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ENVIRONMENTAL BIOTECHNOLOGY

A high constitutive catalase activity confers resistance to methyl viologen-promoted oxidative stress in a mutant of the cyanobacterium *Nostoc punctiforme* ATCC 29133

Lakshmipyari Devi Moirangthem · Sudeshna Bhattacharya · Karin Stensjö · Peter Lindblad · Jyotirmoy Bhattacharya

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Abstract A spontaneous methyl viologen (MV)-resistant mutant of the nitrogen-fixing cyanobacterium *Nostoc punctiforme* ATCC 29133 was isolated and the major enzymatic antioxidants involved in combating MV-induced oxidative stress were evaluated. The mutant displayed a high constitutive catalase activity as a consequence of which, the intracellular level of reactive oxygen species in the mutant was lower than the wild type (*N. punctiforme*) in the presence of MV. The superoxide dismutase (SOD) activity that consisted of a SodA (manganese-SOD) and a SodB (iron-SOD) was not suppressed in the mutant following MV treatment. The mutant was, however, characterised by a lower peroxidase activity compared with its wild type, and its improved tolerance to externally added H_2O_2 could only be attributed to enhanced catalase activity. Furthermore, MV-induced toxic effects on the wild type such as (1) loss of photosynthetic performance assessed as maximal quantum yield of photosystem II, (2) nitrogenase inactivation, and (3) filament fragmentation and cell lysis were not observed in the mutant. These findings highlight the importance of catalase in preventing MV-promoted oxidative damage and cell death in the cyanobacterium *N. punctiforme*. Such oxidative stress resistant mutants of cyanobacteria are likely to be a better source of biofertilisers, as they can grow and fix nitrogen in an unhindered manner in agricultural fields that are often contaminated with the herbicide MV, also commonly known as paraquat.

Keywords Cyanobacteria · Catalase · Methyl viologen resistance · *Nostoc punctiforme* · Superoxide dismutase · Oxidative stress

Introduction

All aerobic organisms face a continuous challenge of having to deal with reactive oxygen species (ROS) that are inevitably produced during normal course of oxygen metabolism. These ROS include singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) (Imlay 2003). In the oxygenic photosynthetic cyanobacteria and other phototrophs, photosynthetic apparatus is a major source of ROS. For instance, $O_2^{\cdot-}$ is generated at both the reducing and acceptor sides of photosystems (PS), PSI and PSII, respectively. H_2O_2 is formed from either $O_2^{\cdot-}$ dismutation at the reducing side of PSI or from incomplete oxidation of water at the water-splitting complex in the donor side of PSII, which in turn may generate highly toxic $^{\cdot}OH$ through Fenton and Haber–Weiss reactions (Latifi et al. 2009; Mittler 2002; Pospíšil 2009; Simon et al. 2005). Additionally, $^1O_2$ is produced from excitation of oxygen ($^3O_2$, ground triplet state) by the reaction center chlorophyll of the triplet excited state in PSII (Latifi et al. 2009; Mittler 2002; Vass 2012). Adverse environmental conditions, e.g., excess light, nutrient starvation, herbicides, and salinity that create an imbalance between photosynthetic electron transport and electron consumption through downstream events leads to excess ROS production (Jeanjean et al. 2008; Kim et al. 2010; Latifi et al. 2005; Ross et al. 2006). Accumulation of excess ROS causes peroxidation of membrane lipids, modification of nucleic acids and oxidative inactivation of proteins, thereby killing

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L. D. Moirangthem · S. Bhattacharya · J. Bhattacharya (✉)
Department of Biotechnology, Mizoram University, PB No. 190, Aizawl 796004, Mizoram, India
e-mail: jyotirmoyb@rediffmail.com

K. Stensjö · P. Lindblad
Department of Chemistry-Ångström Laboratory, Uppsala University, Box 523, 751 20 Uppsala, Sweden

cells. ROS have also been suggested to inhibit PSII activity through direct degradation of PSII core-protein D1, or prevention of its de novo synthesis in phototrophs (Vass 2012).

All photosynthetic organisms, including cyanobacteria, are equipped with an array of both enzymatic and non-enzymatic antioxidants to limit the accumulation of ROS and prevent cell death (Latifi et al. 2009; Mittler 2002). Superoxide dismutase (SOD) constitutes the first line of defense among antioxidative enzymes. SOD detoxifies $O_2^{\cdot-}$ to molecular oxygen and H_2O_2 (Pilon et al. 2011; Simon et al. 2005). The iron- (SodB) and manganese- (SodA) containing SODs are more prevalent in filamentous, nitrogen-fixing cyanobacteria (Priya et al. 2007); biochemical and genetic studies have pointed towards a more effective role of SodA in prevention of oxidative damage (Nomura et al. 2006; Raghavan et al. 2011). An increased susceptibility of photosynthetic apparatus and nitrogen-fixing machinery was noted in a SodA-inactivated mutant of *Anabaena* PCC 7120 under high light induced oxidative stress (Zhao et al. 2007). Conversely, overexpression of SodA in the same cyanobacterium led to increased tolerance to oxidative stress induced by methyl viologen (MV; 1,1′-dimethyl-4,4′-bipyridinium), a redox-cycling herbicide, also commonly known as paraquat (Raghavan et al. 2011). However, the role of SodB remains controversial; a SodB defective mutant of *Synechococcus* PCC 7942 was sensitive to MV (Thomas et al. 1998), whereas its increased expression was found to be detrimental to the growth of *Anabaena* PCC 7120 (Raghavan et al. 2011). Overexpression of SOD alone in chloroplasts of higher plants did not always result in better plant performance under stress (Pilon et al. 2011; Tepperman and Dunsmuir 1990). One of the reasons for such an inconsistency may be a rise in the level of SOD-derived H_2O_2 without a parallel rise in H_2O_2-detoxification activity. Therefore, balanced detoxification of both $O_2^{\cdot-}$ and H_2O_2 is necessary to combat oxidative stress effectively (Latifi et al. 2009; Mittler 2002; Pilon et al. 2011). Indeed, combined overexpression of SOD and ascorbate-dependent peroxidase (H_2O_2 scavenger) has been shown to confer enhanced tolerance not only to oxidative stress but also to high temperature stress in potato plants (Kim et al. 2010). A *Synechocystis* PCC 6803 mutant defective in PSI-reducing side protein PsaE was found to be tolerant to high light as a result of increased expression of SodB and catalase–peroxidase (Jeanjean et al. 2008).

