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Role of calcium in the mitigation of heat stress in the cyanobacterium *Anabaena* PCC 7120

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ABSTRACT

The effects of exogenously added $CaCl_2$ (0.25 mM) on photopigments, photosynthetic O_2-evolution, antioxidative enzyme activity, membrane damage, expression of two heat shock genes (*groEL* and *groES*) and apoptotic features in *Anabaena* 7120 under heat stress (45 °C) for up to 24 h were investigated. Heat stress lowered the level of photopigments; however, Ca^{2+}--supplemented cultures showed a low level reduction in Chl *a* but induced accumulation of carotenoids and phycocyanin under heat stress. Photosynthetic O_2-evolving capacity was maintained at a higher level in cells from Ca^{2+}-supplemented medium. Among the antioxidative enzymes, superoxide dismutase activity was unaffected by the presence or absence of Ca^{2+} in contrast to increases in catalase, ascorbate peroxidase and glutathione reductase activities in cells grown in Ca^{2+}-supplemented medium. Lower levels of lipid peroxidation were recorded in *Anabaena* cells grown in Ca^{2+}-supplemented medium in comparison to cells from Ca^{2+}--deprived medium. Target cells grown in Ca^{2+}-deprived medium developed apoptotic features in the early stages of heat shock, while Ca^{2+} application seemed to interfere with apoptosis because only a few cells showed such features after 24 h of heat exposure, indicating a role for Ca^{2+} in maintaining cell viability under heat stress. There was also continuous up regulation of two important heat shock genes (groEL and groES) in Ca^{2+}-supplemented cultures, exposed to heat shock, again indicating a role for Ca^{2+} in stress management.

1. Introduction

Increases in global temperatures have decreased yields of wheat, maize, barley (Lobell and Field, 2007) and soybean (Schlenker and Roberts, 2009) crops. Recently, it was estimated that wheat output decreased by 6% for every °C rise in temperature (Asseng et al., 2015). There is a prediction that continued increases in temperature would decrease the output of other crops (millet, groundnut and rapeseed) by 2030 in Africa and Asia (Lobell et al., 2008). High temperature affects a wide spectrum of cellular and metabolic activities in plants; these cellular and metabolic processes are variably dependent on stress severity, which is determined by the rate of temperature change, intensity and duration (Sung et al., 2003).

Cyanobacteria, a group of O_2-evolving photosynthetic prokaryotes, originated 2.8 billion years ago (Tandeau-de-Marsac and Houmard, 1993). *Anabaena* is one of the most important nitrogen-fixing cyanobacteria. *Anabaena* are typically found in tropical agroclimatic conditions, where they are major contributor to the nitrogen (N) and carbon (C) economy of the soils. There are reports that cyanobacteria respond to stresses such as heat (Mishra et al., 2005) and dessiccation (Singh et al., 2013) by increasing their antioxidative defense system, including SOD, catalase, ascorbate peroxidase and glutathione reductases activities. Studies on photosynthesis under heat stress are well documented (Inoue et al., 2001; Chaurasia and Apte, 2009). Cyanobacteria and the chloroplasts of higher plants are phylogenetically close; therefore, studies on the former under stress may help elucidate the mechanism(s) of plant adaptability to environmental changes. Thus, the cyanobacterial system may be adopted as a model to understand temperature stress response at the molecular level, as the genome of *Anabaena* PCC 7120 has already been sequenced (Kaneko et al., 2001).

Cyanobacterial adaptation under heat stress correlated with their potential to maintain conformational states of proteins by molecular chaperones such as the Hsp60 family and smaller Hsps (Rajaram et al., 2014). The cyanobacterial Hsp 60 family is charac-

Abbreviations: Chl *a*, chlorophyll *a*; MDA, malonaldehyde; TBARS, 2-thiobarbituric acid-reactive substances; SOD, superoxide dismutase; DAPI, 4′,6-diamidino-2-phenylindole; HSP, heat shock protein; PSI, photosystem I; PS II, photosystem II; EDTA, ethylene diamine tetra-acetic acid.

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terized by the presence of 10 kDa *groES* and two 60 kDa HSP, *groEL*-1 and *groEL*-2 (*cpn 60*) (Kaneko et al., 2001).

Heat-induced oxidative stress was reported to be modulated by calcium in Arabidopsis (Larkindale and Knight, 2002). Calcium (Ca^{2+}) is an important secondary messenger known to be released under a variety of stresses, including heat, and it regulated the stress responses in the plants (Steinhorst and Kudla, 2013). Ca^{2+} is required for heterocyst differentiation in *Anabaena* PCC7120 (Torrecilla et al., 2004). There are many reports regarding the involvement of Ca^{2+} in stress mitigation and acclimation in plants such as peanut (Yang et al., 2015) and wheat (Goswami et al., 2015). Previous studies have shown the involvement of Ca^{2+} in the protection of a subunit of photosystem II (PS II) and latter demonstrated that calcium foliar spray, increased antioxidative activities, resulting in thermotolerance under heat stress but the role of Ca^{2+} in cyanobacteria under heat stress remains relatively unexplored. Therefore, the present study was undertaken using *Anabaena* PCC7120 as a model system to investigate the possible role of Ca^{2+} in the alleviation of heat stress through assessing selected parameters including photosynthetic pigments, antioxidative enzymes, O_2-evolution and expression of *groEL* and *groES* genes.

