J Appl Phycol (2011) 23:887–894
DOI 10.1007/s10811-010-9607-6

Enzymatic antioxidant response to low-temperature acclimation in the cyanobacterium *Arthrospira platensis*

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Received: 14 August 2010 / Revised and accepted: 27 September 2010 / Published online: 16 October 2010

Abstract Changes in antioxidant enzyme activities in response to low-temperature-induced photoinhibition were investigated in the two strains of the cyanobacterium *Arthrospira platensis*, Kenya and M2. When transferred to 15°C from 33°C, cells exhibited an immediate cessation of growth followed by a new acclimated growth rate. Although both strains had similar growth rates at 33°C, once transferred to a lower temperature environment, Kenya had a faster growth rate than M2. There were variations in the antioxidant enzyme activities of both strains during 15°C acclimation. The activity of superoxide dismutase from Kenya was higher than that from M2 and increased remarkably with acclimation time. Catalase activity of both strains increased at first but decreased later in the acclimation process. Ascorbate-dependent peroxidase activity of the Kenya strain declined when transferred to the low-temperature environment while peroxidase activity of M2 decreased in the beginning and then increased with time. The dehydroascorbate reductase activity of both strains was variable during the acclimation period while the glutathione reductase activity was not modified immediately. Our finding may support that the faster growth rate of the Kenya strain at lower temperatures as compared with the M2 strain might be explained by the higher antioxidant enzyme activities of Kenya at lower temperatures and through its ability to apply a more efficient regulatory strategy of enzymatic antioxidant response to low-temperature-induced photoinhibition.

Keywords Catalase · Dehydroascorbate reductase · Enzymatic antioxidant · Glutathione reductase · Low temperature · Peroxidase · Superoxide dismutase

Electronic supplementary material The online version of this article (doi:10.1007/s10811-010-9607-6) contains supplementary material, which is available to authorized users.

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Introduction

Exposure of photosynthetic organisms to low temperature results in an imbalance between the rate of the primary photochemical reactions of photosynthesis and the rate by which the energy is channeled into the biochemical reactions utilizing the energy produced. Such imbalance may bring about the formation of reactive oxygen species (ROS) (Huner et al. 1998; Murata et al. 2007; Takahashi and Murata 2008). Thus any acclimation process to low temperature in photosynthetic organisms requires the development of scavenging mechanisms for the removal of ROS (Mittler 2002; Sung et al. 2003; Foyer and Noctor 2005; Guy et al. 2008).

The major ROS include the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and their derivatives (Foyer and Noctor 2000). Scavenging of ROS occurs mainly through antioxidant pathways that consist of enzymatic and non-enzymatic scavenging systems. Primarily, $O_2^{\cdot -}$ is converted into H_2O_2 by superoxide dismutase (SOD). Two types of SODs have been characterized in cyanobacteria, namely Fe-SOD and Mn-SOD (Asada et al. 1975). Subsequently, H_2O_2

is scavenged by hydroperoxidases which may be involved in three pathways: the catalase (CAT) cycle, the ascorbate (Asc)–glutathione (GSH) cycle, or the glutathione peroxidase (GPX) cycle in respective cyanobacterial species (Obinger et al. 1998; Mallick and Mohn 2000; Regelsberger et al. 2002; Perelman et al. 2003). CAT is thought to be the principal H_2O_2 scavenging enzyme and exists in all cyanobacterial species (Obinger et al. 1998; Mallick and Mohn 2000). In the Asc–GSH cycle, H_2O_2 can be broken down into water by Asc-dependent peroxidase (PX) (Tel-Or et al. 1986; Miyake et al. 1991; Mallick and Rai 1999; Miller et al. 2000; Bhandari and Sharma 2006; Srivastava et al. 2006; Wang et al. 2008), producing Asc derivatives. Furthermore, dehydroascorbate reductase (DHAR), monodehydroascorbate reductase, and glutathione reductase (GR) are involved in depleting Asc derivatives. In the GPX cycle, H_2O_2 is decomposed into water by GPX in conjunction with the oxidation of GSH (El-Sheekh and Rady 1995; Mittler 2002).

