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Carotenoid genes transcriptional regulation for astaxanthin accumulation in fresh water unicellular alga *Haematococcus pluvialis* by gibberellin A3 (GA_3)

Zhengquan Gao[1,a], Chunxiao Meng[1,a,*], Hongzheng Gao[1], Yan Li[2], Xiaowen Zhang[3], Dong Xu[3], Shitan Zhou[1], Banghui Liu[1], Yuanfeng Su[1] and Naihao Ye[3,*]

[1]School of Life Sciences, Shandong University of Technology, Zibo 255049, China

[2]School of Marine & Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia

[3]Yellow Sea Fishery Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

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The fresh water unicellular alga *Haematococcus pluvialis* is a promising natural source of astaxanthin. The present study investigated the transcriptional expression of carotenoid genes for astaxanthin accumulation in *H. pluvialis* using real-time fluorescence quantitative PCR (qRT-PCR). With treatments of 20 and 40 mg/L of gibberllin A_3 (GA_3), five genes *ipi*-1, *ipi*-2, *psy*, *pds* and *bkt*2 were up-regulated with different expression profiles. GA20 (20 mg/L of GA_3) treatment had a greater effect on transcriptional expression of *bkt*2 than on *ipi*-1 *ipi*-2, *psy* and *pds* (>4-fold up-regulation). However, GA40 (40 mg/L of GA_3) induced more transcriptional expression of *ipi*-2, *psy* and *bkt*2 than both *ipi*-1 and *pds*. The expression of *lyc*, *crt*R-B and *crt*O for astaxanthin biosynthesis was not affected by GA_3 in *H. piuvialis*. In the presence of GA_3, astaxanthin biosynthesis genes of *ipi*-1, *pds* and *bkt*2 were up-regulated at transcriptional level, *psy* at post-transcriptional level, whereas *ipi*-2 was up-regulated at both levels. The study could potentially lead to a scale application of exogenous GA_3 in astaxanthin production with *H. pluvialis* just like GAs perform in increasing crops production and it would provide new insight about the multifunctional roles of carotenogenesis in response to GA_3.

Keywords: *Haematococcus pluvialis*, Astaxanthin, Gibberellin A3 (GA_3), Carotenoid genes, Real-time fluorescence quantitative PCR

The freshwater unicellular alga *Haematococcus pluvialis* can accumulate natural astaxanthin (3, 3'-dihydroxy-β, β-carotene-4, 4'-dione) up to 4% of dry weight biomass under abiotic stresses. The higher astaxanthin production is likely a self defense mechanism to protect *H. pluvialis* in adverse conditions[1-3]. Therefore, *H. pluvialis* is a promising organism for the biotechnological production of astaxanthin, which has potential in pharmaceutical, aquaculture, nutraceutical and cosmetic industries[4]. It, therefore, provides a good platform to investigate the regulation of astaxanthin biosynthesis genes in *H. pluvialis.*

Plants have a number of mechanisms, including physical and chemical barriers to deal with unfavorable environmental conditions. Inducible defense systems play a key role in counteracting stresses via a complex network of signaling compounds[5]. Gibberellins (GAs) are a class of plant growth regulators that are essential for normal growth and development[6] and play a role in the response of plants to stress. Besides the three traditional regulatory molecules, namely jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), gibberellin A3 (GA_3) has also been shown to function in response to stress in plants[7]. Seven stress-related genes, including genes for HSP82, PBZ1, beta 1, 3-glucanase, chitinase, SAMT (salicylic acid carboxyl methyltransferase, an enzyme involved in plant defense responses mediated by salicylic acid), salt and metallothionein (MT)-like proteins are found to be down-regulated by exogenous GA_3[8].

