

Relationship Between Endogenous Abscisic Acid and B-carotene Synthesis in Unicellular Green Alga *Dunaliella*

Janat Sarmad, Mansour Shariati and Maryam Madadkar Haghjou

Department of Biology, University of Isfahan, Hezarjarib St., Isfahan, Iran

Abstract: By comparison between two *Dunaliella* species, differing in B-carotene accumulation potential, the interrelationship between B-carotene and ABA production was investigated. Both *D. salina* MUR8 and *D. viridis* MUR29 (as higher and lower B-carotene accumulators, respectively) responded to salt-stress by an increase in ABA levels at initial hours after the imposition of hypersaline stress. But ABA enhancement was considerably higher in *D. salina* than that in *D. viridis*. The high level of ABA was transient in stressed *D. salina* and it had returned near to basal levels, but remained higher than control. In contrast to ABA levels, B-carotene content declined at the point of ABA enhancement in *D. salina* but not in *D. viridis*. After 24 hours exposure to salt stress, B-carotene accumulation was observed in *D. salina*. There were no significant changes of B-carotene in *D. viridis*. These results showed that *D. salina* appears more capable to response to salt stress by producing higher levels of ABA compared with *D. viridis*. This finding indicates an interrelationship between ABA and B-carotene production in *Dunaliella*.

Key words: *Dunaliella* % abscisic acid % B-carotene % ELISA

INTRODUCTION

Salinity is a major factor inducing carotenogenesis in some species of halotolerant green algae *Dunaliella* [1]. Salinity stress also induces *Dunaliella* to produce high levels of ABA [2, 3]. Understanding the way by which ABA levels increase in response to osmotic stress, has been supported by knowledge of the biosynthetic pathway for ABA and the use of various mutants impaired in specific steps in the pathway [4, 5]. ABA is well known as a stress hormone that is active in the adaptation to various forms of environmental stress in higher plants. In contrast, there has been little information about the mechanism that ABA responds to environmental stress, in algae. It might acts as a stress signal compound in some physiological responses in eukaryotic algae [6] as well as in higher plants [7]. Yoshida *et al.* [6] indicated that exogenously added ABA plays a certain physiological role in *Chlamydomonas reinhardtii* in light to increase growth in this alga. The proposed biosynthetic pathway of ABA in higher plant shows close relationship between xanthophylls cycle components and ABA [8]. It has been reported that lycopene can produce B-carotene and subsequent processes will yield violaxanthin [1] that acts as precursor to the ABA biosynthetic pathway, a process involving cleavage of 9-*cis* neoxanthin to yield xanthoxin. This

substance is then converted to ABA via ABA aldehyde [9, 10]. Therefore, ABA biosynthesis is influenced, at least in part, by affecting cleavage of apoxycarotenoid precursor in *Dunaliella* cells [9, 11].

This proposed biosynthesis pathway for ABA in higher plants considers the interrelationship that could exist between B-carotene accumulation and ABA production in *Dunaliella* cells [2, 12]. The results from some studies [1, 2, 8, 12, 13], provided evidences that encouraged us to design present experiments in order to better understanding of the relationship between B-carotene accumulation and ABA production in *Dunaliella* cells.

Among the many strains of the genus *Dunaliella* described, only two species, *D. bardawil* and *D. salina* Teod., have been shown capable of producing a huge amounts of B-carotene under inductive conditions [14]. To enable appropriate comparisons to be made, it was important to measure levels of endogenous ABA in two *Dunaliella* species with different capacity of B-carotene synthesis during salt stress. Therefore, *D. salina* and *D. viridis* as higher and lower B-carotene accumulator species, respectively, were examined. A modification of the EIA (Enzyme Immunoassay) described by Weiler [15] has also been employed to evaluate changes in endogenous levels of ABA in extracts of normal and salt stressed *Dunaliella*.