Most of the studies reported were limited to eukaryotic photosynthetic organisms, and the correlation between different cellular compartments and their regulatory mechanisms remains to be elucidated (Asada 1994; Wilson et al. 2006). Since there is some controversy concerning the location of antioxidant mechanisms between different compartments in eukaryotes, we chose a prokaryotic cyanobacterium for study of the overall antioxidant response to low temperature. In an attempt to provide a better understanding of the acclimation process, the cyanobacterium, *Arthrospira platensis*, which has been used widely in algal biotechnology and is well known to require relatively high temperatures for optimal growth, was employed (Vonshak 1997; Vonshak and Novoplansky 2008). Two *A. platensis* strains marked as Kenya and M2 that grow similarly at optimal temperatures but respond differently to lower temperatures were compared. By examining the Asc–GSH cycle (Supplemental Fig. S1) for changes in the activities of scavenging system antioxidant enzymes, we attempt to assess the regulatory strategy of antioxidant response. The results showed that the activities of Asc-dependent PX, CAT, DHAR, GR, and SOD were functionally regulated at different stages of low-temperature acclimation.

Materials and methods

Two *Arthrospira* strains designated as Kenya isolated from Lake Nakuru in Kenya and M2 isolated from Lake Chad were obtained from the culture collection of the Centro di Studio dei Microrganismi Autotrofi in Florence, Italy, and routinely cultured in Zarouk's medium containing 200 mM sodium bicarbonate (Vonshak et al. 1982). Cells were incubated at 33±2°C in a gyratory shaker (150 rpm) under continuous illumination of 50 μmol photons $m^{-2}s^{-1}$. Low-temperature treatments were performed under these light and shaking conditions. Growth of algal cultures was monitored by measuring the changes in chlorophyll *a* (Chl *a*) content (Hall and Rao 1999).

To prepare cell crude extracts, sufficient algal cultures with at least 1 mg Chl*a* were centrifuged at 1,000×*g* for 5 min, washed three times with 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM EDTA, resuspended in the same buffer, and sonicated (Sonicator XL, Misonix, USA) for four to six cycles at a pulse of 10 sec at 4°C. Unbroken cells and debris were removed by centrifugation at 10,000×*g* for 5 min at 4°C, and the supernatant was collected as cell crude extract. Protein concentration of cell crude extract was measured according to Bradford (1976) using bovine serum albumin as a standard.

Activity staining of SOD was carried out on 10% (*w*/*v*) native PAGE (Lee and Lee 2000). Aliquots of 100 μg proteins from algal cell crude extracts were suspended in a sample buffer of 12.5 mM Tris–HCl (pH 6.8), 0.02% (*w*/*v*) bromophenol blue, and 4% (v/v) glycerol, and loaded into each well. After electrophoresis, the gels were washed with 100 mM phosphate buffer (pH 7.8) for 10 min and then incubated with a solution of 100 mM phosphate buffer (pH 7.8), 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium (NBT), and 0.2% (*w*/*v*) TEMED in the dark at room temperature for 1 h. Gels were washed twice with 100 mM phosphate buffer (pH 7.8) and then exposed to light until colorless bands of SOD activity on the purple-stained gels developed. The reaction was then stopped by immersing the gels into ddH_2O. The isoenzymes of SOD were recognized using different inhibitors of SOD activity: KCN (2 mM) for Cu/Zn-SOD, and H_2O_2 (5 mM) for Fe-SOD and Cu/Zn-SOD (Huang et al. 2006).

The activities of antioxidant enzymes were assayed spectroscopically at room temperature. To prevent interference from Chl *a* absorption, we examined antioxidant enzyme activities with respect to various Chl *a* concentrations of 2, 4, 6, or 8 μg Chl*a* mL^{-1}, and derived them from the slope of the linear regression (Supplemental Fig. S2). SOD was determined spectrophotometrically by measuring the inhibited reduction of cytochrome *c* by the change in absorbance at 549 nm for cytochrome *c* (ε= 21 $mM^{-1}cm^{-1}$) because SOD competes with cytochrome *c* for $O_2{\cdot}^-$ (Azevedo et al. 1998). First, the reduction of cytochrome *c* was measured when 2.5 mU mL^{-1} of xanthine oxidase was added to a reaction mixture of 50 mM potassium phosphate (pH 7.8) in the presence of 50 μM xanthine and 10 μM oxidized cytochrome *c*. After 30 sec, the algal extract was added to the reaction mixture, and cytochrome *c* reduction was monitored. A 50% inhibition of cytochrome *c* reduction was considered as evidence of SOD activity. SOD activity was represented as U μg^{-1} Chl*a* (U, unit, defined as μmol of substrate converted into product per second). CAT activity was determined by measuring the