Lu *et al*[9] found that exogenous GA_3 (2 and 20 mg/L) increases the production of astaxanthin and GA_3 treatments (16 days) increase the transcription of three *bkts* in the WB-1 strain of *H. pluvialis*. Our previous studies have shown that *H. pluvialis* can synthesize and accumulate large quantities of astaxanthin, following exogenous GA_3 treatment[10]. We have also reported that there might be some plant hormone responsive elements, including a GA_3-responsive element (P-box) in the 5'-flanking region of carotenoid genes involved in astaxanthin biosynthesis, such as

*Corresponding authors:
Tel: +0086-5332762265; E-mail: mengchunxiao@126.com (CX Meng)
Tel: 0086-53285830360; E-mail: yenh@ysfri.ac.cn (NH Ye)
[a]These authors equally contributed to this work.

bkt, *crt*O and *ipi*. This suggests that GA_3 could be used as an effective regulator to produce astaxanthin in *H. pluvialis*[11-15].

In order to prove this assumption, this study has been carried out with GA_3 treatment (20 and 40 mg/L) on *H. pluvialis*. We have investigated the expression patterns of eight genes — *ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-B, *bkt2* and *crt*O in combination with astaxanthin accumulation in *H. pluvialis* under GA_3 treatment (20 and 40 mg/L) and have monitored in different physiological stages using real-time PCR. It is anticipated that this study could improve our understanding on molecular regulation mechanism for astaxanthin biosynthesis in *H. pluvialis*.

Materials and Methods

Haematococcus pluvialis strain and cultivation condition

H. pluvialis strain 712 was acquired from the Institute of Oceanology, Chinese Academy of Sciences and preserved in our lab. *H. pluvialis* was cultured in a medium (MCM) using the same cultivation protocols as previously described[16-18].

Stress trial with gibberellin A_3 (GA_3)

When the algal cell reached the logarithmic growth phase, the culture was split into nine aliquots (2000 ml on each), allocated to three treatments of GA_3 (Gibco) with three replicates. GA_3 was first dissolved in ethanol as a stocking solution and then added to the cultures to achieve the experimental concentrations: 20 and 40 mg/L as GA20 and GA40 treatments, respectively. In controls, the similar amount of ethanol was added, instead of GA3 solution. All the cultures were kept in an illuminating incubator (Ningbo Jiangnan Instrument Factory, GXZ-380, Ningbo, China) under a light intensity of 25 μmol photons $m^{-2}s^{-1}$ on a 12 h: 12 h light/dark cycle at 20°C without aeration. All flasks were shaken manually twice at fixed time every day. The 2 ml of algal cells were sampled on days 1, 2, 4, 6, 8, 10, 12, 14 and 16 and preserved for sequent analysis.

Observation of algal cells under microscope and measurement of astaxanthin content

The color change of each sample was observed using a microscope (Nikon Eclipse 80i, Tokyo, Japan) and the proportion of red cells was counted simultaneously. Astaxanthin content was measured using a spectrophotometer (T6 new century, Beijing General Instrument Ltd China) as described previously[16-18]. The major absorption peak in dimethylsulfoxide was at ca. 490 nm and the astaxanthin concentration was calculated according to a formula: C (mg/L) = $(4.5 \times OD_{490} \times Va)/Vb$, where the *Va* and *Vb* represent volumes of dimethylsulfoxide and microalgae samples, respectively. Equal aliquots of culture from each treatment and the control were harvested at different time points and lyophilized. Lyophilized cells were then extracted with dimethylsulfoxide repeatedly until the pellet became colorless.

RNA isolation and RT-PCR

Similar to our previous studies[17,18], the total RNA was extracted from algal cells using TRIzol Reagent (Invitrogen, USA). The gene-specific primers for eight genes (*ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-B, *bkt2* and *crt*O) were designed using Primer 3 software and synthesized by Biosune (China) (Table 1). PCR products were quantified continuously by the ABI StepOne Plus Real-Time PCR System (Applied Biosystems, USA) using SYBR green fluorescence (Takara) according to the manufacturer's instructions. qRT-PCR analysis on these eight genes was performed according to our previous methods[17,18]. The actin gene was used as a reference for calibrating the measurement of total RNA. After the PCR, the data were analyzed using the comparative Ct ($2^{-\Delta\Delta CT}$) method[19].