In cyanobacteria, detoxification of H_2O_2 is catalysed by catalases, catalase–peroxidase, thiol-dependent peroxiredoxins, and ferritin-like proteins (Bernroitner et al. 2009; Latifi et al. 2009; Perelman et al. 2003; Pérez-Pérez et al. 2009; Tichy and Vermaas 1999). A heme-dependent bifunctional catalase–peroxidase present in *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 contributes substantially to H_2O_2-detoxification and is required to protect cells from high concentrations of exogenously added H_2O_2 (Perelman et al. 2003; Tichy and Vermaas 1999). Recently, overexpression of a monofunctional manganese–catalase has also been shown to protect *Anabaena* PCC 7120 from oxidative stress (Banerjee et al. 2012). Unlike in higher plants, ascorbate- and glutathione-dependent peroxidases are not found in cyanobacteria (Tichy and Vermaas 1999). Instead, thiol peroxidases may play an important role in scavenging H_2O_2 and other organic peroxides as they are upregulated under a variety of stress conditions in *Synechococcus* PCC 7942, *Synechocystis* PCC 6803, and *Anabaena* PCC 7120 (Latifi et al. 2009; Perelman et al. 2003; Pérez-Pérez et al. 2009; Tichy and Vermaas 1999).

MV is generally used to decipher oxidative stress tolerance mechanisms in both photosynthetic and non-photosynthetic organisms. MV primarily accepts electrons from the iron–sulphur cluster Fe–S_A/Fe–S_B of PSI and limits NADPH formation and carbon dioxide fixation in photosynthetic organisms (Fujii et al. 1990). A continuous cycle of MV reduction and oxidation converts molecular oxygen to membrane-impermeable $O_2^{\cdot-}$, which on further metabolism produces membrane-permeable H_2O_2 and $^{\cdot}OH$ (Babbs et al. 1989). As MV leads to production of a variety of ROS, it is logical to assume that a mutant able to resist MV-toxicity will show enhanced expression of ROS-detoxifying enzymes and this may help us to understand the nature of antioxidative enzymes that participate in combating severe oxidative stress. Therefore, in this study, we isolated a spontaneous MV-resistant mutant (MV-R) of the nitrogen-fixing cyanobacterium *N. punctiforme* ATCC 29133 to examine the modifications in ROS scavenging antioxidant defence machinery that are responsible for preventing oxidative stress induced by MV.

Materials and methods

Strains and culture conditions

Axenic clonal cultures of the cyanobacterium *N. punctiforme* (wild type (WT)) ATCC 29133 and its MV-R derivative were grown photoautotrophically at 25 °C in $BG11_0$ medium (BG11 without combined nitrogen, N_2-fixing condition, Rippka et al. 1979), pH 7.5. During growth, the cultures were continuously illuminated with cool fluorescent light at a fluence rate of 20–23 μmol photons m^{-2} s^{-1}.

Isolation of the MV-R mutant

Naturally occurring spontaneous MV-R mutants of *N. punctiforme* were isolated by plating cells equivalent to 2 μg chlorophyll *a* (Chl *a*) on solid $BG11_0$ medium containing 1.2 μM MV. The few colonies that survived after 3 weeks of incubation under continuous light, as described above, were picked up and sub-cultured on 1.2 μM MV-containing plates three to five times. Finally, one such mutant colony that

showed normal growth (measured by increase in Chl *a* concentration) in liquid $BG11_0$ medium-containing MV was used for further analyses. Routine maintenance of the mutant was done on MV-containing plates.

Determination of oxidative stress tolerance

Actively growing cultures of WT and its MV-R mutant were inoculated at equal Chl *a* concentration (approximately 0.5 μg/ml) in $BG11_0$ medium and incubated in presence of MV (0–0.5 μM) for 4 days or in presence of 1 mM H_2O_2 for 2 days. Tolerance was assessed by monitoring the growth of such cultures in terms of Chl *a* content (Mackinney 1941).

Microscopy

The number of heterocysts was counted on BX-51 microscope (Olympus, Tokyo, Japan) using five to eight filaments (approximately 500 cells) of WT and its MV-R mutant treated or untreated with MV. The frequency of heterocysts was calculated as percentage of total cells. Images (×400 magnification) of such cyanobacterial cultures were obtained with a ProgRes C3 cool camera (Jenoptik AG, Jena, Germany) fitted with the same microscope.

Determination of ROS level

The intracellular ROS content was quantified using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Life Technologies, Carlsbad, California, USA) (Latifi et al. 2005). CM-H_2DCFDA passively diffuses into cells and is deacetylated to form 2′,7′-dichlorodihydrofluorescein (DCFH). DCFH reacts with H_2O_2, $^{\cdot}OH$ radicals and peroxynitrite anions to form the fluorescent product 2′,7′-dichlorofluorescein (DCF). An increase in DCF fluorescence intensity reflects an increase in ROS concentration. Briefly, WT and its MV-R mutant were grown for 1 day in the presence and absence of 0.3 μM MV, washed twice with 10 mM phosphate buffer (pH 7.0) and incubated with 10 μM CM-H_2DCFDA for 15 min at room temperature in darkness. Fluorescence emission was measured immediately in such cultures at 520 nm after excitation at 488 nm on a spectrofluorometer (SpectraMax M2, Molecular Device, Sunnyvale, California, USA). The total hydroperoxide levels were measured by a modified ferrous oxidation/xylenol orange assay method (Nomura et al. 2006) in cultures grown under same conditions as described above. After MV treatment, cultures were harvested by centrifugation at 2,300×*g* for 5 min and washed with fresh $BG11_0$ medium. The cell pellets were resuspended in 0.8 ml of methanol containing 0.01 % butylated hydroxytoluene (BHT), 0.1 ml of Reagent A (2.5 mM ammonium Fe(II) sulphate, 0.25 M sulphuric acid) and 0.1 ml of Reagent B (40 mM BHT, 1.25 mM xylenol orange in methanol). The mixture was incubated for 30 min at room temperature and then centrifuged at 10,000×*g* to remove any cell debris. The supernatant fraction was used to measure the absorbance at 560 nm and the concentration of hydroperoxides was determined using the extinction coefficient ($\varepsilon_{560}=4.3\times10^4$ M^{-1} cm^{-1}) (Nomura et al. 2006).

Protein extraction and Western blotting

WT and its MV-R mutant grown for 1 day in presence and absence of 0.3 μM MV were harvested by centrifugation at 2,300×*g* for 5 min at room temperature and the cell pellet washed twice with 36 mM potassium phosphate buffer (pH 7.4). For preparation of cell-free extract, the cells were resuspended in the same buffer supplemented with protease inhibitors (phenylmethylsulphonyl fluoride, 1 mM and 5 μM each of leupeptin and pepstatin), and broken by ultrasonication at 4 °C. The cell extracts were then centrifuged at 10,000×*g* for 30 min and total protein concentration was measured in the supernatant fraction by using Bio-Rad (Hercules, California, USA) protein assay kit (Bradford 1976). Twenty micrograms of total soluble protein was run on a 12 % SDS-PAGE gel (Laemmli 1970) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Bio-Rad Mini Trans-Blot Transfer cell. Nitrogenase was detected using anti-nitrogenase (NifH) antibody (1:1,000; Agrisera, Vännäs, Sweden). Antibody dilution was done in TBS (50 mM Tris buffer at pH 7.5, 150 mM NaCl) containing 0.5 % bovine serum albumin. The membrane was incubated in primary antibody for 1 h. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was added as secondary antibody in 1:10,000 dilution and the membrane incubated further for 2 h. Finally, colorimetric staining with a mixture of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt (NBT and BCIP; Merck, Bengaluru, India) was used to detect the nitrogenase protein.

