Transcript levels of phytoene desaturase gene in *Dunaliella salina* Teod. as affected by PbS nanoparticles and light intensity

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ABSTRACT

Phytoene synthase (Psy) and Phytoene desaturase (Pds) are the first two regulatory enzymes in the carotenoids biosynthetic pathway. The genes *Psy* and *Pds* are under transcriptional control in many photosynthetic organisms. In the present study, using quantitative real time-PCR (qRT-PCR), the effects of uncoated and gum-Arabic coated PbS nanoparticles (GA-coated PbS NPs) and light intensity on the mRNA levels of *Pds* were investigated. Relative to mRNA level of *Pds* at 100 µmol photon m⁻² s⁻¹ light intensity (control culture), 2.2-fold increase in transcript levels occurred after 12 h of exposure to higher light intensity, which is significantly (P<0.05) different compared to control. After 48 h of exposure, the mRNA level of *Pds* was reduced to that in control. This indicates that light intensity regulates *Pds* at the mRNA level. In the presence of uncoated and GA-coated PbS NPs, the transcript levels of *Pds* were decreased over time, with uncoated PbS NPs having more inhibitory effects on mRNA levels compared to GA-coated PbS NPs. This shows that PbS NPs have adverse effects on transcription or posttranscriptional processing and coating nanoparticles with biopolymers reduces their toxicity to organisms. Being under control, it seems that genetic manipulation of *Pds* may result in increased biotechnological production of carotenoids by *D. salina*.

Keywords: Phytoene desaturase; qRT-PCR; Carotenoids; Lead sulfide

INTRODUCTION

*Dunaliella salina* Teod. is a unicellular green algae that accumulates large quantities of β-carotene under certain environmental condition such as nutrient limitation, high salinity, high light intensity and temperature stress [1]. It has been suggested that β-
carotene overproduction by high light intensity requires activation of gene(s) encoding β-carotene biosynthetic enzyme(s) [2]. In *Dunaliella*, carotenoids are mainly synthesized in the plastids via the methylerythritol phosphate (MEP) pathway [3, 4]. In this pathway, phytoene synthase (Psy) and phytoene desaturase (Pds) seem to be under transcriptional control and have important role in the regulation of carotenoids biosynthesis [5, 6]. Psy catalyzes the head to tail condensation of two molecules of 20-C compound geranylgeranyl pyrophosphate (GGPP) to produce the 40-C compound phytoene which is desaturated to ζ-carotene by Pds. Through several enzymatic steps ζ-carotene is converted to lycopene and then to β-carotene [7].

There are controversial reports on the protein levels and mRNA of *Psy* and *Pds* in *D. salina* under environmental stresses, such as high light intensity and nutrient limitation. Sanchez-Estudillo et al. [8], reported no change in the steady-state mRNA levels of *Psy* in *D. salina* grown under nitrogen deficiency. In contrast, several fold increase in mRNA levels of *Psy* and *Pds* was observed under nutrient limitation by Coesel et al. [9]. Possible changes in the mRNA levels of *Psy* and *Pds* have been studied in other photosynthetic organisms. In sunflower, the steady-state level of *Psy* was negatively affected by phytoene accumulation [6]. Inhibition of carotenoids synthesis in tomato seedlings resulted in increased *Psy* and *Pds* transcript levels [10], but this up-regulation was not observed in pepper leaves [11].

Carotenoids are essential pigments present in all photosynthetic organisms and protect cells from oxidative damage caused by reactive oxygen species [12]. Nanoparticles cause oxidative damage to macromolecules by mainly increase in ROS production [13]. Therefore, antioxidant defense mechanisms in plants are up-regulated to scavenge ROS and reduce oxidative damage caused by environmental stresses [14].

In the present study, the effects of uncoated and gum-Arabic coated lead sulfide nanoparticles (GA-coated PbS NPs) and light intensity on the mRNA levels of *Pds* in *D. salina* at late logarithmic phase of growth are analyzed by quantitative real time PCR (qRT-PCR) and results are compared with changes in mRNA levels in other photosynthetic organisms.