Statistical analysis

The means ± SD were derived from all data and were statistically analyzed with one-way ANOVA (SPSS 17.0). Fisher's least significance difference (LSD) multiple comparisons tests were adapted to test the significant differences among treatments. *P*-values of less than 0.05 and 0.01 were considered to be statistically significant and highly significant, respectively.

Results

Observation under microscope and measurement of astaxanthin content

Microscopic observation of algal cells showed the initial color changing from green to red in on day 3 after application of GA20 and GA40. On day 4, there were obvious differences between controls and GA groups (Fig. 1a, b, c). On day 16, the percentage of red cells in GA20 was 22.5%, whereas 88.6% was obtained in GA40 (Fig. 1d, e, f). However, about 2.5% of the algal cells were faded in the GA40 treatment.

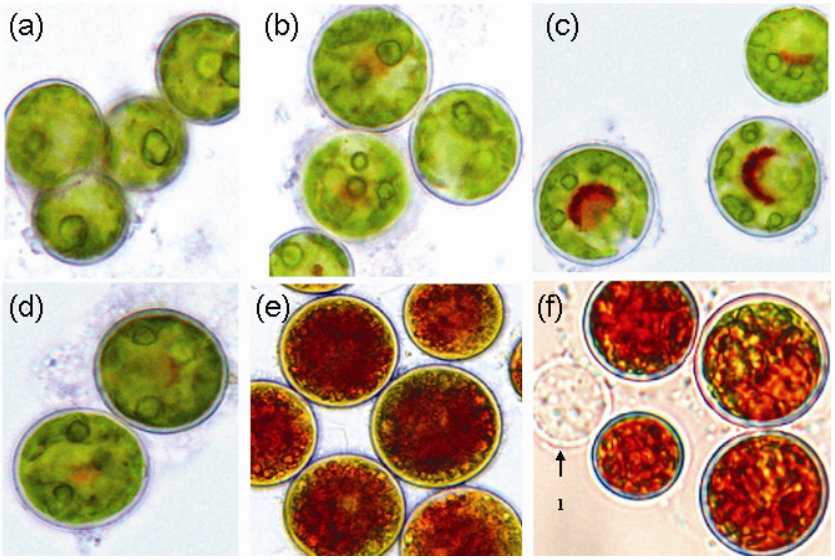

Fig. 1—Microscopic images (400×) of *H. pluvialis* samples within the GA_3 treatments on day 4 and 16 [a, b and c represent the control, GA20 and GA40 samples on day 4; d, e and f represent the control, GA20 and GA40 samples on 16 days; Arrow 1 shows the whitened algal cells]

Results from astaxanthin assays showed increasing accumulation with a peak occurring on day 16 with GA_3 treatments. After 16 days, GA40 treatment had a higher astaxanthin production (2.39 mg/L) than GA20 (1.77 mg/L), while controls had only 0.047 mg/L (Fig. 2). This was in agreement with our previous results, which showed that exogenous phytohormones, such as JA or SA could stimulate astaxanthin accumulation in *H. pluvialis*[17,18].

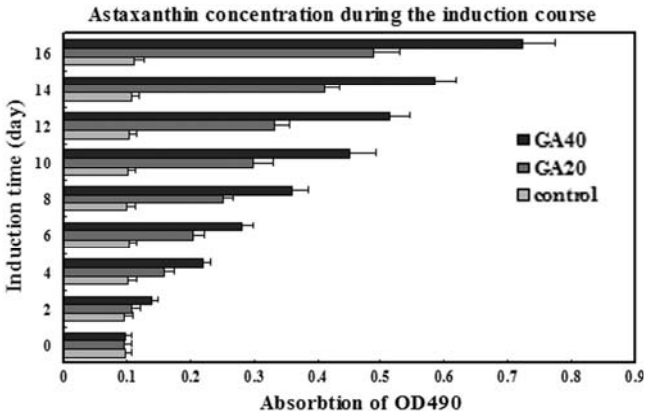

Fig. 2—Astaxanthin measurement in *H. pluvialis* [OD_{490} represents relative astaxanthin content in alga culture solution]