MATERIALS AND METHODS

Algal material and growth condition: Two species from the algal genus *Dunaliella*, *D. salina* MUR8 and *D. viridis* MUR29, utilized in this experiment were a kind gift from Prof. Borowitzka at the Murdoch University, Australia. It is known that *D. salina* is able to accumulate B-carotene higher than *D. viridis* under good inductive condition [14]. Cultures were grown separately in 2000 ml cotton plugged Erlenmeyer flasks containing 500 ml Johnson medium [16], modified according to Shariati and Lilley [17] containing 1.5 M NaCl. The cultures were continuously maintained in a growth cabinet at a temperature of 27°C/24°C (Light/dark), an irradiance of 160 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ with a 16 h/8 h (Light/dark) regime and shaking on an orbital shaker at around 90 rpm. In the logarithmic phase, salt stress was performed from 1.5 M to 3.5 M NaCl by adding NaCl to the medium.

Determination of B-carotene content: The algae were harvested in a linear growth phase. After centrifugation of algae (2500 g for 10 min at 20°C), the level of B-carotene and total chl were determined in the pellet spectrophotometrically (Shimadzu, UV-160A) in 85% acetone [18]. The B-carotene content of *Dunaliella* cells presented on a dry weight basis.

Extraction and purification of endogenous ABA: The levels of ABA and B-carotene were measured in same extract. Endogenous ABA was extracted and purified according to Bopp-Buhler *et al.* [13]. The algae were harvested in a linear growth phase. After centrifugation of algae (2500 g for 10 min at 20°C), the pellet was frozen immediately in liquid N₂, lyophilized (RTO-Olimann 6360 Friedberg-Germany) and weighted. Freeze-dry pellet was extracted with 80% aqueous methanol (4°C for 24 h). After another centrifugation the supernatants were cleared from interfering lipid material by passing the extracts through C₁₈-Sep-Pak cartridges (Waters, Milford, USA) [19]. Afterwards, methanol was removed by evaporation and 1.0 ml ddH₂O added to the residues. The aqueous solutions were acidified to pH 1-3 with 1.0 N HCl and partitioned 3 times against ethyl acetate. The organic solvent was evaporated to dryness under N₂ stream and samples were redissolved in 1.0 ml of methanol and stored at 20°C in the dark until assayed. The samples containing ABA were evaporated under N₂ stream to remove the organic solvent and dissolved in 1.0 ml of Phosphate-buffered Saline (PBS) and subjected to an immunological ABA assay (ELISA). The quantity of ABA in the samples was calculated on the basis of the calibration curve of + (ABA) for each plate.

Determination of endogenous ABA by the enzyme-linked immunosorbent assay (ELISA)

Immunogen synthesis: The (+) enantiomer of *cis*-ABA was coupled to BSA similarly to the procedure previously described by Weiler [19]. The conjugate was lyophilized and stored at -26°C. The coupling ratio was as determined by spectroscopic analysis [19].

Preparation of antibodies: Polyclonal antibodies were raised in rabbits against ABA conjugated to BSA through its carboxyl group (C1) according to Weiler [19]. The immunisation schedule and purification of antibodies followed the procedures described by Daie and Wyse [20]. Sera from all animals had demonstrable ABA binding capacity. Anti-ABA antibodies isolated from the rabbit serum by ammonium sulfate precipitation were lyophilized and stored at -20°C. Competitive ELISA procedure was done according to the modified method of Mohammadnejad *et al* [21]. Amounts of ABA-BSA and polyclonal antibody (PAb) required for optimum conditions were determined by checkerboard titration. In competitive ELISA, free and coated antigens compete for binding sites on the antibody. After washing with phosphate-buffer, the labeled second antibody enzyme was added to measure the amount of conjugated ABA-BSA. Bound enzyme activity was colorimetrically determined after addition of Tetra Methyl Banzidin (TMB). The standard curve of ELISA was obtained by plotting absorbance at 450 nm versus the log of ABA concentration in the assay. Data of this paper largely depend upon the reliability and specificity of the ELISA for ABA. Therefore two types of control were performed to validate the result of this technique [22]. As a first control, a known amount of authentic (+)-ABA was added to purified alga extracts then recovery was evaluated. The second control was done by showing parallelism of extract dilution curves with the standard curve.

Statistical analysis: Statistical analysis were performed using SPSS software and the comparisons were made using ANOVA with Tukey multiple comparisons.