**MATERIALS AND METHODS**

**Algal strain and exposure experiments:** *Dunaliella salina* Teod. strain MSI-3 (GeneBank accession no. KC477401) which previously was isolated, purified and identified in our laboratory, was cultured in nutrient medium as described by Ben-Amotz et al. [15]. The 250 mL Erlenmeyer flasks, each containing 100 mL of the culture with a cell density of 10⁶ cells ml⁻¹ were exposed to continuous illumination with an intensity of 100 µmol photon m⁻² s⁻¹ at 22±2 °C. At late logarithmic phase of growth, uncoated and GA-coated PbS NPs were added to the culture to give 24 µg mL⁻¹ final concentrations. The flasks were immediately transferred to 180 µmol photon m⁻² s⁻¹ light intensity and samples were taken at 3, 6, 12 and 48 hrs time intervals for mRNA analysis by qRT-PCR. The size of the PbS NPs and their coating procedures are as described previously [16].
**Total RNA extraction and cDNA synthesis:** Total RNA was extracted from $10^7$ *D. salina* cells using DENA ZIST Asia Kit according to the protocol provided by the manufacturer. To remove genomic DNA, the extracted RNA was treated with DNase (DNase I- RNase free, Fermentas). Using Nano-Drop ND 1000 spectrophotometer (Wilmington, USA), total RNA was quantified at 260 nm and its quality was determined by the A260/A280 ratio and also by gel electrophoresis. Synthesis of cDNA was performed with the First Strand cDNA Synthesis Kit (Fermentas).

**Quantitative real time PCR:** Relative qRT-PCR was carried out with a Line GeneK thermal cycler (Bioer, China). The reaction mixture in a total volume of 20 µl contained 5 µl cDNA, 10 µl 2x GreenStar TM q-PCR Master Mix (Bioneer) and 10 pmole of each primer. The primers for Pds and internal control (18s rRNA) genes were designed using the sequences obtained in GeneBank and Allele ID 7 software. The sequences of primers for these genes are presented in Table 1. For PCR reaction the following thermal profile was used: 94 °C for 10 min, 40 cycles at 94 °C for 10 s, 60 °C for 25 s and 72 °C for 30 s. Specificity of PCR products were confirmed based on melting curves obtained by heating the amplicons from 50 °C to 90 °C. Relative expression of *Pds* was calculated using the equation $2^{-\Delta\Delta Ct}$ in which $\Delta Ct$ was obtained by subtracting the internal control Ct value from the Ct value of *Pds*.

**Statistical analyses:** Changes in Pds gene expression under different growth conditions are presented as fold-change relative to control. The experiments were carried out in triplicate and the results are presented as mean ± standard error (SE). Duncan's multiple range tests was used to compare the expression levels. SPSS 16.0 was employed for statistical analyses and *p*<0.05 was considered as statistically significant.

**RESULTS**

Figure 1 shows changes in the expression levels of the gene encoding Pds in *D. salina* after the algal cells grown at 100 µmol photon m$^{-2}$ s$^{-1}$ light intensity (control culture) were transferred to 180 µmol photon m$^{-2}$ s$^{-1}$ intensity and simultaneously exposed to uncoated and GA-coated PbS NPs. In the absence of PbS NPs, after 3 h of exposure to higher light intensity, the mRNA level of *Pds* increased by 1.2 fold compared to the control culture, but the increase was not statistically significant at *p*<0.05. In the presence of the uncoated and GA-coated PbS NPs, significant decrease in the mRNA levels of *Pds* was observed. Exposure to uncoated PbS NPs had more inhibitory effect on the expression level of *Pds*.

**Table 1:** Nucleotide sequences of primers and size of the products in real time PCR amplification

| Genes  | Primer sequences (5'--->3') | Amplicon length (bp) |
|--------|-----------------------------|----------------------|
| Pds F  | CTATGACCGTGTGCTAA           | 128                  |
| Pds R  | CCTGGAAGTGAAGTGTGCTAA       | 128                  |
| 18S rDNA F | AGTGTTGGGCAAGTGGAC      | 148                  |
| 18S rDNA R | TAGAAATAGCGAGCGAGCG     | 148                  |

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than GA-coated NPs. The expression ratios significantly reduced to about 0.6 and 0.3 in the presence of coated and uncoated NPs, respectively (BOX A).

**Figure 1:** Effects of uncoated and gum-Arabic coated lead sulfide nanoparticles (GA –coated PbS NPs) and light intensity on mRNA levels of phytoene desaturase (*Pds*) in *Dunaliella salina* Teod. Cells grown at 100 µmol photons m⁻² s⁻¹ light intensity with no PbS NPs (control culture) were transferred to 180 µmol m⁻² s⁻¹ intensity and exposed to PbS NPs. Samples were taken at 3 h (Box A), 6 h (Box B), 12 h (Box C) and 48 h (Box D) after shift to higher light intensity for mRNA analysis.