Superoxide dismutase activity measurements

SOD activity was determined by in-gel analysis (Beauchamp and Fridovich 1971). WT and its MV-R mutant grown for 1 day in the absence and presence of 0.3 μM MV were harvested, and cell-free extracts were obtained as described above. Total protein equivalent to 40 μg were separated by electrophoresis on 10 % nondenaturing polyacrylamide gel containing 10 % glycerol, and then the gel was soaked in 28 μM *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine and 28 μM riboflavin in 36 mM potassium phosphate buffer (pH 7.6) for 30 min. The gel was further soaked for 10 min in 2.5 mM nitroblue tetrazolium and illuminated with cool fluorescent white light at an intensity of 500 μmol m^{-2} s^{-1} for colour

development. The SOD activity was observed as an achromatic zone on a purple-blue background of the gel. Individual isoforms were identified by 30 min of treatment with 10 mM H_2O_2 before staining of the gel. SOD isoforms were quantified by densitometry using Genesys tools software (Syngene, Cambridge, UK) and results are expressed as a percentage of each isoform compared with *N. punctiforme* WT, arbitrarily considered 100 %.

Catalase and peroxidase activity measurements

Catalase activity (H_2O_2 detoxification activity) was determined in cell-free extracts of WT and its MV-R mutant (both grown for 1 day in the absence and presence of 0.3 μM MV, respectively) by monitoring the rate of H_2O_2 detoxification at 240 nm using an extinction coefficient of 43.6 M^{-1} cm^{-1} (Beers and Sizer 1952). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and 50 μg of total protein. Peroxidase activity was assayed in cell-free extracts by following the rate of pyrogallol oxidation at 430 nm using an extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance and Maehly 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM pyrogallol, 0.1 mM H_2O_2, and 50 μg of total protein.

Pulse amplitude modulated fluorometry of Chl *a*

PSII-mediated electron transport rate (ETR) was measured using a Dual-Pulse Amplitude Modulated-100 fluorometer (Waltz, Effeltrich, Germany) in the cultures of WT and its MV-R mutant. Actinic light intensities were increased stepwise from 0 to 1976 μmol photons m^{-2} s^{-1}, and the steady-state (F) and maximal (F_m') fluorescence levels were determined after a 3-min adaptation period after each step. The maximal efficiency of PSII photochemistry was determined as F_v/F_m, where $F_v=(F_m–F_0)$ and F_m and F_0 are the maximal and minimal fluorescence yields in the dark adapted state, respectively (Genty et al. 1989). F_0 was determined from the cultures of WT and its MV-R mutant grown for 1 day in the absence and presence of 0.3 μM MV after a 15-min dark adaptation followed by illuminating the sample with a low-irradiance measuring light (0.24 μmol m^{-2} s^{-1}). A saturating pulse of white light (1.6 s, 1,000 μmol photons m^{-2} s^{-1}) was then applied to determine F_m.

Bioinformatic analysis of *N. punctiforme* SODs

The amino acid sequences of SOD isoforms were retrieved from cyanobase (http://genome.microbedb.jp/cyanobase) and their physico-chemical properties were analysed using ProtParam tool at Expasy (http://www.expasy.ch/tool/ProtParam).

Statistical analysis

Results are presented as mean±standard error of mean from two to three independent experiments with two replicates. Significant differences in photosynthetic performance between control and test samples were analysed with one-way analysis of variance using Statistical Package for the Social Sciences (SPSS 11.5, SPSS Inc. Chicago, Illinois, USA). The probability (P) values less than 0.05 were considered significantly different.

Results

Growth and morphology of *N. punctiforme* WT and MV-R mutant

Growth response of the WT and its MV-R mutant was evaluated in the absence and in the presence of MV in $BG11_0$ medium for 4 days. In the absence of MV, the growth of the MV-R mutant was similar to its WT (Fig. 1a, b). However, growth of the WT was severely affected in the presence of

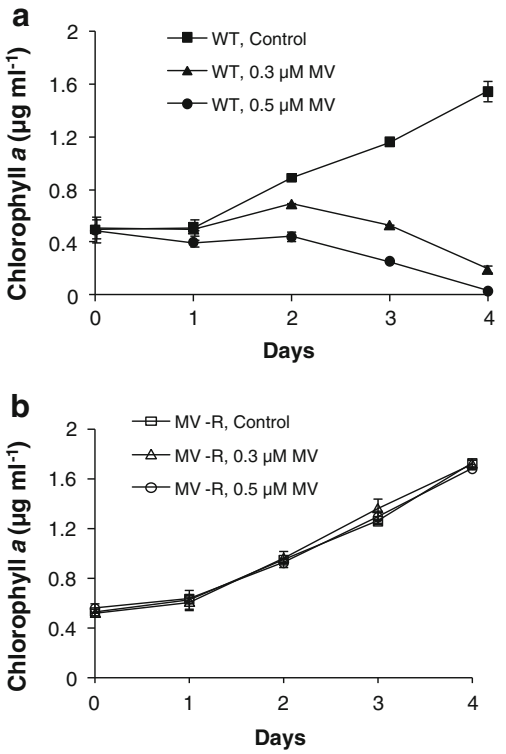

Fig. 1 Effect of MV on growth of *N. punctiforme* WT and its MV-R mutant. The actively growing cultures of WT and MV-R mutant were inoculated at equal Chl *a* concentration (approximately 0.5 μg ml^{-1}) in fresh $BG11_0$ medium containing increasing concentrations of MV (0–0.5 μM) and the growth of WT (**a**) and MV-R mutant (**b**) was assessed at periodic intervals by measuring Chl *a* content. *Error bars*, sometimes smaller than the symbols, represent standard error of two biological and technical replicates

MV. Compared with control (untreated WT), the growth dropped to 45 and 21 % at the end of 3 days and further to 13 and 2 % at the end of 4 days following treatment with 0.3 and 0.5 μM MV, respectively. As opposed to the WT, similarly treated MV-R mutant showed no reduction in growth during the same time period. Light microscopy examination of 3-day-old and 0.3-μM MV-treated WT cultures revealed massive fragmentation of filaments and cell lysis, whereas size and shape of cells in filaments was found to be fully preserved in the MV-R mutant (Supplementary Fig. S1). These results indicate that acquisition of resistance to MV is not accompanied by any apparent morphological change in the MV-R mutant. All further investigations were performed on cyanobacterial cultures incubated for one day with 0.3 μM MV.