RESULTS

Kinetic of B-carotene accumulation in *Dunaliella* cells: The B-carotene content of *Dunaliella* cells during the 7 days following salt stress were determined and expressed on a dry weight basis (Fig. 1-3). As are shown in Fig. 1 and 2, the amounts of B-carotene in salt-stressed cells of *D. salina* and *D. viridis* were compared with controls of both species, respectively. The high salinity

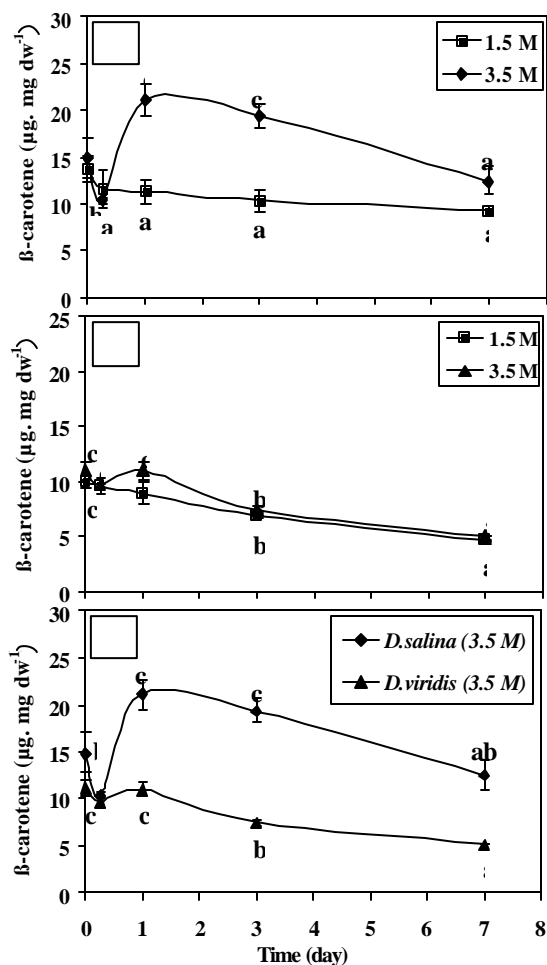


Fig. 1-3: Kinetics of β -carotene accumulation by cells of *D. salina* (Fig 1) and *D. viridis* (Fig 2) exposed to increased salinity stress (1.5 to 3.5 M NaCl) and comparison between β -carotene content changes in salt-stressed cells (3.5 M NaCl) of both strains (Fig. 3). Salt stress was done from 1.5 M to 3.5 M NaCl by adding NaCl to the medium. Data are expressed as the means of three replicates \pm SD. Different letters above the upper line in each figure, indicate significant differences in β carotene contents between various sampling times in stressed cells and different letters under the lower line in each figure, indicate significant differences between various sampling times in controls at $P<0.05$ according to Tukey test

