (B), 24 h (C), 48 h (D), and 72 h (E).

2.2 Astaxanthin Content Changes

Figure 3 [Figure 3: see original paper] illustrates astaxanthin content changes during nitrogen depletion. The nitrogen-depleted group consistently showed higher astaxanthin content than the control throughout the experiment, with a dramatic increase occurring between 12–24 h of stress treatment—the period of maximum accumulation rate. Thereafter, astaxanthin accumulation in the nitrogen-depleted group slowed and stabilized. The greatest difference between nitrogen-depleted and control groups occurred at 72 h, when the nitrogen-depleted group' s astaxanthin content reached 2.64-fold that of the control.

2.3 MSAP Analysis of Haematococcus pluvialis DNA Under Nitrogen Depletion

Eleven selective amplification primer pairs were used for genome-wide methylation analysis of *H. pluvialis* DNA samples (Figure 4 [Figure 4: see original

paper]). M: marker. The figure shows groups of six lanes, with each group representing the same primer combination. Within each group, the first three samples represent DNA from nitrogen-depleted algae at 0 h, 24 h, and 72 h digested with HpaII, while the last three samples represent DNA from the same time points digested with MspI.

Figure 4 Representative MSAP profiles of *Haematococcus pluvialis* DNA under nitrogen depletion for 0, 24, and 72 hours.

After band scoring and classification according to Tang et al. (2014), methylation patterns were categorized as shown in Table 2 .

Table 2 Classification of methylation banding patterns

HpaII/MspI Pattern	Type	Description
I (Non-methylated)	(1,1)	Bands present in both HpaII and MspI digests, indicating no methylation
II (Full-methylation)	(0,1)	Band absent in HpaII but present in MspI, indicating internal cytosine full methylation
III (Semi-methylation)	(1,0)	Band present in HpaII but absent in MspI, indicating external cytosine hemimethylation
IV (Hyper-methylation)	(0,0)	Bands absent in both digests, indicating hypermethylation

Statistical analysis revealed a total of 1,265 bands, from which 291 methylation polymorphic loci were identified. Type I (non-methylated) was most abundant, with 194, 176, and 203 loci at 0 h, 24 h, and 72 h of nitrogen depletion, respectively. Type II showed minimal variation across the three time points. Type III decreased by 86.49% between 24 h and 72 h, representing a significant change. Type IV remained relatively stable, reaching 64 loci at 72 h—the highest number among the three time points.

Methylation rates were calculated by considering Types II and IV as full methylation (Table 3).

Table 3 Methylation rates

Parameter	0 h	24 h	72 h
Total amplified loci	291	291	291
Total methylated loci (II+III+IV)	97	115	88
Full-methylated loci (II+IV)	81	78	83
Total methylation rate	33.33%	39.52%	30.24%
Full-methylation rate	27.84%	26.80%	28.52%
Semi-methylation rate	5.50%	12.71%	1.72%

Note: Methylation rate (%) = [(II+III+IV)/(I+II+III+IV)] × 100; Full-methylation rate (%) = [(II+IV)/(I+II+III+IV)] × 100; Semi-methylation rate (%) = [III/(I+II+III+IV)] × 100

Methylation rate analysis indicated that total methylated loci peaked at 115 (39.52% methylation rate) after 24 h of nitrogen depletion, while the minimum (88 loci, 30.24% methylation rate) occurred at 72 h, representing a 23.48% down-regulation of genome-wide methylation during 24–72 h. Full-methylation loci showed minimal variation across 0 h, 24 h, and 72 h, with rates ranging from 26–28%. Semi-methylation loci numbered 16, 37, and 5 at 0 h, 24 h, and 72 h, respectively, with the semi-methylation rate reaching its maximum (12.71%) at 24 h—an increase of 131.25% compared to 0 h but a decrease of 86.47% compared to 72 h, indicating significant dynamic changes.

Patterns of cytosine methylation and demethylation changes are summarized in Table 4 .