Effect of MV on heterocyst frequency and nitrogenase

Heterocysts are specialised cells in the filaments of *N. punctiforme* that harbour nitrogenase, an enzyme necessary for nitrogen fixation. The frequency of heterocysts in the filaments of the WT and its MV-R mutant was similar (5-6 %) in the absence of MV. After treatment with MV, WT filaments showed a 50 % drop in heterocyst frequency, but the mutant retained its normal heterocyst level (Fig. 2a). The nitrogenase levels were analysed by immunodetection of NifH protein in cell-free extracts of the same cultures. An approximately 32.5 kDa NifH polypeptide of similar intensity was identified in the WT and its mutant. MV treatment resulted in a severe decline in the level of this polypeptide in the WT, whereas it remained unaffected in the mutant (Fig. 2b). These results indicate that the enzyme nitrogenase is more sensitive to MV than is the stability of heterocysts in the WT. A normal heterocyst frequency coupled with no loss of nitrogenase, and unabated growth of the MV-treated mutant in $BG11_0$ medium suggests that its ability to fix atmospheric nitrogen remains intact.

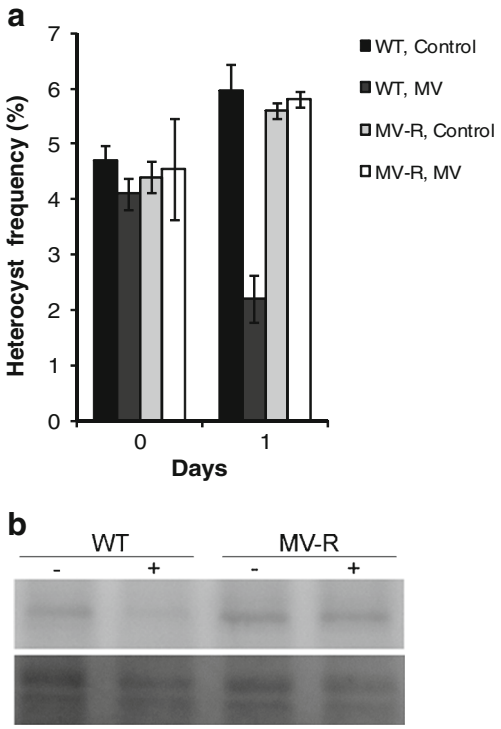

Fig. 2 Effects of MV on heterocyst frequency (**a**) and nitrogenase level (**b**) in 1-day-old 0.3-μM MV-treated or untreated cultures of *N. punctiforme* WT and MV-R mutant. Heterocyst frequency was counted from approximately 500 cells under the light microscope (×400 magnification). *Error bars* represent standard error of three independent experiments. For immunoblot analysis, solubilised protein extracts containing 20 μg of protein were resolved on 12 % SDS-PAGE before being electroblotted on PVDF membrane. NifH protein (approximately 32.5 kDa) was detected using rabbit anti-nitrogenase antibody. A part of the photograph of SDS-PAGE analysis is included in the bottom panel to show that each sample contained equal amounts of proteins. The *plus* and *minus* symbols denote the growth of cultures ($BG11_0$ medium) in the presence and absence of MV, respectively

Photosynthetic performance of WT and MV-R mutant

Photosynthetic efficiency was monitored in the WT and its MV-R mutant before and after treatment with MV. The maximal quantum yield of PSII (F_v/F_m) decreased significantly (30 %; $P<0.05$) in MV-treated WT cultures compared with control cultures after 1 day (Fig. 3a). The untreated mutant displayed a slightly lower (7 %) photochemical activity than the WT (the difference is statistically not significant; $P>0.05$), which remained unaffected in the presence of MV. We compared the relative ETR of the WT and its MV-R mutant as a function of increasing light intensities (Fig. 3b). The highest ETR, for both the WT and its MV-R mutant, was obtained at a light intensity of 240 μmol m^{-2} s^{-1}, whereas at 1311 μmol photons m^{-2} s^{-1} and above, the ETRs were completely abolished in both strains. These results suggest that the photosynthetic efficiency of PSII, including ETR activity of the mutant is comparable to that of WT.

Effect of MV on intracellular ROS content

Since MV stimulates harmful ROS production, the intracellular content of ROS was determined in the WT and MV-R mutant. A cell-permeable probe, CM-H_2DCFDA, which reflects the intracellular levels of H_2O_2, ·OH radicals, and peroxynitrite anions, was used for this purpose. As shown in Fig. 4a, DCF fluorescence in the WT and its MV-R mutant was not significantly different from each other under normal growth conditions. However, exposure to MV produced a marked increase in DCF fluorescence in the WT, which was 2-fold higher than that in the mutant. We also compared total hydroperoxide levels between the two strains grown under the same conditions (Fig. 4b). Consistent with DCF fluorescence results, the total hydroperoxide level in the WT was found to

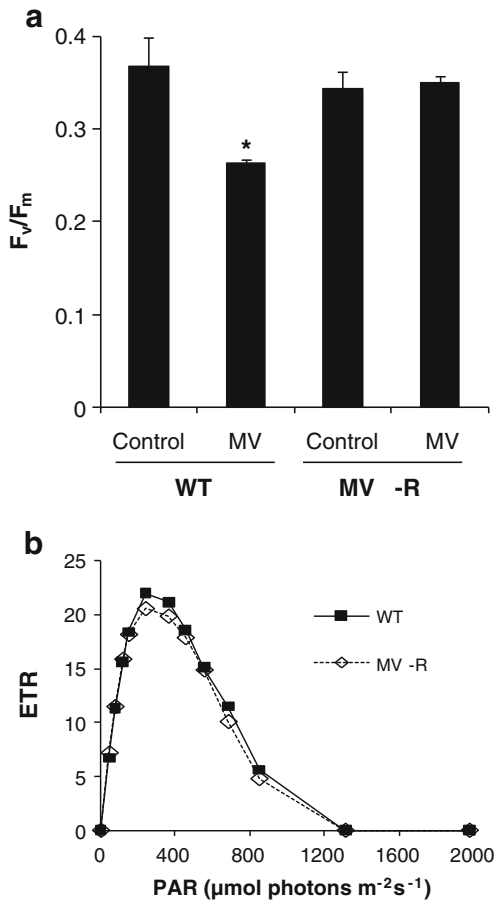

Fig. 3 PSII photochemical efficiency (**a**) and ETR (**b**) of *N. punctiforme* WT and MV-R mutant after 1 day of growth in the absence (control) and presence of 0.3 μM MV. Photochemical efficiency was measured in cultures (10 μg Chl *a* ml^{-1}) as F_v/F_m at a light intensity of 1,000 μmol m^{-2} s^{-1} after 15 min dark incubation. *Error bars* represent standard error of two biological and technical replicates. *$P<0.05$, significant difference of F_v/F_m value between the treated and untreated cultures of WT. The measurement of ETR in the WT and its mutant was performed at increasing light intensities (0–1976 μmol m^{-2} s^{-1}) in a stepwise manner. A 3-min adaptation period followed each step. The ETR assay was repeated two times under similar conditions and similar results were obtained