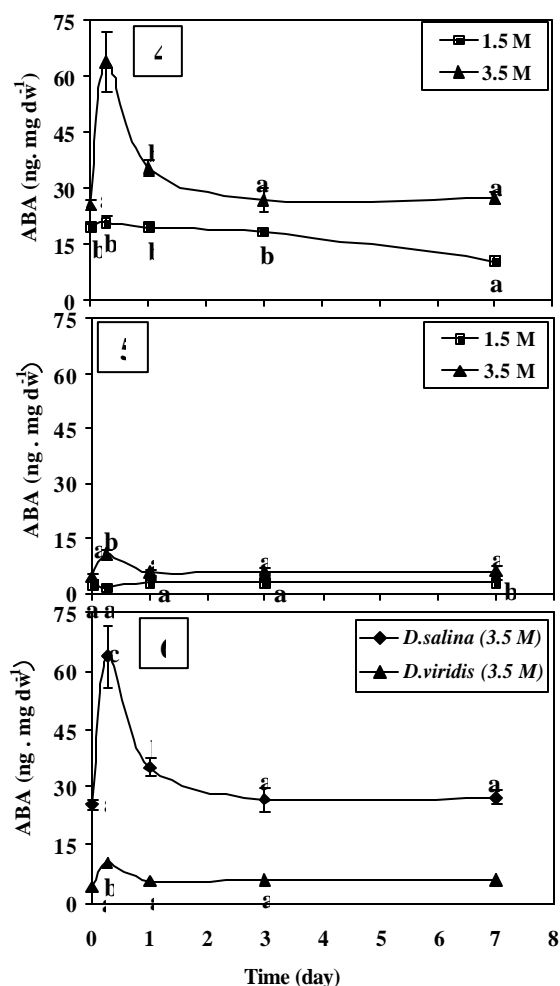


Fig. 4-6: Kinetics of ABA accumulation by cells of *D. salina* (Fig. 4), *D. viridis* (Fig. 5) exposed to increased salinity (1.5 to 3.5 M NaCl) and comparison between ABA accumulations in salt-stressed cells (3.5M NaCl) of both strains (Fig. 6). Salt stress was done from 1.5 M to 3.5 M NaCl by adding NaCl to the medium. Data are expressed as the means of three replicates \pm SD. Different letters above the upper line in each figure, indicate significant differences in ABA contents between various sampling times in stressed cells and different letters under the lower line in each figure, indicate significant differences between various sampling times in controls at $P<0.05$ according to Tukey test

(3.5 M NaCl) markedly reduced B-carotene after 6 h salt treatment in *D. salina* (Fig. 1) but not in *D. viridis* (Fig. 2), compared with those in their controls (1.5 M NaCl).

Thereafter, B-carotene levels in *D. salina* increased up to 1.5 to 2.0 folds and decreased after 24 h, to basal levels. The control of *D. salina* did not exhibit significant changes in B-carotene content and it remained nearly constant during initial days of experiment (Fig. 1). In *D. viridis*, B-carotene showed declining in rate gradually, in stressed cells which was consistent to that in control (Fig. 2).

A comparison between salt-stressed cells of both species was made in Fig. 3. As is shown in this figure, after 24 h, a significant ($P < 0.05$) stress stimulated B-carotene accumulation, was observed in *D. salina* that there was not in *D. viridis*.

The amounts of B-carotene when were expressed on a cell number basis ($\text{pg} \cdot 10^6 \text{cells}$), showed the same results (data not shown).

Overall these results suggest that, in *D. salina* (having higher potential for B-carotene accumulation), salt treatment strongly induce a time dependent B-carotene production which was resulted in an accumulation after an initial decline in B-carotene content.

Kinetic of ABA accumulation in *Dunaliella* cells: The ABA content of *Dunaliella* cells during the 7 days following salt stress were determined and expressed on a dry weight basis (Fig. 4-6).

Figure 4-5, showed changes in endogenous ABA levels of both salt-stressed (3.5 M NaCl) and controls cells (1.5 M NaCl) of *D. salina* and *D. viridis*. Results indicate that, in controls of both species, low levels of endogenous ABA remained nearly constant, whereas levels of ABA in stressed cells enhanced 2 to 3 folds after 6 h by hypersaline condition and reached 11 and 65 $\text{ng mgG}^{-1} \text{dw}$. for *D. viridis* and *D. salina*, respectively (Fig. 4-5). The high level of ABA was transient and it was decreased to basal levels after initial hours in stressed *D. salina* cells (Fig. 4). These changes were lower for *D. viridis* (Fig. 5). The changes in ABA contents from salt-stressed cells of both species are compared in Fig. 6. As is shown in this figure, there was a significant ($P < 0.05$) and higher increase in the levels of ABA in *D. salina* than *D. viridis*, when they were exposed to salinity stress.

Overall, these data suggests that the *D. salina* (containing higher B-carotene) respond to salt stress condition by producing significant and higher amounts of ABA compared with that in *D. viridis*.

DISCUSSION

Results from the present investigation indicate that cells of both *D. salina* and *D. viridis* produce ABA as an endogenous compound in response to hypersaline conditions. However there was a marked increase in ABA concentration in species with higher potential for B-carotene accumulation (*D. salina*) compared with low B-carotene accumulator species (*D. viridis*). This can suggest a relationship between the ability of B-carotene accumulation and ABA production in salt-stressed cells of *Dunaliella*.