decomposition of H_2O_2. The reaction was initiated by the addition of algal extract to a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) with addition of 10 mM H_2O_2 (Lee and Lee 2000; Miller et al. 2000; Bhandari and Sharma 2006). The change in absorbance at 240 nm for H_2O_2 (ε=0.0436 $mM^{-1}cm^{-1}$) was monitored. Asc-dependent PX was assayed in a buffer of 100 mM potassium phosphate (pH 7.5), 5 mM EDTA, and 4 mM Asc in the presence of 10 mM H_2O_2 (Miller et al. 2000; Bhandari and Sharma 2006). The change in absorbance at 290 nm for Asc (ε=2.8 $mM^{-1}cm^{-1}$) was monitored for 2 min. DHAR activity was determined by measuring the oxidation of GSH (reduced glutathione) to GSSG (oxidized glutathione). The reaction was initiated by the addition of the algal extract to the reaction mixture containing 100 mM phosphate (pH 6.5) and 5 mM EDTA buffer in the presence of 4 mM GSH and 0.2 mM dehydroascorbate (DHA) (Butow et al. 1997). The oxidation of GSH was measured as the change in absorbance at 265 nm for GSSG (ε=14 $mM^{-1}cm^{-1}$). DHAR activity was calculated by subtracting the activity of both the non-enzymatic reaction and the reaction in the absence of GSH. GR activity was determined by the dehydrogenation of NADPH. The reaction was initiated by the addition of 0.2 mM GSSG into the reaction mixture containing 100 mM potassium phosphate (pH 7.5), 5 mM EDTA, and 0.2 mM NADPH (Butow et al. 1997). The dehydrogenation of NADPH was measured as the change of absorbance at 340 nm for NADPH (ε=6.2 $mM^{-1}cm^{-1}$).

Results

Growth of the two *A. platensis* strains, Kenya and M2, under control (33°C) and low temperature (15°C) was studied by following the changes in concentration of Chl *a* in the two cultures (Fig. 1). At the 33°C control stage (indicated as I), both strains had a similar specific growth rate. When transferred to 15°C, a complete inhibition of growth was observed (indicated as II), and the specific growth rates of both strains dropped to zero (Table 1). After 6 days, both strains resumed growth, which is referred to as the acclimated stage (indicated as III). At this stage, the two strains grew at the much lower specific growth rates, while Kenya was acclimated with 60% higher growth rate (0.22 day^{-1}) than M2 (0.14 day^{-1}).

In order to evaluate whether the exposure to low temperature leads to the generation of extra $O_2{\cdot}^-$ (Huner et al. 1998; Mittler 2002), the SOD activity of Kenya and M2 during the 15°C acclimation was analyzed by activity staining on native PAGE. Upon exposure of the cells to 15°C, the SOD activities of Kenya and M2 gradually increased with time, as one major colorless band on each well of the purple-stained gel became brighter and broader

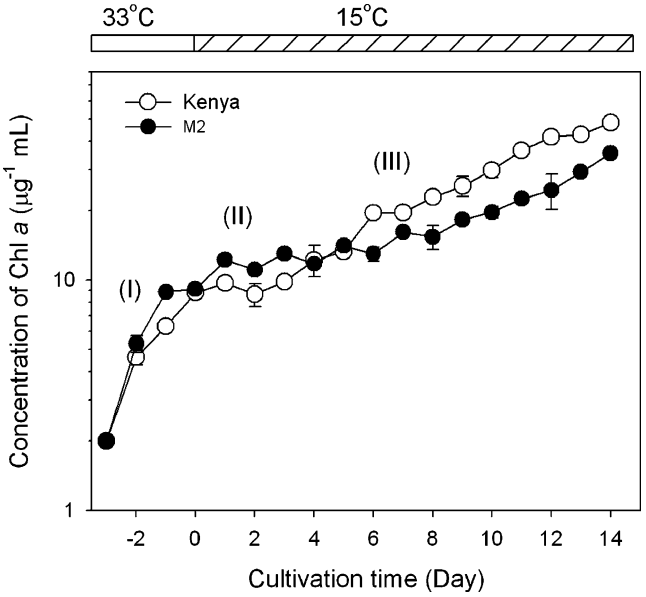

Fig. 1 Growth of *A. platensis* Kenya and M2 indicated as increases in Chl concentration during cultivation at 33°C and then shifted to 15°C. The temperature shift was performed when the culture reached 10 μg Chl*a* mL^{-1}. *Empty circles*, Kenya; *filled circles*, M2. *Day 0* was defined as the time of cold acclimation. *I*, *II*, and *III* refer to the control, inhibitory, and acclimated stages, respectively. Data are means±SE (n=4)