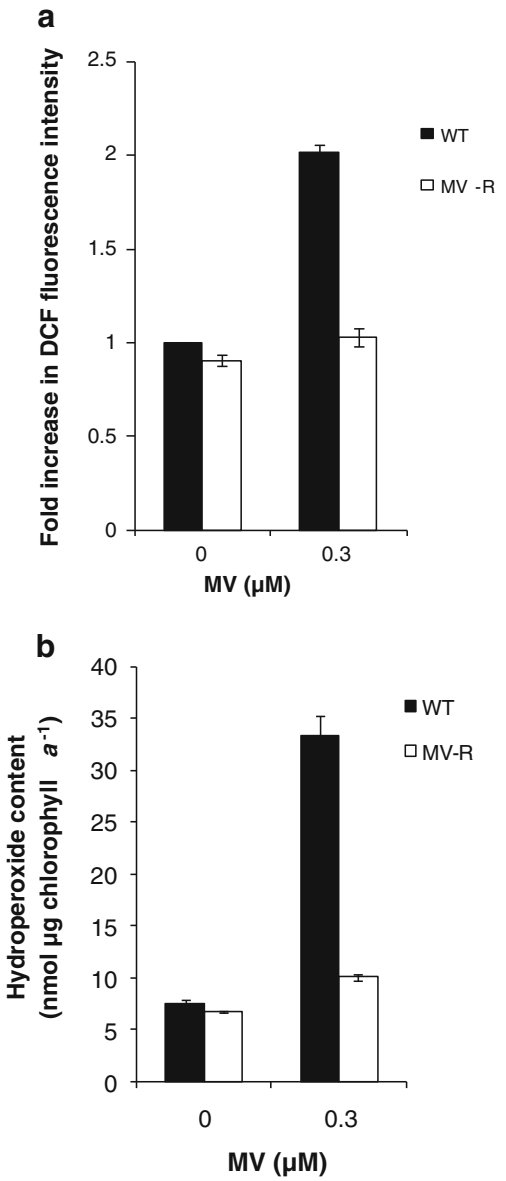

Fig. 4 MV-induced ROS production in the *N. punctiforme* WT and MV-R mutant. The cultures of WT or MV-R mutant were inoculated at 1 μg Chl *a* ml^{-1} in fresh $BG11_0$ medium and grown for 1 day in absence and presence of 0.3 μM MV. Such cells were washed with 10 mM phosphate buffer and used for DCF fluorescence (**a**) and total hydroperoxide (**b**) measurements. Fluorescence intensity is expressed as fold increase compared with WT control cultures. *Error bars*, sometimes smaller than the symbols, represent standard error of two biological and technical replicates

be much higher (3-fold) than in the MV-R mutant after incubation with MV, whereas untreated cultures of both strains showed similar hydroperoxide levels. These results indicate that the accumulation of ROS normally executed by MV is prevented in the MV-R.

Superoxide dismutase activity of WT and MV-R mutant

As SOD is the primary enzyme responsible for dismutation of MV-induced $O_2^{\cdot-}$ into molecular oxygen and H_2O_2, total SOD activity was compared between the WT and its MV-R mutant by in-gel analysis. The results presented in Fig. 5a show two SOD activities in control cultures of the WT. The fast migrating band was identified as SodB based on its sensitivity to H_2O_2, whereas the H_2O_2-insensitive slow migrating band was SodA (Fig. 5b). The activities of both SOD isoforms were more or less similar in untreated cultures of the WT and the mutant. MV exerted a differential impact on the activities of both SODs in the WT; SodB being more sensitive than SodA (78 % reduction in SodB activity compared with 50 % reduction in SodA activity as revealed by densitometry of bands, Supplementary Table S1). However, unlike the WT, both SOD activities were retained in the MV-treated mutant, suggesting that it can efficiently detoxify MV-induced $O_2^{\cdot-}$.

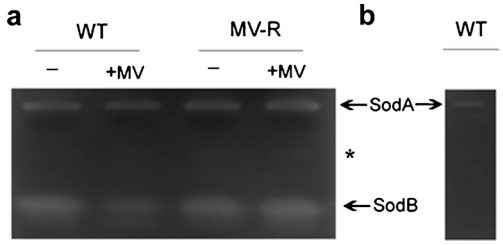

Fig. 5 In-gel assay of SOD activity in the *N. punctiforme* WT and its MV-R mutant after 1 day of incubation in the absence and presence of 0.3 μM MV. **a** Protein extracts from the cultures (40 μg total protein) were electrophoresed on 10 % native gels and stained for SOD activity. *Asterisk* indicates possible SodA-B heterodimer (refer to the discussion section). **b** Isoforms were identified by incubating the gel in 10 mM H_2O_2 for 30 min prior to staining. The SOD assay was repeated at least five times with similar results, and a representative gel is shown

Catalase and peroxidase activities of WT and MV-R mutant

SOD-derived H_2O_2 must be detoxified by catalase, and/or peroxidase activities to prevent formation of toxic $^{\cdot}OH$. Analysis of catalase activity, as determined by the decomposition of H_2O_2, revealed a 3-fold higher activity in the mutant compared with its WT under normal growth condition (Fig. 6a). The mutant sustained the same high activity after MV treatment. Although WT cultures showed nearly 1.8-fold increase in catalase activity as a response to MV, the activity was still lower than that in the mutant. As shown in Fig. 6b, the peroxidase activity in MV-untreated and treated cultures of the WT were similar (0.31±0.014 and 0.325±0.007 unit mg protein^{-1}, respectively). However, the mutant had a lower peroxidase activity compared with the WT, irrespective of the presence or absence of MV (0.22±0.014 and 0.195±0.02 unit mg protein^{-1}, respectively). The mutant was more tolerant to H_2O_2 compared with its WT (Fig. 6c), most likely as a result of the much higher H_2O_2 detoxification activity (Fig. 6a).

Discussion

Cyanobacteria are equipped with various enzymatic antioxidants, including SODs, catalases, peroxidases and DNA-binding proteins from starved cells (Dps) that maintain intracellular ROS at a low level (Bernroitner et al. 2009; Ekman et al. 2014; Latifi et al. 2009; Pérez-Pérez et al. 2009; Priya et al. 2007). However, excess ROS accumulation, as in the presence of the herbicide MV may become lethal to cells (Nomura et al. 2006; Raghavan et al. 2011; Thomas et al. 1998; Tichy and Vermaas 1999). A large body of evidence indicates that increased expression of SODs in combination with catalase or peroxidase helps to protect photosynthetic organisms from oxidative stress (Fujibe et al. 2004; Jeanjean et al. 2008; Kim et al. 2010). In an effort to investigate the constituents of ROS-detoxification machinery that participates during progression of MV-induced oxidative stress in the