Elevation in endogenous ABA might reflect enhanced rates of either biosynthesis and/or stress-induced inhibition of ABA degradation [12]. But most evidence suggests that general rate of metabolism is enhanced following the imposition of stress [23]. The results also showed that a large increase in ABA concentration in *D. salina* was along with a decrease in B-carotene content in this species. In other words, the rate of ABA and B-carotene changes (compared to each other) in salt-stressed *D. salina* (higher B-carotene accumulator), were in reverse order during the experiment. In contrast, *D. viridis* (lower B-carotene accumulator) exhibited only a slight increase in ABA levels as a response to salinity stress and there was no noticeable change in B-carotene concentration in this species. Therefore it seems that ABA biosynthesis in *Dunaliella* cells could be, at least partly, due to B-carotene destruction. This ABA might influence one or more physiological process, there by allowing cells to tolerate prevailing stressful conditions. Elevation of ABA under this situation is found to increase the generation of antioxidants in anti-oxidative defense [24].

Our results also indicate that, ABA levels in cells had returned to basal levels after reaching a maximum (6 h after exposure to salt stress). Cowan and Rose [2] showed that the rate of appearance of labeled ABA in the incubation medium increased dramatically within 30 min of exposure of *Dunaliella* to salinity stress. Thus the decrease of ABA in *Dunaliella* cells after 6 h exposure to stress in this study could be due to preferential partitioning of ABA into the culture medium.

B-carotene also accumulated in salt-stressed cells of *D. salina* after a decline at initial hours of stress. As ABA production preceded the accumulation of B-carotene, one possibility is that ABA might be involved in the induction of B-carotene production. The chemical bioinduction of carotenogenesis by ABA has been demonstrated in the fungus *Blackesslea trispora* [25]. Also a possible role for

ABA as a regulator of carotenogenesis in *Dunaliella* is suggested [2]. Therefore this accumulation might be mediated by initial increase in ABA content, an enhanced conversion of ABA to subsequent compounds and release of these compounds into the culture medium [2]. ABA could function to initiate the accumulation carotenoids, in particular, B-carotene through gene activation and/or alternations in enzyme synthesis and /or activity [26] which is occurred in the higher B-carotene accumulator *Dunaliella* species. Therefore a greater amount of ABA in *D. salina* at high stress level might raise its ability to B-carotene synthesis compared with *D. viridis*. Overall, these results showed an interrelationship between B-carotene and ABA production in *Dunaliella* cells. Furthermore, enhanced ABA metabolism in *D. salina* seems to be a marked and early response whereas B-carotene accumulation seems to represent late response to salt stress in this alga. In contrast, *D. viridis* had only an early response to stress by minor increase in ABA content.

ACKNOWLEDGMENTS

This work was supported by the Faculty of Postgraduate Studies, University of Isfahan, Isfahan, IRAN. We would also like to thank Dr Mohamad Javad Rasaee, Dept. of Medical Biotechnology, University of Tarbiat Modares, Tehran for technical helps for measuring endogenous abscisic acid.