After 6 h of exposure to higher light intensity, in the absence of NPs, the expression level of *Pds* significantly augmented to 1.7. In the presence of NPs, greater reduction in *Pds* expression was observed when compared to 3 h of exposure; the expression ratios decrease to about 0.4 and 0.2 for coated and uncoated NPs, respectively (BOX B). *D. salina* culture exposed to 180 µmol photon m⁻² s⁻¹ light intensity for 12 h, up-regulated *Pds* expression by 2.2 fold compared to cells grown at 100 µmol photon m⁻² s⁻¹ intensity (control culture). Continued reduction in mRNA levels in the presence of NPs is noticeable (BOX C). Finally, in the absence of PbS NPs, after 48 h of exposing algal culture to higher light intensity, the mRNA level of *Pds* reduced to that in control culture. Continued reduction in the expression levels of the target gene was observed in the presence of NPs with uncoated PbS NPs essentially abolished *Pds* expression (BOX D).
DISCUSSION

Phytoene desaturase (Pds) and phytoene synthase (Psy) play important role in the regulation of carotenoids biosynthetic pathway in many photosynthetic organisms [17, 18]. In this study, Pds transcript level continued to increase with time reaching a maximum of 2.2-fold, 12 h after shift to higher light intensity. Since D. salina was sampled at late logarithmic phase of growth, in addition to higher light intensity, nutrient depletion may have contributed to increase in Pds mRNA levels. Increase in pds transcript levels due to high light intensity and under nutrient limitation is reported by Coesel et al. in D. salina [9]. About 4.5-fold increase in Pds mRNA level was observed 48 hr after shift to high light intensity. Combined effects of high light intensity and nutrient limitation caused 8-fold increase in pds mRNA after 24 hr of exposure. In addition to Pds, high light intensity and nutrient deficiency caused up-regulation of Psy.

Contrary findings have been reported in D. salina var. bardavil [19]. No significant increase in Pds mRNA levels or protein level was observed upon exposure of culture to high light intensity. As pointed out by Coesel et al. [9], nutrient levels of algal culture at the time of sampling for mRNA analysis may be responsible for the observed differences in Pds response to high light intensity. The up-regulation of carotenoids biosynthetic genes have been demonstrated in other photosynthetic organism. In the Cyanobacterium Synechococcus sp. Psy and Pds were shown to be under transcriptional control. The promoter activity at the Pds/Psy operon was higher under strong light [20]. In the unicellular green algae C. reinhardtii Psy and Pds showed a fast up-regulation in response to light [21]. In the green algae Haematococcus pluvialis, increase in the Pds protein level, was accompanied by increase in Pds mRNA level during the accumulation of ketocarotenoids [22]. It was concluded that Pds is regulated at the mRNA level, most likely by transcriptional control.

In accordance with the large production and use of nanomaterials, the number of publications addressing their potential hazards to living organisms and to the environment, especially aquatic environment, has increased rapidly [23]. Generation of ROS has been considered as a general toxicity mechanism for several types of NPs [24, 25]. Coating NPs with substances like gum-Arabic reduces the toxicity of NPs to organisms [16]. In this work, reduction in Pds expression occurred in the presence of PbS NPs. Uncoated NPs had more inhibitory effects on Pds mRNA levels compared to the coated NPs. In general, anti-oxidative genes are up-regulated in the presence of NPs [26, 27]. In A. thaliana, genomic responses to TiO2 and CeO2 NPs were up-regulation of genes involved in oxidative stress. CeO2 NPs also resulted in down-regulation rather than up-regulation of several genes associated with photosynthesis [14]. Since Pds seems to be under transcriptional control in the green algae D. salina, genetic manipulation of this gene may result in higher biotechnological production of carotenoids.

Acknowledgments: The authors would like to thank the Shiraz University Research Council for supporting this research.

Conflict of Interest: No competing interests are declared by any of the authors.
REFERENCES

1. Borowitzka MA, Borowitzka LJ, Kessly D. Effects of salinity increase on carotenoid accumulation in the green alga *Dunaliella salina*. J Appl Phycol 1990;2:111-119.

2. Lers A, Biener Y, Zamir A. Photoinduction of massive beta-carotene accumulation by the alga *Dunaliella bardawil*: kinetics and dependence on gene activation. Plant Physiol 1990;93:389-395.

3. Bartley GE, Scolnik PA. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell 1995;7:1027-1038.

4. Phillips MA, Leon P, Boronat A, Rodriguez-Concepcion M. The plastidial MEP pathway: unified nomenclature and resources. Trends Plant Sci 2008;13:619-623.

5. Campisi L, Fambrini M, Michelotti V, Salvini M, Giuntini D, Pugliesi C. Phytoene accumulation in sunflower decreases the transcript levels of the phytoene synthase gene. Plant Growth Regul 2006;48:79-87.

6. Salvini M, Bernini A, Fambrini M, Pugliesi C. cDNA cloning and expression of the phytoene synthase gene in sunflower. J Plant Physiol 2005;162:479-484.

7. Cazzonelli, C.I., and Pogson, B.J. Source to sink: regulation of carotenoid biosynthesis in plants. Trends Plant Sci 2010;15:266-274.