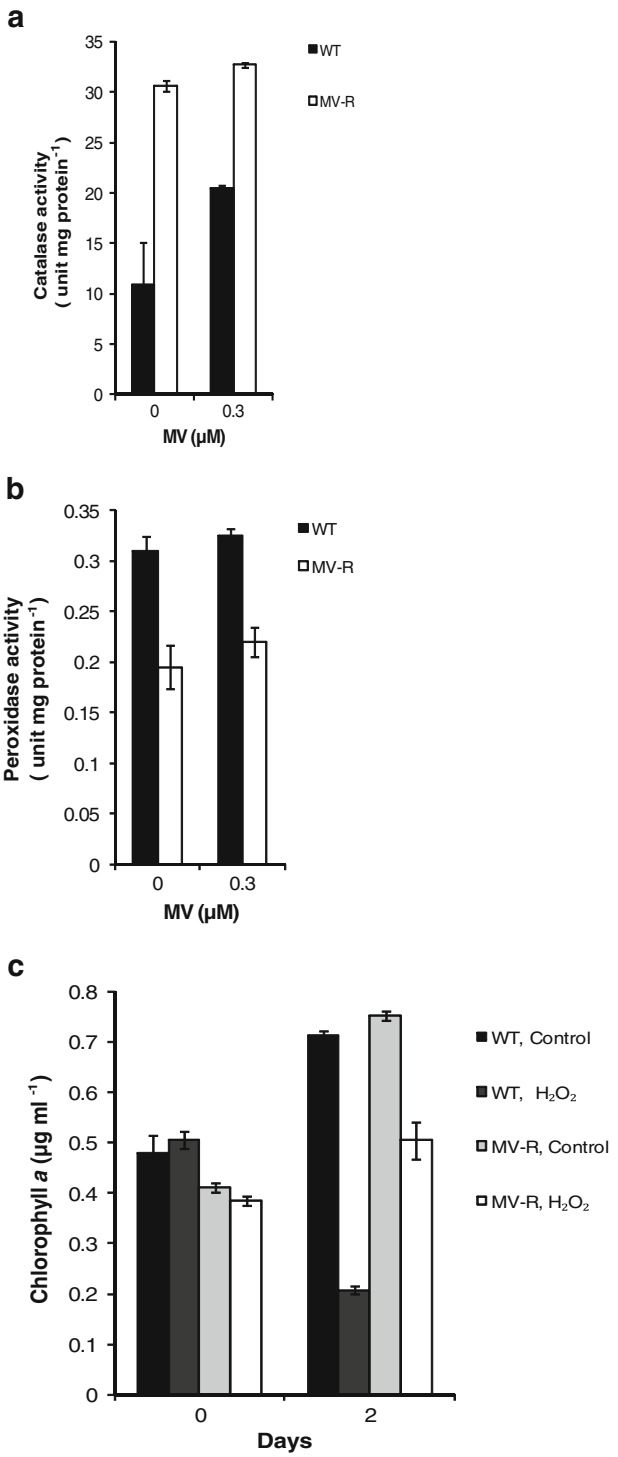

Fig. 6 H_2O_2-detoxification activity and H_2O_2 tolerance of the *N. punctiforme* WT and MV-R mutant. The cultures of WT or MV-R mutant were inoculated at 1 μg Chl *a* ml^{-1} in fresh BG11$_0$ medium and grown for 1 day in absence and presence of 0.3 μM MV. Such cells were washed with 50 mM phosphate buffer (pH 7) and broken by ultrasonication in the same buffer. The crude cell extracts obtained after centrifugation at 2,300×*g* were used (50 μg total protein) for determination of catalase (**a**) and peroxidase (**b**) activities. **c** Growth responses of the WT and MV-R mutant were measured by Chl *a* content after 2 days of incubation in BG11$_0$ medium with or without 1 mM H_2O_2. *Error bars* represent standard error of three independent experiments

nitrogen-fixing cyanobacterium *N. punctiforme*, we isolated a mutant (MV-R) that can survive prolonged exposure to MV. MV primarily promotes $O_2^{\cdot-}$ formation, and subsequently other ROS within cells (Babbs et al. 1989; Fujii et al. 1990). SOD detoxifies $O_2^{\cdot-}$ into molecular oxygen and H_2O_2 (Imlay 2003; Pilon et al. 2011; Simon et al. 2005). A SodB and a SodA defective mutant of *Anabaena* PCC 7120 (Agervald et al. 2010; Zhao et al. 2007), and a SodB inactivated strain of the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 have earlier been shown to be sensitive to oxidative stress (Thomas et al. 1998). We observed two SOD isoforms, namely SodA and SodB in WT *N. punctiforme*. Activities of both SodA and SodB isoforms declined in 0.3 μM MV-exposed WT cells; the latter was found to be more sensitive than the former (Fig. 5a). These findings indicate that SODs are MV-sensitive components of the *N. punctiforme* antioxidant machinery, which is consistent with a study performed by Raghavan et al. (2011), which showed a progressive loss of SodA and SodB activities in *Anabaena* PCC 7120 cells exposed to 0.2 μM MV. In the case of the MV-R mutant, activities of both SODs were similar to WT under normal growth conditions. Moreover, the SodA and SodB activities were retained in the mutant in the presence of MV, suggesting that MV-generated $O_2^{\cdot-}$ is efficiently detoxified by SODs in the mutant. We also noted a weak band (marked with an asterisk in Fig. 5a) in addition to SodA and SodB, which migrated faster than SodA but slower than SodB. Currently, the exact identity of this band is not known. Using a quantitative shotgun proteomics strategy, three SODs have been shown to be expressed in *N. punctiforme* of which two are predicted to belong to the SodA family (Ow et al. 2009). A bioinformatic analysis of *N. punctiforme* genome revealed that the open reading frames (ORFs) Npun_F5478 and Npun_F1605 encodes for the two SodA isoforms. The ORF Npun_F5478 encodes a subunit with theoretical molecular mass of 28.773 kDa and isoelectric point (p*I*) of 9.16, and ORF Npun_F1605 encodes a subunit with molecular mass of 30.0772 kDa and p*I* of 9.66. The SodB isoform with a subunit molecular mass of 22.429 kDa and p*I* of 5.67 is encoded by the ORF Npun_R6491. Provided that each of the two SodA proteins (with little difference in their molecular mass and pI values) are homodimers in their native form (SODs from *Anabaena* PCC 7120 are homodimers, Li et al. 2002; Regelsberger et al. 2002), one would expect that the mobility of these proteins would be very similar and hence they will be located in close proximity to each other on native gels. The weak band observed by us is unlikely to be the second SodA, but possibly a heterodimer of SodA and SodB, as it was observed midway between the homodimers of SodA and SodB. Such SOD activity bands have also been observed in *Anabaena* PCC 7120 (Li et al. 2002; Raghavan et al. 2011), predicted to be heterodimers/oligomers of SodA and SodB. However, the reason for lack of visibility of a second SodA activity is not clear, but this may be due to low expression of the protein under the experimental conditions used during this study or inadequate separation of the two SodA isoforms.