with the increasing exposure time and the decreasing temperature (Fig. 2). This SOD is about 100 kDa according to un-denatured protein markers and recognized as the Fe-SOD isoenzyme (designated as Fe-SOD1 in accordance with the order of discovery) since the activity was inhibited by H_2O_2 but was not sensitive to KCN (Lee and Lee 2000). The densities of the Fe-SOD1 bands were quantified with "NIH Image" software and displayed in Fig. 3. The activity of Fe-SOD1 in Kenya and M2 gradually increased with time at stage (II), while in the acclimated stage (stage III), the activity increases in Kenya whereas it became stable in M2 (Fig. 3).

After $O_2{\cdot}^-$ was converted by SOD into H_2O_2, CAT and Asc-dependent PX are considered to be in charge of scavenging this second line of oxidative stress (Foyer and Noctor 2000; Mittler 2002). We thus followed the changes

Table 1 Specific growth rates at different stages during 20°C and 15°C acclimation

Stage	15°C acclimation		
	I	II	III
Kenya	0.60	0.01	0.22
M2	0.64	0.00	0.14

Assuming Chl *a* to be a quantitative measure of algal biomass, the specific growth rates (μ, day^{-1}) were derived from the data at different stages in Fig. 1 using linear regression analysis

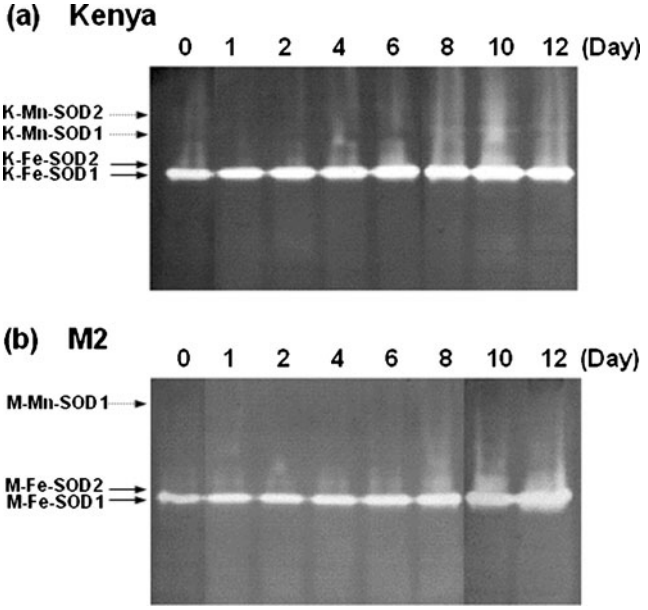

Fig. 2 Native-PAGE analysis of SOD from *A. platensis* Kenya (**a**) and M2 (**b**) during 15°C acclimation. Algal extracts of 100 μg were loaded into each well and separated on a 10% native PAGE. Gels were then developed with NBT in the presence of riboflavin. At least two Fe-SODs and four Mn-SODs were observed. *Numbers* represent the order of discovery

in H_2O_2 to assess the activities of CAT in the Kenya and M2 strains during the temperature shift (Fig. 4). When transferred to 15°C, CAT activity of Kenya increased by about fourfolds and M2 by 50% over the control on day 1. CAT activity of both strains increased to a peak on day 4. The results showed that CAT activity in Kenya increased notably with the resumption of cell growth but distinctly declined after the resumption to the same level as M2.

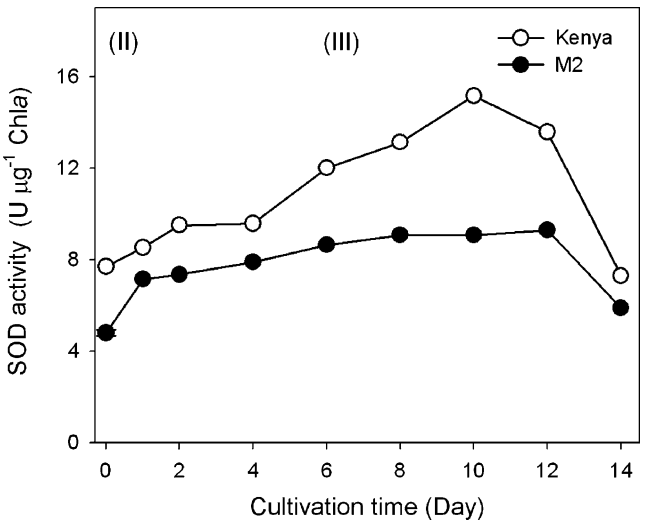

Fig. 3 Changes in the activity of SOD from *A. platensis* Kenya and M2 during 15°C acclimation, compared with those at 33°C. *Empty circles*, Kenya; *filled circles*, M2. Data were derived from Fig. 2 after the quantitative analysis of SOD active staining gels using the NIH image program

