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Differential expression of antioxidant proteins in the drought-tolerant cyanobacterium *Nostoc flagelliforme* under desiccation

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Abstract

Nostoc flagelliforme is a terrestrial nitrogen-fixing cyanobacterium that is distributed in arid or semi-arid steppes in Western and North-western of China. This species shows a strong ecological adaptability to xeric drought environments. 2DE coupled with MALDI-TOF-TOF/MS was used to analyze the differentially expressed antioxidant proteins in *N. flagelliforme* under desiccation. A total of five antioxidant proteins (eight protein spots) were successfully identified, including peroxiredoxin (Prx), Mn-containing catalase (Mn-CAT), iron superoxide dismutase (Fe-SOD), superoxide dismutase (SOD) and ferritin. These proteins were all significantly down-regulated except for Prx that was up-regulated, when *N. flagelliforme* was subjected to 24-h desiccation. The colonies maintained very limited amounts of water (15%) and very low physiological activity to resist severe drought. These proteins may serve as biochemical markers for *N. flagelliforme* colonies under desiccation because of their significant changes in expression level. In addition, the content of superoxide anions increased when the colonies were exposed to desiccating conditions for 6 hours. However, the content dropped when the desiccation treatment lasted for 24 hours. The activities of SOD, CAT and POD showed the same up- and down-regulation pattern. Hydrogen peroxide (H_2O_2) levels constantly increased during 24 h desiccation treatment, while the ascorbic acid content continuously decreased. These characteristics may contribute to our understanding of its survival strategy in dry habitats.

Keywords: Antioxidant protein; Desiccation; Differential expression; *Nostoc flagelliforme.*
Abbreviations: CAT_catalase; IEF_isoelectric focusing; MALDI_matrix assisted laser desorption ionization; MS_mass spectrometry; POD_peroxidase; Prx_peroxiredoxin; ROS_reactive oxygen species; SOD_superoxide dismutase; TOF_time of flight.

Introduction

Drought is one of the most serious abiotic stress factors which has a negative impact on plant growth and development due to the accumulation of reactive oxygen species (ROS), such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl (OH^-) and H_2O_2 (Mahajan and Tuteja, 2005; de Carvalho, 2008; Lyudmila et al., 2009; Gill et al., 2010; Harb et al., 2010; Benešová et al., 2012; Choudhury et al., 2013). Although ROS can act as secondary messengers, involving in the stress signal transduction pathway, but excessive ROS production can cause oxidative stress to the photosynthetic apparatus and seriously impair the normal function of cells (Foyer et al., 2009; Dietz et al., 2011). Drought might promote the production of ROS damages cellular structures, proteins, lipids and nucleic acids leading to cell death (Gill et al., 2010; Uzilday et al., 2012). To protect themselves against these toxic ROS, plants have evolved specific adaptation mechanisms in response to drought stress, such as ROS-scavenging antioxidative enzymes (Møller et al., 2007; Bian and Jiang, 2009; Ashraf and Harris, 2013). Previous reports indicated that enhancement of anti-oxidative protection correlates with better drought resistance and adaptation (Sairam and Srivastava, 2001; Chen et al., 2004; Khanna-Chopra and Selote, 2007). The capacity of the antioxidative defense system will determine the fate of cells (Qiu et al., 2003). ROS detoxifying enzymes such as SOD, POD, CAT, and ascorbate peroxidase (APX) have important functions in the defense against ROS (Lyudmila et al., 2009; Wang et al., 2014). SODs, a group of metalloenzymes are considered as the first defense against ROS, being responsible for the dismutation of O_2^- to H_2O_2 and O_2. CAT, APX, POD are enzymes that catalyze the conversion of H_2O_2 to H_2O and O_2 (Gratão et al., 2005). Previous research showed that enhanced antioxidant enzyme activities could increase plant resistance to drought stress in many species, such as rice (Basu et al., 2010), cotton (Deeba et al., 2012). *Nostoc flagelliforme* is a terrestrial nitrogen-fixing cyanobacterium that is distributed in arid or semiarid steppes in Western and North-western in China (Gao, 1998), where the species is often subjected to extreme drought (Qian et al., 1989). This cyanobacterium can survive for several decades under xeric conditions and its physiological and metabolic functions are rapidly resumed after reabsorbing water (Cameron, 1962). Studies of drought-resistant mechanisms in *N. flagelliforme* indicated that the cyanobacterium is enveloped by a gelatinous sheath which absorbs water quickly and loses it slowly (Qiu and Gao, 2002; Gao and Ye, 2003). The structures of the cells remain stable in response to dehydration and rehydration treatments except that