2. Materials and methods

2.1. Strain and culture conditions

Anabaena strain PCC7120 was grown in BG-11 medium (Rippka et al., 1979) under continuous tungsten plus fluorescent illumination (14.4 Wm^{-2}) at 27 ± 1 °C. Cultures were maintained under a 16/8 h light/dark regime. For every experimental set up, glassware was soaked overnight in 20 mM EDTA and *Anabaena* pellets were washed with 10 mM EDTA to chelate all divalent metallic cations, including calcium (Giraldez-Ruiz et al., 1997). Target cyanobacteria were kept in two groups, one with Ca^{2+} (equal to BG-11 medium; 0.25 mM) and the other without Ca^{2+} each having density 0.7–0.8 OD at A_{730} nm. These groups were exposed to heat (45 °C) for 24 h. A third group was cultured in Ca^{2+}--deficient medium at 28 °C for 24 h. Samples were harvested periodically from each culture for analysis of parameters adopted in the experiment.

2.2. Chlorophyll a, carotenoid and phycocyanin content

Three major photopigments, chlorophyll *a*, carotenoid and phycocyanin, were extracted from homogenized *Anabaena* cells in 80% acetone after incubation at 4 °C overnight. The optical density of the supernatant was measured at 665 and 460 nm for chlorophyll *a* and carotenoid, respectively, and the contents were determined according to the method of Myers and Kratz (1955). Phycocyanin content was estimated by resuspending the remaining blue residue in deionized water through 2–3 cycles of freezing and thawing, and then the absorbance was measured at 620 nm (Brody and Brody, 1961).

2.3. Photosynthetic O_2-evolution

Anabaena PCC7120 cells from both Ca^{2+}-supplemented and Ca^{2+}--deprived media were sampled (1 mL) into air tight reaction vessel of O_2--electrode (Digital Oxygen System, Model 10, Rank Brothers, UK) at a constant temperature (28 °C). Photosynthetic O_2 evolution in PAR light of 360 μmol photon m^{-2} s^{-1} was measured for 5 min. The O_2 -evolution was expressed as μmol O_2 evolved mg^{-1} Chl *a* h^{-1}.

2.4. Determination of antioxidative enzymes

Cyanobacterial cells from Ca^{2+}-supplemented and Ca^{2+}--deprived groups incubated at 45 °C were crushed in liquid nitrogen and homogenized in 100 mM EDTA phosphate buffer (pH 7.8) for superoxide dismutase (SOD) activity and 100 mM sodium phosphate buffer (pH 7.8) for catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX) activity, with the addition of 1 mM ascorbate in the case of the APX assay. Homogenates were centrifuged (12,000 rpm for 15 min), and the supernatants were used immediately for different enzymatic assays. SOD activity of cells was assayed by measuring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm in a reaction mixture (3 mL) containing 0.05 mM sodium phosphate buffer (pH 7.8), 33 μM NBT, 10 mM L-methionine, 0.66 mM Na_2EDTA, 0.0033 mM riboflavin and 200 μL of enzyme extract (Beauchamp and Fridovich, 1971). One unit of SOD is defined as the amount of the enzyme that inhibits 50% NBT photoreduction. CAT activity was calculated by measuring the consumption of H_2O_2 at 240 nm (Aebi, 1984). The reaction mixture (3 mL) contained 50 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 100 μL of enzyme extract. GR activity was determined by estimating the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) (Schaedle and Bassham, 1977) in a reaction mixture containing 50 mM potassium phosphate buffer (pH-7.8), 2 mM Na_2 EDTA, 0.15 mM NADPH, 0.5 mM glutathione oxidized (GSSG) and the enzyme extract (200 μL). The reaction was initiated by adding NADPH, and corrections were made for the background absorbance at 340 nm without NADPH. APX activity was determined by measuring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min (Nakano and Asada, 1981). The reaction mixture (3 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and enzyme extract (200 μL).

2.5. Lipid peroxidation assay

Lipid peroxidation was measured as total 2-thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA) (Dhindsa et al., 1981). Target cells were harvested and homogenized in 3 mL of 20% TCA containing 0.5% TBA followed by incubation of homogenates for 30 min (100 °C). Reaction mixtures were cooled quickly and centrifuged (10,000 rpm, 10 min) and the optical density was measured at 532 nm. Corrections were made by deducting the absorbance at 600 nm for unspecific turbidity. The MDA content was calculated using extinction coefficient 155 mM^{-1} cm^{-1}.

2.6. Measurement of apoptosis by DAPI staining

Anabaena cells were transferred to fresh tubes and centrifuged at 5000 rpm (5 min) at room temperature. The supernatant was discarded and the pellet was washed twice in sterile phosphate-buffered saline (PBS). Cells were stained with DAPI (0.5 μg mL^{-1}), incubated (15 min) and washed twice with PBS. A smear of stained cells was prepared on a clean glass slide. Cells were mounted with DABCO and observed under a Nikon ECLIPS 80i fluorescence microscope, captured with a Nikon DS-Ri1 camera (Tokyo, Japan) with original magnification (X600) under oil immersion at room temperature. Images were further processed by LSM image browser and Adobe Photoshop 7.0 software.

2.7. RNA extraction, primer design and RT-PCR

RNA from *Anabaena* cultures was isolated using TRIzol reagent (Sigma Aldrich) as per the instructions given in the manufacturer's