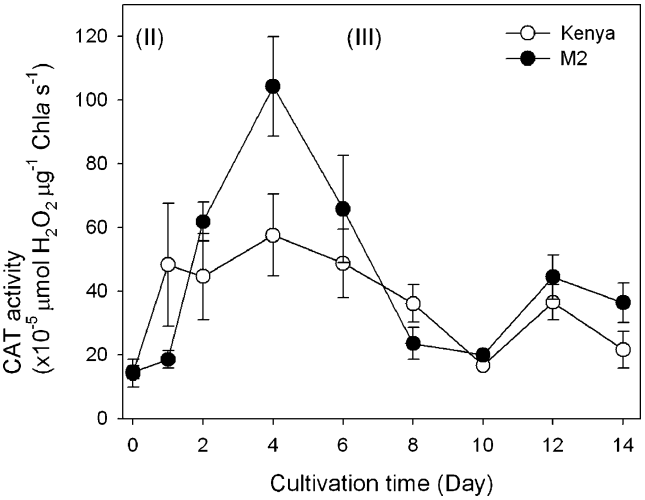

Fig. 4 Changes in the activity of CAT from *A. platensis* Kenya and M2 during 15°C acclimation, compared with those at 33°C. *Empty circles*, Kenya; *filled circles*, M2. Data are means±SE (n=4)

A significant difference between the strains was observed when following the Asc-dependent PX activity during the acclimation process to low temperatures (Fig. 5). Asc-dependent PX of Kenya exhibited twofolds higher activity than M2 at day 0 with continuous reduction throughout all stages and reaching a decrease by 70% on day 8, whereas in the M2 strain after a decline on the first day of exposure, a recovery in the activity took place and stayed at the same level as in the 33°C grown culture.

Consequently, DHAR, which was involved in depleting Asc derivatives, was examined as well (Fig. 6). At 15°C,

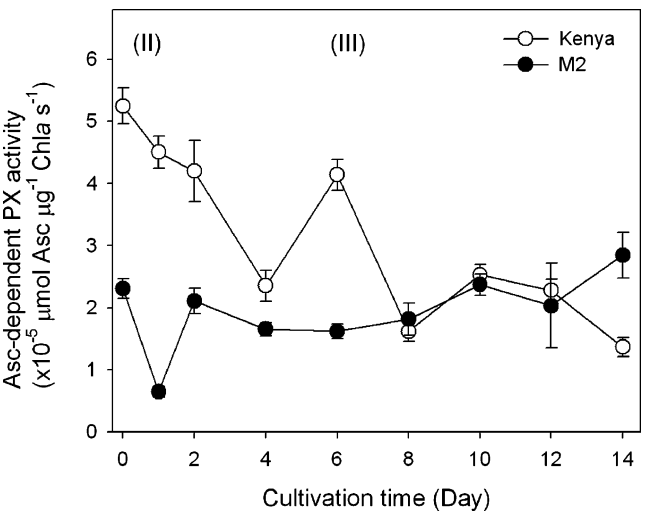

Fig. 5 Changes in the activity of Asc-PX from *A. platensis* Kenya and M2 during 15°C acclimation, compared with those at 33°C. *Empty circles*, Kenya; *filled circles*, M2. Data are means±SE (n=4)

the DHAR activity of Kenya increased up to threefolds during the acclimation period (II), while M2 increase twofolds and then both of them leveled off once cell resumed a logarithmic growth phase (III).

The activities of GR were examined because it participated in the utilization of NADPH, which may correlate to the dissipation of excessive excitation energy during low-temperature acclimation (Polle 2001; Mittler 2002). When transferred to 15°C, the GR activities of Kenya exhibited stable level while those of M2 exhibited a wide fluctuation as a double rate of the control was observed on day 8 (Fig. 7).