the sheath and vacuole shrink during desiccation (Liang et al., 2012). A total of 32 differentially expressed proteins were identified in dehydration/rehydration cycle (Liang et al., 2012). Some reports showed that photosynthesis, nitrogen fixation and respiration rates significantly increase after water absorption (Bi and Hu, 2003; Zhao et al., 2008; Liang et al., 2012). However, limited data is available on the changes in expression levels of antioxidant proteins in *N. flagelliforme* during desiccation. The present study aims to investigate differentially expressed antioxidant proteins associated with drought resistance of *N. flagelliforme* and the effects of drought resistance on protein expression levels and physiological characteristics. This work will provide new insights into the mechanisms of detoxifying ROS in *N. flagelliforme* under desiccation.

Results

Differentially expressed proteins and antioxidant protein identification

2DE was applied to obtain protein gel images of *N. flagelliforme* of different degrees of dehydration (Fig. 1). Each protein sample was repeated three times to minimize error during electrophoresis. More than 900 protein spots were detected in each gel by ImageMaster software analysis. Thirty-eight differentially expressed protein spots showed more than two-fold increases in abundance. The identified proteins by MALDI-TOF-TOF/MS are listed in Table 1, Table S1 and Fig. 2. The results indicated that eight antioxidant protein spots, namely, peroxiredoxin (Prx; spots 1 and 3), Mn-CAT (spot 2), Fe-SOD (spot 4), SOD (spot 8), ferritin and Dps family protein (oxidative damage protectant) (spots 5, 6, and 7) were detected in the three 2DE gel images (Fig. 1, 2 and Table 1). All of these protein spots showed a decrease in expression level during desiccation except for Prx (spot 1). Fig. 3 shows the enlarged areas of the differentially expressed antioxidant protein spots (the number marking for the protein spots are the same as those in Fig. 1).

Changes of ROS levels, antioxidant enzyme activities and ascorbic acid content

Superoxide anion content and antioxidant enzyme activities were assayed in different dehydrated *N. flagelliforme*. The results showed significant increase of superoxide anion content in 6-h dehydrated colonies (223% water content) but dramatical decrease of the content was shown in 24-h dehydrated colonies (15% water content) (Fig. 4a). H_2O_2 levels constantly increased during 24-h desiccation treatment (Figs. 4b). Antioxidant enzyme activities such as SOD, CAT and POD increased in 6-h dehydrated colonies but decreased ($P < 0.01$) in 24-h dehydrated colonies (Figs. 4c to 4e). However, ascorbic acid content continuously decreased while the colonies were subjected to 24 h desiccation (Fig. 4f).

Confirmation of expression abundance of Mn-CAT by Western blot analysis

In the current study, the Mn-CAT expression pattern shown in 2DE (Figs. 1 and 2, Table 1) during the dehydration was further confirmed by immunoblot analysis. Proteins of *N. flagelliforme* were separated by one-dimensional SDS-PAGE and immunoblot analysis was performed for Mn-CAT. In agreement with the changes in protein abundance observed by 2-DE, Mn-CAT showed a decreased amount of cross-reacting polypeptide bands in response to the process of desiccation (Fig. 5).