Table 4 Methylation pattern changes

Pattern Change	Type Transition	0→24 h	24→72 h	0→24 h (%)	24→72 h (%)
Methylation	I→II, I→III, I→IV	46	73	15.81%	25.09%
Demethylation	II→I, III→I, IV→I	41	84	14.09%	28.87%
No change	Various	204	134	70.10%	46.05%

Analysis revealed three patterns of cytosine methylation changes under nitrogen depletion: (1) No change in methylation status, with 204 and 134 loci (70.10% and 46.05% of total methylated loci) showing no changes during 0–24 h and 24–72 h, respectively. (2) De novo methylation, with 46 loci (15.81%) methylated during 0–24 h and 73 loci (25.09%) during 24–72 h. (3) Demethylation, with 41 loci (14.09%) demethylated during 0–24 h and 84 loci (28.87%) during 24–72 h. Among the 291 polymorphic loci, 87 (29.90%) underwent methylation or demethylation changes during 0–24 h, while 157 (53.95%) showed such changes

during 24–72 h. These results demonstrate that both methylation and demethylation occurred throughout nitrogen depletion, with more extensive changes occurring during the later experimental period (24–72 h).

Discussion

Nitrogen is an essential and quantitatively dominant element for plant growth and development, playing critical roles in cell structure, division, and expansion, and is therefore termed the "life element" (Chen et al., 2013). Previous studies have shown that nitrogen is the most important factor affecting growth and lipid accumulation in various microalgae (Griffiths et al., 2009). In this study, nitrogen-depleted *H. pluvialis* exhibited reduced growth rate, consistent with observations by Zhuang et al. (2000) who reported "significantly delayed growth in nitrogen-depleted treatment groups 3 days after inoculation." Huang et al. (2009) found that under nitrogen deficiency, *H. pluvialis* accumulated astaxanthin without extensive cyst formation, instead accumulating astaxanthin during the motile cell stage, which aligns with our microscopic observations showing no thick-walled cyst formation at 72 h.

Although nitrogen depletion slowed *H. pluvialis* growth, astaxanthin content continuously increased, indicating that nitrogen deficiency promotes astaxanthin synthesis—a finding consistent with numerous studies (Zhuang et al., 2000; Borowitzka et al., 1991). The underlying mechanism may involve nitrogen depletion-induced increases in intracellular reactive oxygen species (ROS) levels (Mendesferreira et al., 2010), creating oxidative stress that triggers rapid, massive astaxanthin synthesis to scavenge ROS and maintain redox homeostasis (Wang et al., 2012). Additionally, nitrogen deficiency increases lipid body content in certain green algae (Hirooka et al., 2014), and lipid bodies serve as the storage site for astaxanthin in *H. pluvialis* cytoplasm (Kobayashi, 2003), representing another mechanism for astaxanthin accumulation under nitrogen stress.

MSAP results showed that among the four methylation types, Type I (non-methylated) was most abundant, followed by Type IV (hypermethylation). Notably, the semi-methylation rate peaked at 12.71% after 24 h of nitrogen depletion, coinciding with the period of maximum astaxanthin accumulation (12–24 h) and the lowest full-methylation rate (26.80%). This suggests that during the initial phase of nitrogen depletion, astaxanthin accumulation may be predominantly regulated by genomic DNA hemimethylation. At 72 h, when astaxanthin accumulation had slowed, the full-methylation rate reached its maximum, indicating a shift toward full-methylation-mediated regulation.

DNA methylation and demethylation can regulate gene expression and may silence exogenous DNA to maintain genome integrity, enabling normal growth and metabolism and facilitating environmental adaptation (Liu, 2013). Generally, gene methylation suppresses expression, while demethylation enhances transcription and promotes gene expression (Ik et al., 1997). In this study, to

adapt to nitrogen depletion, algal DNA showed more methylation than demethylation events during 0–24 h, suggesting that activated genes (such as those in the astaxanthin synthesis pathway) were outnumbered by suppressed genes. Thus, nitrogen depletion primarily inhibited gene expression. Wang et al. (2015) similarly found in tall fescue that nitrogen stress resulted in higher methylation than demethylation rates, indicating that adaptation to nitrogen deficiency involves not only activating stress-resistance genes but also silencing certain gene expressions. During 24–72 h, demethylation events exceeded methylation events, with demethylation becoming predominant. As astaxanthin accumulation slowed, related genes may have been sequentially activated through demethylation. These findings demonstrate that astaxanthin accumulation under nitrogen depletion is associated with genomic DNA methylation, which regulates gene expression and thereby influences astaxanthin synthesis.

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Note: Figure translations are in progress. See original paper for figures.