In filamentous cyanobacteria, SodA is normally associated with thylakoid membranes, which houses the photosynthetic apparatus, whereas SodB is localised in the cytosol (Latifi et al. 2009; Li et al. 2002; Priya et al. 2007). It has been shown that inactivation of SodA results in oxidative damage to photosynthetic apparatus in *Anabaena* PCC 7120 (Zhao et al. 2007). Consistent with this finding, an increased activity of a membrane-bound SOD (presumably SodA) has been found to prevent loss of photosynthetic activity in presence of MV and high light in a cytochrome oxidase defective mutant (*ctaDII*) of *Synechococcus* PCC 7002 (Nomura et al. 2006). We found that the maximal photochemical efficiency of PSII (F_v/F_m) was affected in WT cells after MV treatment. However, the MV-R mutant exhibited only a marginal decrease in F_v/F_m values compared with its WT under normal growth conditions and its activity was not affected by MV (Fig. 3a). The ETR in the mutant was also found to be more or less similar to that of the WT at increasing light intensities suggesting that functional efficiency of PSII is intact in the mutant (Fig. 3b). A sustained SodA activity in the presence of MV is probably involved in protecting PSII of the MV-R mutant from MV-induced $O_2^{\cdot-}$ damage. However, H_2O_2 derived from SOD activity may directly or indirectly (through $^{\cdot}OH$ formation by Fenton reaction) affect vital cellular components, including SodB, and nitrogenase in cyanobacteria (Raghavan et al. 2011; Tözüm and Gallon 1979). We did not observe any significant decrease in SodB activity (Fig. 5a), heterocyst frequency or in nitrogenase levels in MV-treated mutant as opposed to its WT (Fig. 2a, b), thus raising the possibility that H_2O_2 or other ROS levels are possibly kept low in the mutant by enhanced expression of other enzymatic antioxidants. Indeed, measurement of DCF fluorescence and total hydroperoxide level indicated a much lower intracellular ROS pool in the mutant compared with its WT following MV exposure (Fig. 4a, b). As a result of this, bleaching of photosynthetic pigments, fragmentation of filaments, cell lysis, and cell death was observed in the WT but not in the mutant (Fig. 1a, b; Supplementary Fig. S1). However, it may be noted that the two ROS detection methods used in the present study produced a differential increase in ROS levels in MV-treated WT cells; a 5-fold increase was observed in hydroperoxide levels, whereas a 2-fold increase was detected in DCF fluorescence intensity compared with their respective control cultures, possibly due to lipid hydroperoxides, which are detected by the former but not by the latter method.

In cyanobacteria, H_2O_2 generated by MV is detoxified by peroxidases, catalases, and catalase–peroxidises but not by ascorbate-dependent peroxidase as the gene encoding for such an activity is absent from cyanobacterial genomes (Bernroitner et al. 2009; Latifi et al. 2009; Perelman et al. 2003; Pérez-Pérez et al. 2009; Tichy and Vermaas 1999). Evidence for catalase-mediated oxidative stress protection has earlier been

presented in cyanobacteria, and it has been shown that overexpression of a monofunctional non-heme manganese catalase enhances the tolerance of *Anabaena* PCC 7120 to oxidative stress (Banerjee et al. 2012). A similar protective role against oxidative stress was also noted in transgenic tobacco plants overexpressing a catalase (*KatE*) from *Escherichia coli* (Miyagawa et al. 2000). Another group of proteins of importance for protection against oxidative stress in cyanobacteria are the ferritin-like proteins including Dps proteins (Chiancone and Ceci 2010; Latifi et al. 2009). Ferritin-like proteins from *Anabaena* PCC 7120 have also been shown to detoxify H_2O_2 (Wei et al. 2007) as well as to protect cells against oxidative stress when recombinantly expressed in *E. coli* (Narayan et al. 2010). Moreover, inactivation by gene deletion of one of the Dps proteins in *N. punctiforme* has suggested an important physiological role for this protein in the tolerance of cells against peroxide (Ekman et al. 2014). We found that the peroxidase activity in the WT was more or less similar regardless of the presence or absence of MV, whereas the mutant exhibited a lower peroxidase activity in comparison to its WT under both conditions (Fig. 6b). The reason for low peroxidase activity in the mutant is not known; nevertheless it is possible that H_2O_2-detoxifying enzymes other than peroxidase, e.g., catalase, may be important for detoxification of MV-derived H_2O_2 and protection against MV. Accordingly, an enhanced constitutive catalase activity was observed in the mutant compared with its WT (Fig. 6a). This high constitutive H_2O_2 detoxification activity in the MV-R mutant also improved its tolerance to exogenous H_2O_2 over WT (Fig. 6c). The possibility that this H_2O_2 detoxification activity may originate from ferritin-like proteins, including Dps proteins cannot be ruled out entirely, and needs further investigation. *N. punctiforme* does not possess a bifunctional catalase–peroxidase that detoxifies exogenous H_2O_2 as in unicellular cyanobacteria, but possess three ORFs encoding for two manganese-dependent (Npun_F0233 and Npun_R4582) and one iron-dependent catalase (Npun_F5237) (Bernroitner et al. 2009). Current efforts are now directed towards identifying the gene/enzyme, which is responsible for the H_2O_2 detoxification activity that is upregulated in the MV-R mutant of *N. punctiforme*.

Taken together, our findings suggest that a high constitutive catalase activity, and not peroxidase activity, seems to be the basis of resistance to MV and improved tolerance to externally added H_2O_2 in the MV-R mutant of the cyanobacterium *N. punctiforme*. As a consequence, the mutant was also able to sustain SodA and SodB activities in the presence of MV and displayed low levels of intracellular ROS, normal photosynthetic performance and nitrogenase levels, lack of filament fragmentation and cell lysis. Such oxidative stress resistant mutants of cyanobacteria are likely to be highly useful as biofertilisers because they can grow and fix atmospheric nitrogen in the presence of MV, a common and widely used herbicide in agricultural fields.