Discussion

Both strains of *A. platensis* were capable of acclimating to the low-temperature stress as reflected by their ability to establish a new steady state of logarithmic growth once transferred to 15°C from 33°C. The growth rates of the two strains were similar at 33°C; however, once acclimated to lower temperatures, Kenya strain had a faster growth rate than M2 (Fig. 1 and Table 1). Higher antioxidant enzyme activities may be the primary explanation for the faster growth rate as the SOD, Asc-dependent PX, and DHAR activities of Kenya were about 1.5, two, and 1.3 times, respectively, higher than those of M2 in the control stage (I). The other reason may be that Kenya engaged a more efficient enzymatic antioxidant defense strategy than M2 upon exposure to low temperature. As soon as the cultures shifted to 15°C, the SOD, CAT, DHAR, and GR activities of Kenya were amplified and higher than those of M2. This suggested that SOD was induced to a level matching the requirement for excess $O_2\cdot^-$ to be totally scavenged. Successively, the CAT rate was enhanced, but the Asc-dependent PX rate, which was high at 33°C, gradually reduced, indicating that CAT was the principal H_2O_2 scavenging enzyme in Kenya strain at low temperature (Figs. 4 and 5). CAT may be more effective than Asc-dependent PX in diminishing H_2O_2 because it has a very high reaction rate and does not consume reducing power, whereas PX requires a reductant source. Subsequently, an increased DHAR depleted the Asc derivatives (Fig. 6), though the GR activity of both strains insignificantly changed (Fig. 7). With the reduction of DHA, the small increases of GR and DHAR implied that the part of the dissipated excessive excitation pressure through the utilization of NADPH was insubstantial. All these strategies applied by the Kenya strain during stage II may enable it to overcome the low-temperature stress. In summary, our results demonstrated that the antioxidant enzyme activities of Kenya and M2 were regulated at different periods of acclimation. We did not observe any single pattern of change for the activity of any of the antioxidant enzymes measured during acclimation.

In most of our experiments, the initial Chl *a* concentration of cultures exposed to 15°C was 10 μg Chl*a* mL^{-1} since at a lower initial Chl *a* concentration (6 μg Chl*a* mL^{-1}) a greater inhibitory effect on growth was observed (Fig. S3). This effect may be a result of a combined effect of the low-temperature and a supper imposed photoinhibitory stress. Under such conditions, Fe-SOD activity appeared to be amplified and accompanied with the induction of multiple SOD isoenzymes and revealed another significant difference between the two strains (Fig. 8). In both strains, the major colorless band of Fe-SOD1 was greatly enhanced after

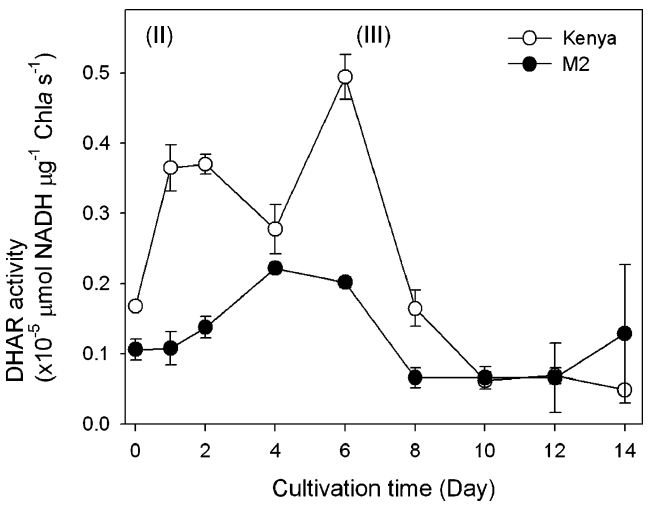

Fig. 6 Changes in the activity of DHAR from *A. platensis* Kenya and M2 during 15°C acclimation, compared with those at 33°C. *Empty circles*, Kenya; *filled circles*, M2. Data are averages±SD (n=2)