Discussion

Drought stress can cause oxidative stress. Development of oxidative stress is a result of an imbalance between the formation and detoxification of ROS (Mittler, 2002; Zimmermann and Zentgraf, 2005). Oxidative stress, structural damage, function loss and programmed cell death in plant cell may be caused by excessive ROS production (Mundree et al., 2002). This study identified the antioxidant proteins, such as Prx, Mn-CAT, Fe-SOD, SOD and ferritin, by 2DE and MALDI-TOF-TOF/MS. All of these proteins were significantly down-regulated when the *N. flagelliforme* were subjected to 24-h desiccation except for Prx (Figs. 1 and 2). The aforementioned results were confirmed by Mn-CAT immunoblotting. These proteins may serve as biochemical markers for *N. flagelliforme* subjected to continuous desiccation because of their significant characteristic changes in expression level. SOD, CAT and POD are the most important antioxidant enzymes in plant cells. Active Fe-SOD is the third most abundant soluble protein in rehydrating cells of *N. commune* CHEN/1986 colonies that were desiccated for 13 years (Shirkey et al., 2000). Moreover, the photosynthetic apparatus in *N. flagelliforme* may be damaged by desiccation-induced oxidative stress (Qiu et al., 2003; Liang et al., 2012). In the present study, the observed tendency of superoxide anion level and SOD, CAT and POD activities initially increased at 6-h desiccation and then remarkably decreased after the colonies were subjected to 24-h desiccation (Figs. 4a, 4c, 4d and 4e). It implies that the desiccated colonies become dormant with very low physiological activities due to the low water content (15 %, Table 2). However, H_2O_2 level increased and ascorbic acid content decreased with 0-24h desiccation (Fig. 4b, 4f). These results indicate that the antioxidant enzyme system plays an important role in the ecological adaptation in this species. Ascorbate is a main component against oxidative stress found in cyanobacteria (Asada, 2000). Dai et al. (1991) reported that *N. flagelliforme* contains ascorbate using the redox reaction with Fe (III), but Qiu et al. (2003) suggested that ascorbate cannot be detected in *N. flagelliforme* using ascorbate oxidase. The present study showed clearly that ascorbate was detectable in *N. flagelliforme* using ascorbate oxidase (Fig. 4f). Therefore, we suggest that ascorbate is an important component against oxidative stress in *N. flagelliforme*. Prx (spots 1 and 3) was identified and changes in expression level were shown in Fig.2. Prx is an anti-oxidation protein superfamily widely found in prokaryotes and eukaryotes, it has a vital function in scavenging ROS (Kroll, 2002; Sagadevan et al., 2002). Régine et al. (2008) identified seven types of Prx in *Chlamydomonas reinhardtii*. Previous studies also showed that Prx is involved in the cytokine signal cascade and other functions by regulating the intracellular concentration of H_2O_2 (Wood et al., 2003; Rhee et al., 2005). Liang et al. (2013) speculated that Prx has different functions in modulating cell growth and/or H_2O_2 concentration in *N. flagelliforme* during the diurnal cycle. Our results indicated that the expression quantity of Prx (spot 3) showed a positive correlation with Mn-CAT and ferritin but a negative correlation with H_2O_2 level. However, the expression quantity of Prx (spot 1) showed diametrically opposite results. This finding suggests that spots 1 and 3 may be two types of Prx, each of which has a different function associating with Mn-CAT and ferritin for regulating H_2O_2 in *N. flagelliforme* under desiccation. However, this assumption needs further

Table 1. Differentially expressed antioxidant proteins in *Nostoc flagelliforme* identified by MALDI-TOF-TOF/MS and database searching.

Spot No. [a]	Accession No. [b]	Homologous protein name[c]	NMP [d]	Coverage [e] (%)	Protein score [f]	Theoretical Mr (kDa)/ pI [g]	Observed Mr(kDa) / pI[h]
1	gi|17232133	Peroxiredoxin [*Nostoc* sp. PCC 7120]	9	58.62	353/79	22.6/4.87	25.1/4.85
2	gi|23127951	COG3546: Mn-containing catalase [*Nostoc punctiforme* PCC 73102]	13	80.87	154/79	25.6/5.15	26.1/4.97
3	gi|23126633	COG0450: Peroxiredoxin [*Nostoc punctiforme* PCC 73102]	13	85.38	284/79	23.81/ 4.92	27.3/5.04
4	gi|56182538	iron superoxide dismutase [*Nostoc commune* CHEN]	9	45.50	216/79	22.5 /5.52	23.6/5.37
5	gi|186463002	Ferritin, Dps family protein (oxidative damage protectant) [*Nostoc punctiforme* PCC 73102]	8	62.01	102/79	20.1/5.03	20.7/5.16
6	gi|186463002	Ferritin, Dps family protein (oxidative damage protectant) [*Nostoc punctiforme* PCC 73102]	5	39.66	81/79	20.1/5.03	20.1/5.24
7	gi|186463002	Ferritin, Dps family protein (oxidative damage protectant) [*Nostoc punctiforme* PCC 73102]	7	58.66	94/79	20.1/5.03	19.5/5.57
8	gi|23126626	COG0605: superoxide dismutase [*Nostoc punctiforme* PCC 73102]	8	45.50	117/79	22.4/5.67	23.9/5.44

Putative protein identification and accession number of the closest match in NCBI database are indicated.
[a]Number of each protein spot of 2DE, as indicated in Fig. 1 and Fig. 2.
[b]Accession number was recorded as a reference for the identification in NCBI database.
[c]Protein description and species from homologous protein as in NCBI database (release date: 2012.08.2).
[d]Number of matched peptides.
[e]Percentage of predicated protein sequence covered by matched sequence.
[f]MOWSE score probability (protein score) for the entire protein and for ions complemented by the percentage of the confidence index (C.I.).
[g]Theoretical molecular mass and isoelectric point (*pI*).
[h]Experimental molecular mass and *pI*.