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References

Agervald Å, Baebprasert W, Zhang X, Incharoensakdi A, Lindblad P, Stensjö K (2010) The CyAbrB transcription factor CalA regulates the iron superoxide dismutase in *Nostoc* sp. strain PCC 7120. Environ Microbiol 12:2826–2837

Babbs CF, Pham JA, Coolbaugh RC (1989) Lethal hydroxyl radical production in paraquat-treated plants. Plant Physiol 90:1267–1270

Banerjee M, Ballal A, Apte SK (2012) Mn-catalase (Alr0998) protects the photosynthetic, nitrogen-fixing cyanobacterium *Anabaena* PCC 7120 from oxidative stress. Environ Microbiol 14:2891–2900

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287

Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195: 133–140

Bernroitner M, Zamocky M, Furtmüller PG, Peschek GA, Obinger C (2009) Occurrence, phylogeny, structure, and function of catalase and peroxidases in cyanobacteria. J Exp Bot 60:423–440

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chance B, Maehly AC (1955) Assay of catalase and peroxidase. Methods Enzymol 2:764–775

Chiancone C, Ceci P (2010) The multifaceted capacity of Dps proteins to combat bacterial stress conditions: Detoxification of iron and hydrogen peroxide and DNA binding. Biochim Biophys Acta 1800:798–805

Ekman E, Sandh G, Nenninger A, Oliveira P, Stensjö K (2014) Cellular and functional specificity among ferritin-like proteins in the multicellular cyanobacterium *Nostoc punctiforme*. Environ Microbiol. doi:10.1111/1462-2920.12233

Fujibe T, Saji H, Arakawa K, Yabe N, Takeuchi Y, Yamamoto KT (2004) A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive rcd1, is tolerant to supplemental ultraviolet-B irradiation. Plant Physiol 134:275–285

Fujii T, Yokoyama E, Inoue K, Sakurai H (1990) The sites of electron donation of photosystem I to methyl viologen. Biochim Biophys Acta 1015:41–48

Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990:87–92

Imlay JA (2003) Pathways of oxidative damage. Annu Rev Microbiol 57: 395–418

Jeanjean R, Latifi A, Matthijs HCP, Havaux M (2008) The PsaE subunit of photosystem I prevents light-induced formation of reduced oxygen species in the cyanobacterium *Synechocystis* sp. PCC 6803. Biochim Biophys Acta 177:308–316

Kim MD, Kim KYH, Kwon KSY, Yun DJ, Kwak SS, Lee HS (2010) Enhanced tolerance to methyl viologen-induced oxidative stress and high temperature in transgenic potato plants overexpressing the CuZnSOD, APX and NDPK2 genes. Physiol Plant 140:153–162

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Latifi A, Jeanjean R, Lemeille S, Havaux M, Zhang CC (2005) Iron starvation leads to oxidative stress in *Anabaena* sp. strain PCC 7120. J Bacteriol 187:6596–6598

Latifi A, Ruiz M, Zhang CC (2009) Oxidative stress in cyanobacteria. FEMS Microbiol Rev 33:258–278

Li T, Huang X, Zhou R, Liu Y, Li B, Nomura C, Zhao J (2002) Differential expression and localization of Mn and Fe superoxide dismutases in the heterocystous cyanobacterium *Anabaena* PCC 7120. J Bacteriol 184:5096–5103

Mackinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140:315–322

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Miyagawa Y, Tamoi M, Shigeoka S (2000) Evaluation of the defense system in chloroplasts to photooxidative stress caused by paraquat using transgenic tobacco plants expressing catalase from *Escherichia coli*. Plant Cell Physiol 41:311–320

Narayan OP, Kumari N, Rai LC (2010) Heterologous expression of *Anabaena* PCC 7120 all3940 (a Dps family gene) protects *Escherichia coli* from nutrient limitation and abiotic stresses. Biochem Biophys Res Commun 394:163–169

Nomura CT, Toshio S, Bryant DA (2006) Roles of heme-copper oxidases in extreme high-light and oxidative stress response in the cyanobacterium *Synechococcus* sp. PCC 7002. Arch Microbiol 185:471–479

Ow SY, Noirel J, Cardona T, Taton A, Lindblad P, Stensjö K, Wright PC (2009) Quantitative overview of N_2 fixation in *Nostoc punctiforme* ATCC 29133 through cellular enrichments and iTRAQ shotgun proteomics. J Proteome Res 8:187–189

Perelman A, Uzan A, Hacohen D, Schwarz R (2003) Oxidative stress in *Synechococcus* sp. strain PCC 7942: various mechanisms for detoxification with different physiological roles. J Bacteriol 185:3654–3660

Pérez-Pérez ME, Mata-Cabana A, Sánchez-Riego AM, Lindahl M, Florencio FJ (2009) A comprehensive analysis of the peroxiredoxin reduction system in the cyanobacterium *Synechocystis* sp. strain PCC 6803 reveals that all five peroxiredoxins are thioredoxin dependent. J Bacteriol 191:7477–7489

Pilon M, Ravet K, Tapken W (2011) The biogenesis and physiological function of chloroplast superoxide dismutases. Biochim Biophys Acta 1807:980–998

Pospíšil P (2009) Production of reactive oxygen species by photosystem II. Biochim Biophys Acta 1787:1151–1160

Priya B, Premanandh J, Dhanalakshmi RT, Seethalakshmi T, Uma L, Prabaharan D, Subramanian G (2007) Comparative analysis of cyanobacterial superoxide dismutases to discriminate canonical form. BMC Genomics 8:435

Raghavan PS, Rajaram H, Apte SK (2011) Nitrogen status dependent oxidative stress tolerance conferred by overexpression of MnSOD and FeSOD proteins in *Anabaena* sp. strain PCC 7120. Plant Mol Biol 77:407–417

Regelsberger G, Atzenhofer W, Rüker F, Peschek GA, Jakopitsch C, Paumann M, Furtmüller PG, Obinger C (2002) Biochemical characterization of a membrane-bound manganese-containing superoxide dismutase from the cyanobacterium *Anabaena* PCC 7120. J Biol Chem 277:43615–43622

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61

Ross C, Santiago-Vázquez L, Paul V (2006) Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcyctis aeruginosa*. Aquat Toxicol 78:66–73

Simon FW, Grzebyk D, Schofield O (2005) The role and evolution of superoxide dismutases in algae. J Phycol 41:453–465

Tepperman JM, Dunsmuir P (1990) Transformed plants with elevated levels of chloroplastic SOD are not more resistant to superoxide toxicity. Plant Mol Biol 14:501–511

Thomas DJ, Avenson TJ, Thomas JB, Herbert SK (1998) A cyanobacterium lacking iron superoxide dismutase is sensitized to oxidative stress induced with methyl viologen but not sensitized to oxidative stress induced with norflurazon. Plant Physiol 116:1593–1602

Tichy M, Vermaas W (1999) In vivo role of catalase–peroxidase in *Synechocystis* sp. strain PCC 6803. J Bacteriol 181:1875–1882

Tözüm SRD, Gallon JR (1979) The effects of methyl viologen on *Gloeocapsa* sp. LB795 and their relationship to the inhibition of acetylene reduction (nitrogen fixation) by oxygen. J Gen Microbiol 111:313–326

Vass I (2012) Molecular mechanisms of photodamage in the Photosystem II complex. Biochim Biophys Acta 1817:209–217

Wei X, Mingjia H, Xiufeng L, Yang G, Qingyu W (2007) Identification and biochemical properties of Dps (starvation-induced DNA binding protein) from cyanobacterium *Anabaena* sp. PCC 7120. IUBMB Life 59:675–681

Zhao W, Guo Q, Zhao J (2007) A membrane associated Mn-superoxide dismutase protects the photosynthetic apparatus and nitrogenase from oxidative damage in the cyanobacterium *Anabaena* PCC 7120. Plant Cell Physiol 48:563–572