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Research article

The induction of menadione stress tolerance in the marine microalga, *Dunaliella viridis*, through cold pretreatment and modulation of the ascorbate and glutathione pools

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ABSTRACT

The effect of cold pretreatment on menadione tolerance was investigated in the cells of the marine microalga, *Dunaliella viridis*. In addition, the involvement of ascorbate and glutathione in the response to menadione stress was tested by treating cell suspensions with L-galactono-1,4-lactone, an ascorbate precursor, and buthionine sulfoximine, an inhibitor of glutathione synthesis. Menadione was highly toxic to non cold-pretreated cells, and caused a large decrease in cell number. Cold pretreatment alleviated menadione toxicity and cold pretreated cells accumulated lower levels of reactive oxygen species, and had enhanced antioxidant capacity due to increased levels of β-carotene, reduced ascorbate and total glutathione compared to non cold-pretreated cells. Cold pretreatment also altered the response to L-galactono-1,4-lactone and buthionine sulfoximine treatments. Combined L-galactono-1,4-lactone and menadione treatment was lethal in non-cold pretreated cells, but in cold-pretreated cells it had a positive effect on cell numbers compared to menadione alone. Overall, exposure of *Dunaliella* cells to cold stress enhanced tolerance to subsequent oxidative stress induced by menadione.

1. Introduction

Dunaliella, a unicellular, wall-less green micro alga is a unique organism that has evolved to live in extreme environmental conditions (Rothschild and Mancinelli, 2001). This alga has frequently been studied as a model system because of its physiological plasticity under changing environmental conditions (Avron and Ben-Amotz, 1992; Cowan et al., 1992; Jimenez and Niell, 1990). Under harsh conditions, *Dunaliella* naturally produces high levels of carotenoids, especially β-carotene, and other antioxidant molecules to protect against oxidative stress (Jahnke and white 2003, Salguero et al., 2003). As some *Dunaliella* species have high potential for β-carotene production, the production and accumulation of this antioxidant in response to different stresses has been extensively studied (Jahnke, 1999; Jimenez and Pick, 1993; Lamers at al. 2008, Mendoza et al., 1996; Salguero et al., 2003; Shaish et al., 1993) but recent investigations have shown the induction of other antioxidants such as ascorbic acid (AsA) and glutathione (GSH) in *Dunaliella* under oxidative stress conditions (Abd El-Baky et al., 2004; Haghjou et al., 2009; Jahnke and White, 2003; Madadkar Haghjou, 2012).

Ascorbate and glutathione are abundant antioxidants, and are efficient scavengers of reactive oxygen species (ROS) both directly and indirectly as co-substrates for enzymes such as ascorbate peroxidase and glutathione peroxidase. They belong to a complex collection of antioxidants found in plant cells, and their antioxidant functions can be closely integrated *via* the ascorbate–glutathione cycle (Foyer and Noctor, 2000, 2011; Latowski et al., 2010; Noctor, 2006). Both AsA and GSH are considered to play important roles in redox signaling, and in regulation of the cell cycle (Potters et al., 2002). In addition, both antioxidants have independent roles. For example, GSH is important for sulfur homeostasis, heavy metal sequestration and thiol-disulfide exchange in the regulation of protein activity (Noctor et al., 2012; Rouhier et al., 2008), whilst AsA is a cofactor for a number of enzymes, including enzymes involved in the biosynthesis of abscisic acid, gibberellic acid, ethylene and anthocyanins (Arrigoni and De Tullio, 2002; Foyer and Noctor,

Abbreviations: AsA, ascorbic acid; BSO, buthionine sulfoximine, GSH synthesis inhibitor; Chl, chlorophyll; DHA, dehydroascorbic acid; GalL, L-galactono-1,4-lactone, ascorbate biosynthesis precursor; GSH, glutathione; GSSG, glutathione disulphide; ROS, reactive oxygen species.

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2011; Smirnoff and Wheeler, 2000). Up-regulation of antioxidant defences is a widely-reported event in response to elevated levels of ROS due to exposure to abiotic stress conditions, and is a key factor in the ability to survive such stresses (Mittler, 2002).

Menadione (2-methyl-1, 4-naphthoquinone, vitamin K_3), is commonly used to induce superoxide formation in oxidative stress studies. One electron reduction of menadione results in the formation of semiquinone radicals, which are very unstable and can react rapidly with oxygen to form superoxide (Nutter et al., 1992). Superoxide is converted to H_2O_2 by superoxide dismutase (SOD) activity and H_2O_2 is reduced by ascorbate peroxidase or glutathione peroxidase in the presence of AsA or GSH (Foyer and Noctor, 2000; Kayanoki et al., 1996). Menadione can also directly react with cellular thiols such as GSH (McAmis et al., 2003). Therefore, AsA and GSH may be involved in the antioxidant response to menadione stress in higher plant and human cells.

In this research, tolerance of *Dunaliella viridis* to menadione was studied in combination with pretreatments which modulate the cellular AsA and GSH levels. *D. viridis* is not a β-carotene hyperaccumulator (García et al., 2007), and was selected for this study so that the role of AsA and GSH in stress responses could be investigated without the influence of high levels of β-carotene. The pretreatments comprised cold stress, which could induce AsA and GSH accumulation (Haghjou et al., 2009) and presumably many other acclimatory changes, L-galactono-1,4-lactone (GalL), which is a biosynthetic precursor of AsA, and buthionine sulfoximine (BSO), an inhibitor of GSH synthesis. The pretreatments were applied separately and in combination prior to treatment with menadione. Pretreatment with cold stress increased the tolerance of *D. viridis* to menadione stress.