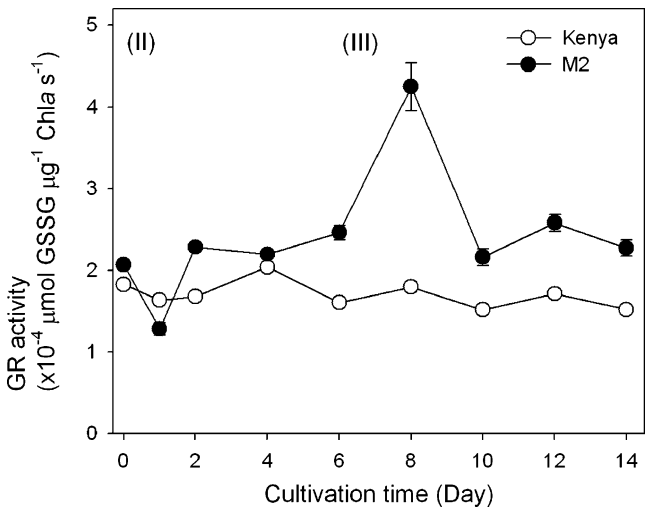

Fig. 7 Changes in the activity of GR from *A. platensis* Kenya and M2 during 15°C acclimation, compared with those at 33°C. *Empty circles*, Kenya; *filled circles*, M2. Data are means±SE (n=4)

12 days of acclimation. It is worth noting that there were at least four Mn-SODs and two Fe-SODs existing in Kenya cells after exposure to the low temperature for 12 days (Fig. 8a) while only one Mn-SOD and two Fe-SODs were observed in the M2 cells after the same time of exposure to the low temperature (Fig. 8b) Obviously, a greater number of SOD isoenzymes were induced in Kenya than in M2. Another observation to be pointed out about the difference between the cultures that were exposed to low temperature at 10 μg Chl*a* mL^{-1} compared with the one exposed at 6 μg Chl*a* mL^{-1} is revealed when comparing the results in Figs. 2 and 8. Some Mn-SODs were expressed in the 6 μg Chl*a* mL^{-1} treatment and not observed in the 10 μg Chl*a* mL^{-1} treatment. This may imply that these Mn-SODs are induced by the combination of low temperature and light stress because a lower concentration of algal cells may be exposed to higher photon density (Li et al. 2002). It is interesting to note that Cu/Zn-SOD was not found in either strain.

The data displayed in Fig. 9 summarize the stimulation of Fe-SOD1 at inhibitory and recovery stages during 15°C acclimation (Figs. 2 and 8) and illustrate that Fe-SOD1 was particularly responsive to temperature (Thomas et al. 1999). It is of interest to note that the activities of Fe-SOD1 were enhanced nonlinearly as a function of increasing rate over low-temperature acclimation at inhibitory and acclimated stages, regardless of the cultivation concentration. These data fit an exponential curve, implying that the induction of the main Fe-SODs and the formation of $O_2\cdot^-$ may be in a pseudo-first-order manner. This result does not follow the model of Polle (2001) whereby $O_2\cdot^-$ immediately reaches a steady-state concentration soon after photosystems receive excessive excitation energy and subsequently generate a fixed rate. Instead, our results may be compatible with the phenomenon of biphasic superoxide generation (Johnson et al. 2003) or temperature dependency (Perelman et al. 2006).

The existence of Asc-dependent PX, associated with the Asc–GSH cycle, in cyanobacteria has been suspected, and genetic analysis of peroxidases has shown that sequenced cyanobacteria do not possess a gene similar to the plant ascorbate peroxidase (APX) genes (Regelsberger et al. 2002). Nevertheless, some cyanobacteria have indeed exhibited Asc-dependent PX activity (Tel-Or et al. 1986; Miyake et al. 1991; Rozan et al. 1992; Mallick and Rai 1999; Miller et al. 2000; Bhandari and Sharma 2006; Srivastava et al. 2006; Wang et al. 2008). In our study, Asc-dependent PX of both *Arthrospira* strains was detectable. We suggested that this Asc-dependent PX of *Arthrospira* may be a catalase–peroxidase (KatG)-like protein that represents bifunctional CAT and PX activities (Obinger et al. 1999; Regelsberger et al. 2002; Singh et al. 2008). The genetic investigations of Zámocky et al. (2001) showed that KatGs have little sequence homology to typical heme-containing catalayses but that they have homology to yeast cytochrome *c* peroxidase and plant APX. However, KatG has not been found in the genomes of *Anabaena* PCC 7120, *Nostoc punctiforme*, or *Prochlorococcus marinus* MED4