8. Sánchez-Estudillo L, Freile-Pelegrin Y, Rivera-Madrid R, Robledo D, Narvaez-Zapata JA. Regulation of two photosynthetic pigment-related genes during stress-induced pigment formation in the green alga, *Dunaliella salina*. Biotechnol Lett 2006;28:787-791.

9. Coesel SN, Teles LM, Ramos AA, Henriques NM, Cancela L, Varela JCS. Nutrient limitation is the main regulatory factor for carotenoids accumulation and for *Psy* and *Pds* steady state transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress. Mar Biotechnol 2008;10:602-611.

10. Simkin AJ, Laboure AM, Kuntz M, Sandmann G. Comparison of carotenoid content, gene expression and enzyme levels in tomato (*Lycopersicon esculentum*) leaves. Z Naturforsch [C] 2003;58:371-380.

11. Simkin AJ, Zhu C, Kuntz M, Sandmann G. Light-dark regulation of carotenoid biosynthesis in pepper (*Capsicum annuum*) leaves. J Plant Physiol 2003;160:439-443.

12. Racchi ML. Antioxidant defenses in plants with attention to *prunus* and *citrus* spp. Antioxidant 2013;2:340-369.

13. Perrault F, Popovic R, Dewez D. Different toxicity mechanisms between bare and polymer coated copper oxide nanoparticles in *Lemma gibba*. Environ. Pollut 2014;185:219-227.

14. Andersen C, King G, Plocher M, Storm M, Rygiewicz P, Tumburu L, Reichman J. Effect of nTiO2 and nCeO2 nanoparticles on gene expression, germination, and early development in plants. International Congress on Safety of Engineered Nanoparticles and Nanotechnologies, Helsinki, Finland. 2015.

15. Ben-Amotz A, Shaish A, Moradhay A. Mode of action of massively accumulated beta-carotene of *Dunaliella bardawill* in protecting the alga against damage by excess irradiation. Plant Physiol 1989;91:1040-1043.
16. Zamani H, Moradshahi A, Jahromi HD, Sheikh MH. Influence of PbS nanoparticle polymer coating on their aggregation behavior and toxicity to the green algae Dunaliella salina. Aquatic Toxicol 2014;154:176-183.

17. Li F, Vallabhaneni R, Wurtzel E. PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and regulator of abiotic stress-induced root carotenogenesis. Plant Physiol 2008;146:1333-1345.

18. Cazzonelli CI, Pogson BJ. Source to sink: regulation of carotenoid biosynthesis in plants. Trends in Plant Sci 2010;15:266-274.

19. Rabbani S, Beyer P, Von Lintig J, Hugueney P, Kleinig H. Induced beta-carotene synthesis driven by triacylglycerol deposition in the unicellular alga Dunaliella bardawil. Plant Physiol 1998;116:1239-1248.

20. Schaeffer L, Sandmann M, Woitsch S, Sandmann G. Coordinate up-regulation of carotenoid biosynthesis as a response to light stress in Synechococcus PCC7942. Plant Cell Environ 2006;29:1349-1356.

21. Bohne F, Linden H. Regulation of carotenoid biosynthesis genes in response to light in Chlamydomonas reinhardtii. Biochim Biophys Acta 2002;1579:26-34.

22. Grunewald K, Eckert M, Hirschberg J, Hagen C. Phytoene desaturase is localized exclusively in the chloroplast and upregulated at the mRNA level during accumulation of secondary carotenoids in Haematococcus pluvialis (Volvocales, Chlorophyceae). Plant Physiol 2000;122:1261-1268.

23. Peralta-Videa JR, Zhao L, Lopez-Moreno ML, de la Rosa G, Hong J, Gardea Torresdey JL. Nanomaterials and the environment: a review for the biennium 2008–2010. J Hazard Mater 2011;186:1-15.

24. Kumari M, Khan SS, Pakrashi S, Mukherji A, Chandrasekaran N. Cytogenetic And genotoxic effects of zinc oxide nanoparticles on root cells of Allium cepa. J Hazard Mater 2011;190:613-621.

25. Xia T, Kovochich M, Liong M, Madler L, Gilbert B, Shi H, Yeh J, Zink J, Nel AE. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 2028; 2:2124-2134.

26. Lee SH, Pie j, Kim Y, Lee HR, Son SW, Kim M. Effects of zinc oxide nanoparticles on gene expression profile in human keratinocytes. Mol Cell Toxicol 2012;8:113-118.

27. Kaveh R, Li YS, Ranjarb S, Tehrani R, Brueck CL, Van Aken B. Changes in Arabidopsis thaliana gene expression in response to silver nanoparticles and silver ions. Environ Sci Technol 2013;47:10637-44.