Transcriptional patterns of carotenogenic genes induced by GA_3

Overall, the transcriptional expression level and change pattern were different among eight genes. The first increased transcriptional expression which was also the maximum transcriptional *ipi*-1 levels in both GA20 (2.4-fold of mRNA expression increase than the control) and GA40 (2.2-fold) was observed on day 16, respectively (Fig. 3a). The *ipi*-2 expression in GA20 was increased to 3.3-fold on day 2, followed by a slight decline and then reached 3.9-fold on day 16. However, the maximum transcriptional levels of *ipi*-2 (5.9-fold) in GA40 reached on day 2 (Fig. 3b). Similar to *ipi*-2, the highest transcriptional levels of *psy* also occurred on day 2 (3.0-fold in GA20 and 5.4-fold in GA40, Fig. 3c). Interestingly, the transcriptional levels of *pds* started to increase on day 16, with 2.6-fold in GA20 and 3.6-fold in GA40 (Fig. 3d). The increased transcriptional expression was also observed in *lyc*, with the maximum expression on day 8 (1.7-fold and 1.4-fold in GA20 and GA40, respectively), suggesting that GA_3 treatment had little influence on the transcriptional expression of *lyc* (Fig. 3e). Although *crt*R-B and *crt*O showed similar expression patterns to *lyc* in both GA_3 treatments, but expression patterns were not much different from the control (Fig. 3f and g). In both GA_3 treatments, the increase transcriptional expression of *bkt2* occurred on day 1 (2.1- and 2.0-folds in GA 20 and GA40, respectively), but declined afterwards. It was worth noting that *bkt2* had the highest expression level on day 4, when 9.7- and 7.6-fold was achieved in GA20 and GA40, respectively (Fig. 3h).

With GA_3 treatment, five genes *ipi*-1 *ipi*-2, *psy*, *pds* and *bkt*2 were up-regulated, showing different expression patterns. In comparison,