REFERENCES

1. Eonseon, J., J.E.W. Polle, H.K. Lee, S.M. Hyun and M. Chang, 2003. Xanthophylls in microalgae: From biosynthesis to biotechnological mass production and application. *Journal of Microbiology and Biotechnology*, 13: 165-174.
2. Cowan, A.K. and P.D. Rose, 1991. Abscisic acid metabolism in salt-stressed cells of *Dunaliella salina*. *Plant Physiology*, 97: 798-803.
3. Hirsch, R., W. Hartung and H. Gimpler, 1989. Abscisic acid content of algae under stress. *Botanica Acta*, 102: 326-334.
4. Giraudat, J., 1995. Abscisic acid signaling. *Current Opinions in Cell Biology*, 7: 232-238.
5. Giraudat, J. and J. Schroeder, 2001. Cell signaling and gene regulation-plant signal transduction pathways: Graying of the black boxes-Editorial overview. *Current Opinions in Cell Biology*, 4: 379-381.
6. Yoshida, K., E. Igarashi, K. Mukai, K. Hirata and K. Miyamoto, 2003. Induction of tolerance to oxidative stress in the green alga, *Chlamydomonas reinhardtii*, by abscisic acid. *Plant Cell and Environment*, 26: 451-457.
7. Bray, E.A., 2002. Abscisic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. *Plant Cell and Environment*, 25: 153-161.
8. Sharma, P.K., S. Sankhalkar and Y. Fernandes, 2002. Possible function of ABA in protection against photodamage by stimulating xanthophylls cycle in sorghum seedlings. *Current Science*, 82: 167-171.
9. Milborrow, B.V., 2001. The pathway of biosynthesis of abscisic acid in vascular plants: A review of the present state of knowledge of ABA biosynthesis. *Journal of Experimental Botany*, 52: 1145-1164.
10. Schwartz, S.H., B.C. Tan, D.A. Gage, J.A.D. Zeevaart and D.R. Micarthy, 1997. Specific oxidative cleavage of carotenoids by VP 14 of maize. *Science*, 276: 1872-1874.
11. Jiang, X.Y., T. Rustem, S. Omarov, Z. Yesbergenova and M. Sagi, 2004. The effect of molybdate and tungstate in the growth medium on abscisic acid content and the Mo-hydroxylases activities in barley (*Hordeum vulgare* L.). *Plant Sciences*, 167: 297-304.
12. Cowan, A.K., P.D. Rose and L.G. Horne, 1992. *Dunaliella salina*: Model system for studying the response of plant cells to stress. *J. Exp. Bot.*, 43: 1535-1547.
13. Bopp-Buhler, M.L., P. Wabra, W. Hartung and H. Gimpler, 1991. Evidence for direct ABA synthesis in *Dunaliella* (Volvocales). *Cryptogamic Botany*, 2/3: 192-200.
14. Shaish, A. Ben-Amotz and M. Avron, 1992. Biosynthesis of B-carotene in *Dunaliella*. In: *Methods in Enzymology*. (ed: Packer, L.). Academic Press Inc, Sandiago, 70: 439-444.
15. Weiler, E.W., 1982. An enzyme-immunoassay for cis-(+)-abscisic acid. *Physiology of Plant*, 54: 510-514.
16. Johnson, M.K., Johnson, R.D. Mc Elroy, H.L. Speer and B.S. Bruff, 1968. Effect of salt on the halophilic alga *Dunaliella viridis*. *Journal of Bacteriology*, 95: 1461-1468.
17. Shariati, M. and R.McC. Lilley, 1994. Loss of intracellular glycerol from *Dunaliella* by electroporation at constant osmotic pressure: subsequent restoration of glycerol content and associated volume changes. *Plant, Cell and Environment*, 17: 1295-1304.

18. Eijkelhoff, C. and J.P. Dekker, 1997. A routine method to determine the chlorophyll a, pheophytin a and B-carotene contents of isolated photosystem II reaction center complexes. *Photosynthetic Research*, 52: 69-73.
19. Weiler, E.W., 1980. Radioimmunoassay for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. *Planta*, 148: 262-272.
20. Daie, J. and R. Wyse, 1982. Adaptation of the enzyme-linked immunosorbent assay (ELISA) to the quantitative analysis of abscisic acid. *Analytical Biochemistry*, 119: 365-371.
21. Mohammadnejad, J., M.J. Rasaee, B. Saqhafi, M. Rajabibazl, F. Rahbarizadeh and K. Omidfar, 2006. A new competitive enzyme linked immunosorbent assay (MRP83-CA15-3) for MUC1 measurement in breast cancer. *Journal of Immunology and Immunochemistry*, 27: 139-149.
22. Hallaj, S., M.J. Rasaee, M. Haerian, M. Paknejad, S. Kashanian, F. Rahbarizadeh, K. Omidfar and M. Malekaneh, 2003. A heterologous enzyme linked immunosorbent assay of morphine using penicillinase as label. *Iranian Journal of Biotechnology*, 1: 239-249.
23. Zeevaart, J. and R.C. Creelman, 1998. Metabolism and physiology of Abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology*, 39: 439-473.
24. Nayyar, H. and D. Gupta, 2006. Differential sensitivity of C₃ and C₄ plants to water deficit stress: Association with oxidative stress and antioxidants. *Environmental and Experimental Botany*, 58: 106-113.
25. Dandekar, S. and V.V. Modi, 1980. Involvement of cyclic AMP in caroteneogenesis and cell differentiation in *Blakeslea trispora*. *Biochemistry and Biophysics Acta*, 628: 398-406.
26. Lers, A.Y. Biener and A. Zamir, 1990. Photoinhibition of massive carotene accumulation by the alga *Dunaliella bardawil*. Kinetics and dependence on gene activation. *Plant Physiology*, 93: 389-395.