2. Materials and methods

2.1. Growth and treatment conditions

Dunaliella viridis was obtained from algal collection of Isfahan University, Iran. Cells were grown in medium, described by Shariati and Lilley (1994), containing 1.7 M NaCl as optimum salt concentration (Avron and Ben-Amotz, 1992). Cultures were grown under photoperiodic conditions of 16 h cool-white fluorescent light (50 μmol m^{-2} s^{-1})/8 h dark, at 28°C/24 °C ± 0.5 °C. Cells were sampled during the logarithmic phase of growth and were divided into two flasks. From this point cell suspensions were grown under continuous light (50 μmol m^{-2} s^{-1}) to eliminate the effects of applying treatments at different points in the photoperiod. One flask was incubated at 28 °C for 24 h (C_0). After 24 h the cell suspension was divided into five equal parts and transferred to flasks. The following treatments were added: 5 mM GalL (CGM), 1 mM BSO (CBM) and 5 mM GalL + 1 mM BSO (CGBM), no treatments were added to the two remaining C_0 flasks. The flasks were incubated at 28 °C, and after 6 h 0.16 μM menadione was added to CGM, CBM, CGBM and one of the C_0 flasks (CM). The remaining C_0 flask was untreated (C). The flasks were incubated for a further 18 h. The cell suspension in the second flask was immediately divided into five Erlenmayer flasks and treatments were added: 5 mM GalL (LG_0), 1 mM BSO (LB_0) and 5 mM GalL + 1 mM BSO (LGB_0) to three of the flasks. No treatments were added to the remaining two flasks (L_0). The flasks were incubated at 14 °C for 24 h (cold pretreatment) prior to addition of 0.16 μM menadione to LG_0 (LGM), LB_0 (LBM), LGB_0 (LGBM) and one of the L_0 (LM) flasks. The remaining L_0 flask was untreated (L). The flasks were incubated at 28 °C for 24 h. Since low temperature may cause a decrease in the rate of cellular metabolic processes (Madadkar Haghjou and Shariati, 2007; Zhou et al., 2007), GalL and BSO were added to the cold pretreated cultures 24 h before menadione addition, but for the culture incubated at normal temperature (28 °C) this time was reduced to 6 h. All data were collected after 24 h and 48 h at 50 μmol m^{-2} s^{-1}continuous light and different temperatures. The experimental design is summarized in Fig. 1.

2.2. Determination of cell number and fresh weight

Dunaliella cells were counted after 24 and 48 h using a hemocytometer following cell inactivation by solid iodine. To determine fresh weight, 40 mL of cultures were centrifuged at 1500 g (Sanyo MSE Mistral 2500i, Loughborough, UK) for 10 min, the pellet was resuspended in a small volume of fresh medium (1.7 M) and transferred to pre-weighed 1.0 mL microcentrifuge tube and recentrifuged at 2500 g (Eppendorf 5415C) for 3 min. The supernatant was removed by vacuum, and the pellet was washed with 0.2 M NaCl culture medium to remove residual salt prior to weighing.

2.3. Ascorbate and glutathione measurements

Forty mL of cell cultures were centrifuged, and the pellet extracted with 500 μL of 100 mM HCl containing 1 mM EDTA. The extracts were centrifuged at 13,000 g (Eppendorf 5415C) for 4 min. The supernatants were frozen in liquid nitrogen, and stored at −80 °C until analysis the next day. Total ascorbate and reduced ascorbate (AsA), were determined by the method of Kampfenkel et al. (1995) using a microtiter plate reader (Versa, Molecular Devices, Wokingham, UK), at 520 nm. Dehydroascorbic acid (DHA) content was estimated from the difference between assays with and without dithiothreitol. Part of the same extract was used for GSH/glutathione disulphide (GSSG) assay based on the method of Baker et al. (1990). The concentration of GSH was estimated from the difference between total glutathione and GSSG assays.

2.4. Pigment measurements

Pigments were extracted from 1.0 ml of cell cultures, which were centrifuged at 12,000 g (Eppendorf 5415C) for 4 min with 1 mL of 80% acetone. All pigment concentrations were determined spectrophotometrically according to the methods of Eijckelhoff and Dekker (1997).

2.5. Measurement of in vivo ROS production

Intracellular ROS generation in *D. viridis* cells in response to cold and menadione treatment was measured using dihydrofluorescein diacetate. Cell suspensions were incubated at 28 °C (control) or 14 °C (cold pretreated) for 24 h prior to treatment with 0.16 μM menadione for 6 h. ROS production was measured in control and cold-pretreated suspensions both before and after menadione treatment. 20 μL of 0.125 mM dihydrofluorescein diacetate and equal volumes of cell suspensions were combined in a microtiter plate well and the increase in fluorescence (excitation wavelength of 485 nm and an emission wavelength of 520 nm) was recorded over 10 min using a microtiter plate reader (FLUOstar OPTIMA −BMG-LABTECH spectrofluorophotometer). Ten μL H_2O_2 (0, 25 or 200 μM) was included as a positive control to assess whether dihydrofluorescein diacetate was a reliable probe for ROS production in the cell suspensions (Wang et al., 2011). Algal medium was used instead of cell suspensions for blank measurements, and in addition background fluorescence was measured in cell suspensions in the absence of dihydrofluorescein diacetate, and the background was subtracted from the sample fluorescence with dihydrofluorescein diacetate. The influence of light on ROS production was measured in cell suspensions treated with 0.16 μM menadione or 5 mM GalL for 6 h. For the dark conditions, the