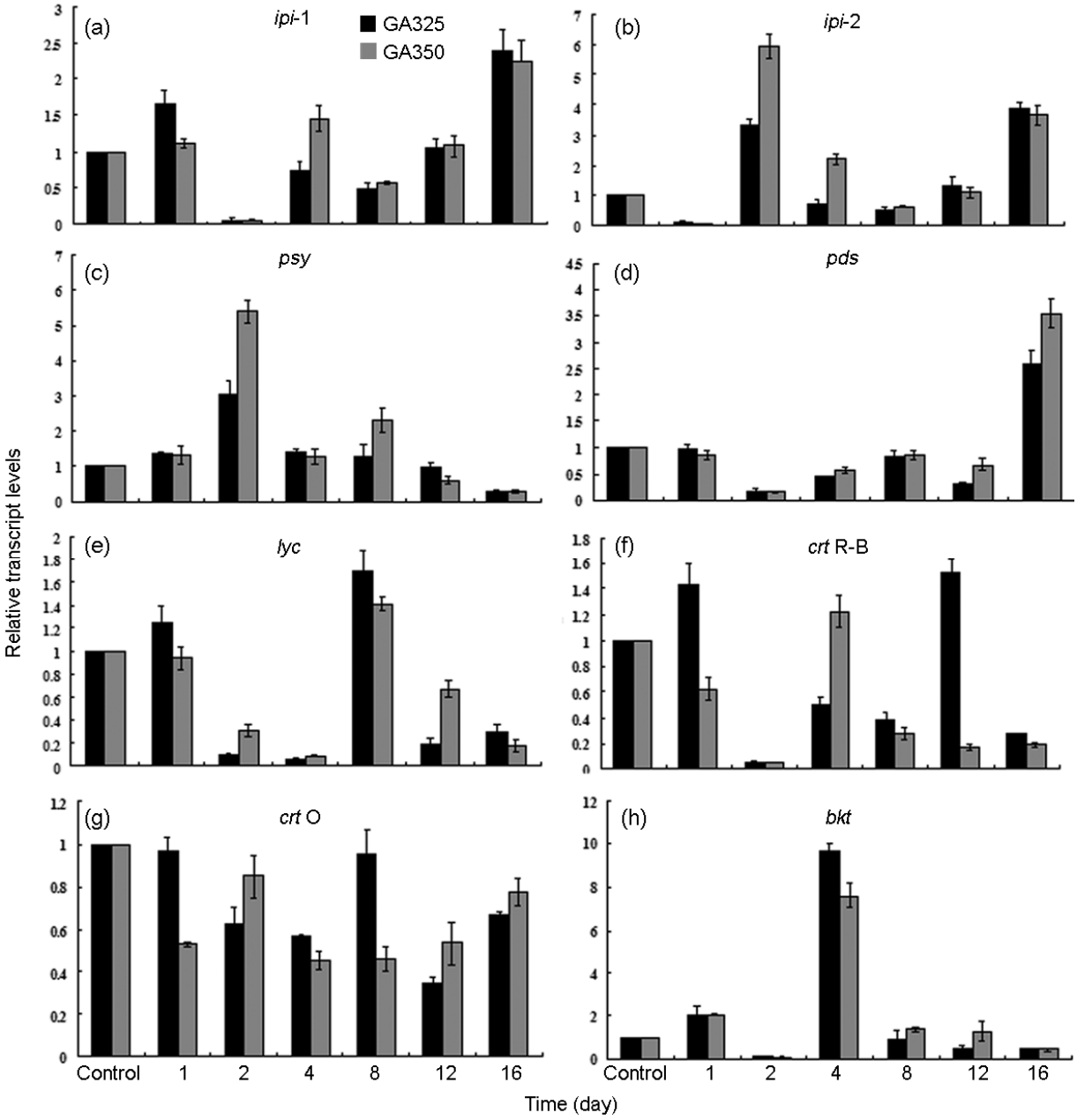

Fig. 3—Effects of GA_3 on the transcript expression kinetics of eight carotenogenic genes in *H. pluvialis* [a, b, c, d, e, f, g & h represent transcript levels expression of *ipi*-1, *ipi*-2, *psy*, *pds*, *lyc*, *crt*R-B, *crt*O and *bkt*, respectively]

GA20 treatment had a greater effect on transcriptional expression of *bkt*2 than that on *ipi*-1 *ipi*-2, *psy* and *pds*, concomitant with more than 4-fold more up-regulation. However, GA40 treatment induced more up-regulated transcriptional expression on *ipi*-2, *psy* and *bkt*2, which was a more than 4-times that of *ipi*-1 and *pds*. Apparently, GA_3 had little or less effect on *lyc*, *crt*R-B and *crt*O genes for astaxanthin biosynthesis.

Discussion

Similar to previous work, the induction of astaxanthin in *H. pluvialis* by GA_3 stress was also observed in this study. However, the transcriptional mechanism of astaxanthin biosynthesis related genes is not fully studied yet. In response to GA_3 induction, this study proved that some of carotenoid genes were involved in astaxanthin biosynthesis and accumulation. Thus, the regulation information of

these genes-induced by GA_3 in this study also could improve our knowledge on astaxanthin biosynthesis mechanism in *H. pluvialis.*

GA_3 is involved in regulating a wide range of growth and development processes, such as inducing the synthesis of carotenoid pigments in cucumber flowers[20] and stimulating *chs* transcription expression in the corolla of *Petuniu kybridu* [21]. The *tpp* gene is also reported to be down-regulated by GA_3[22]. GAmyb mRNA levels are also increase transiently in response to GA_3, which is 5-fold higher than control by 12 h and decline by 50% by 24 h, suggesting that GAmyb expression in isolated barley aleuronic layers is up-regulated by GA_3[23]. It is also shown that though several enzymes are regulated by GA, the sensitivity of individual genes may vary[24]. GA_3 promotes *chs* expression and sucrose uptake independently[25]. It is also shown that *EPB-1* is more tightly regulated by GA than EPB-2[26]. GA induction of GASA1 occurs at the transcriptional level[27].