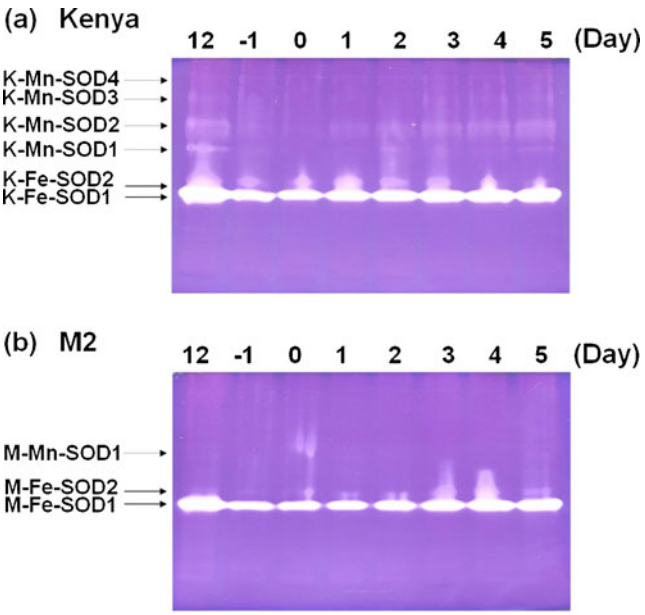

Fig. 8 Induction of multiple SOD isoenzymes in *A. platensis* **a** Kenya and **b** M2 with low algal cultivation concentration during 15°C acclimation. The temperature shift to 15°C was performed when the culture reached 6 μg Chl*a* mL^{-1}. Algal extracts of 100 μg were loaded into each well and separated on a 10% native PAGE. Gels were then developed with NBT in the presence of riboflavin

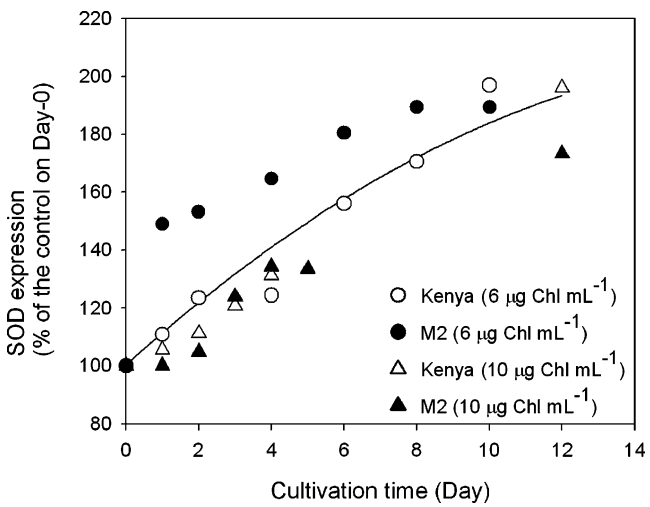

Fig. 9 Induction of Fe-SOD1 during low-temperature acclimation. Data were derived from Figs. 2 and 8 in which the major bands of Fe-SOD1 on the gels were quantified by using the NIH image program. Each point represents a percentage of the control on day 0. *Empty circles*, 15°C (Kenya) with initial concentration of 6 μg Chl*a* mL^{-1} culture; *filled circles*, 15°C (M2) with initial concentration of 6 μg Chl*a* mL^{-1}; *empty triangles*, 15°C (Kenya) with initial concentration of 10 μg Chl*a* mL^{-1}; *filled triangles*, 15°C (M2) with initial concentration of 10 μg Chl*a* mL^{-1}

(Regelsberger et al. 2002; Perelman et al. 2003). Hence, the molecular mechanisms for H_2O_2 detoxification in distinct cyanobacteria require further clarification.

In conclusion, we would like to point out that *A. platensis*, like other photosynthetic organisms, have developed an extensive set of mechanisms in order to be able to acclimate to changes in their growth environments. The data presented in this study provide for the first time an elaborated scheme of the enzymes involved in the protection from oxidative stress in this species. Furthermore, it tries to point out that differences between strains reflected in their ability to better acclimate to environmental stress may be reflected in their ability to employ a better enzymatic cascade of enzymes that can protect the cell from ROS. Such a screen process may also be useful in the selection of strains suitable for large-scale production in algal biotechnology.

Acknowledgments This work was supported by a fellowship to Lee-Feng Chien from the Blaustein Center for scientific collaboration in the Jacob Blaustein Institutes for Desert Research, Israel. We would like to acknowledge the significant contribution of Ms. Nurit Novsplanski for reading and preparing the paper for publication, and for technical assistance. The secretarial help from Ilana Saller is also greatly appreciated.

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