GA differentially regulates the *bri1*-201 expression levels of *Arabidopsis ecotype* mutant and also modulates GA_5 expression, at least in part, at the transcriptional level[28]. GA causes 2-fold increase in the rate of GAMYB transcription and its effect can be blocked by abscisic acid (ABA)[29]. GA also promotes *Arabidopsis* seeds germination by down-regulating *RGL2* expression[30]. GA and nitrate delay the reduction of *DXS* expression and the induction of *PaO* and *PSY* transcript accumulation in *C. clementina*, while no differences in CHLP are observed[31]. GA_3 (5 µM, 24 h) treatment up-regulates 29 unique cDNA clones and down-regulates 42 unique cDNA clones from a cDNA microarray in rice seedlings.

Earlier Lu *et al*[9] reported that GA_3 is involved in the astaxanthin accumulation of *H. pluvialis* is likely due to the up-regulation of the key enzymes in the astaxanthin biosynthetic pathway. They reported GA_3 treatment increases the transcription of three β-carotene ketolase genes (*bkts*), with 5.5- and 3.1-fold increase in the transcripts of *bkt*2 and *bkt*3 on day 16 when treated with GA2 and GA20, respectively. This observation was inconsistent with our present results (Fig. 3h). We found that the expression of *bkt*2 was lower than control levels on day 16, whereas it was higher on day 4 with 9.7- and 7.6-fold increase in expression in GA20 and GA40, respectively. The contradiction might be due to different strains used in the study.

Although previous study has revealed the up-regulation of *bkts* on day 16 under GA_3 (2 and 20) treatments[9], information on carotenogenesis during GA_3-induced accumulation of astaxanthin is limited. Therefore, a more detailed study involving a long time course rather than only one time point of the *bkt* would likely provide more insight into the mechanism of GA_3 on the transcript levels. The correlation between transcriptional peaks of eight carotenoid genes and fast astaxanthin accumulation reported in the present study was as suggested by previous reports[3,17,18,32]. Carotenoid genes were up-regulated for astaxanthin accumulation at transcriptional, post-transcriptional, or both levels. It was reflected by the correlation between transcriptional peaks of eight carotenoid genes and fast astaxanthin accumulation.

The rapid accumulation of astaxanthin began at day 4 in both GA_3 treatments (Fig. 2). When the maximum transcript of carotenoid genes appeared before day 4, these genes were regarded as controlling astaxanthin biosynthesis at the post-transcriptional level. Otherwise, if the transcript peak of carotenoid genes was lagged behind or just on day 4, these genes were considered as regulating astaxanthin biosynthesis at the transcriptional level[3,17,18,32]. Therefore, in GA20 treatment, the mRNA peaks of *ipi*-1, *ipi*-2, *pds* and *bkt*2 were individually obtained on day 16, 16, 16 and day 4, later than or just on the time point of fast astaxanthin accumulation (day 4). This indicated that these four genes might up-regulate astaxanthin biosynthesis at transcriptional level. However, the mRNA peak of *psy* was on day 2, preceding the initial fast accumulation of astaxanthin on day 4, thus *psy* might up-regulate astaxanthin biosynthesis at post-transcriptional level. In GA40 treatment, *ipi*-1, *pds* or *bkt*2 likely up-regulated astaxanthin biosynthesis at transcriptional level, since their mRNA peaks occurred on day 16, 16 and day 4, respectively. With regard to the increase of *ipi*-2 and *psy* on day 2, it was conceived that these genes might up-regulate astaxanthin biosynthesis at post-transcriptional level. However, GA_3 had no effect on the expression levels of *lyc*, *crt*R-B and *crt*O.

In conclusion, the efficient biosynthesis of astaxanthin in *H. piuvialis* exposed to the exogenous GA_3 induction was associated with the up-regulation of *ipi*-1, *pds* and *bkt* at the transcriptional level, *psy* at the post-transcriptional level and *ipi*-2 at both transcriptional and post-transcriptional levels.

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