Table 2. The treatments of *N. flagelliforme* samples.

Sample	Temperature (℃)	Relative humidity (%)	Light intensity (μmol m^{-2} s^{-1})	Light period (light/dark)	Rehydration time (h)	Dehydration time (h)	Water content [a] (%)
D0	25 ± 2	55 ± 5	200	Absolute light	4	0	863 ± 10.5
D6	25 ± 2	18 ± 1	200	light	4	6	223 ± 5.6
D24	25 ± 2	15 ± 1	200	light	4	24	15 ± 1.3

[a]Water content = $(W_W-W_D)/ W_D\times100\%$, where W_W is wet weight, and W_D is dry weight.

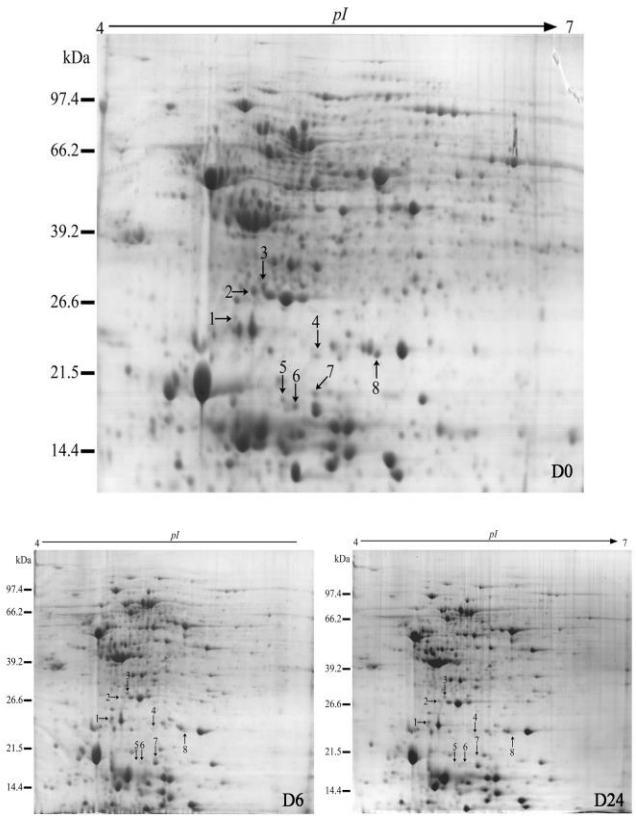

Fig 1. 2DE images of total protein extracts from *N. flagelliforme*. D0, rehydrated 4 hours, and then rehydrated 0 hours (water content 863%); D6, rehydrated 4 hours, and then dehydrated 6 hours (water content 223%); D24, rehydrated 4 hours, and then dehydrated 24 hours (water content 15%). All samples were run in biological triplicate gels. 1.3 mg protein was separated on the 24 cm, pH 4-7 linear gradient IPG strips. SDS-PAGE was performed with 12.5% gels. Protein spots were visualized by CBB R-250 staining. Arrows indicate the 8 differential antioxidant protein spots. Identity of these protein spots are given in Table 2.

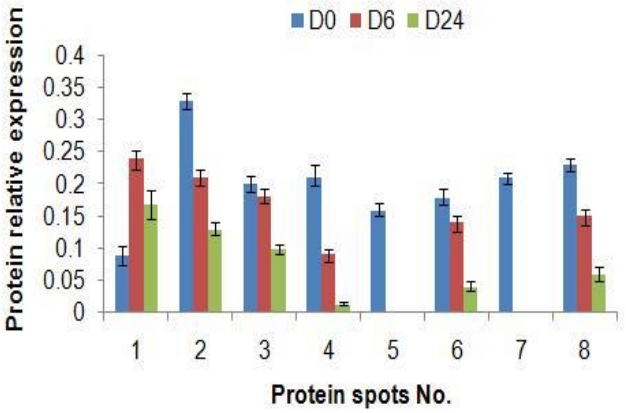

Fig 2. Relative expression levels of 8 differentially expressed proteins of *N. flagelliforme* under desiccation. Changes in protein expression under desiccation were calculated by PDQuest software 7.3. Mean of relative protein abundance and standard error. Three treatments were performed.

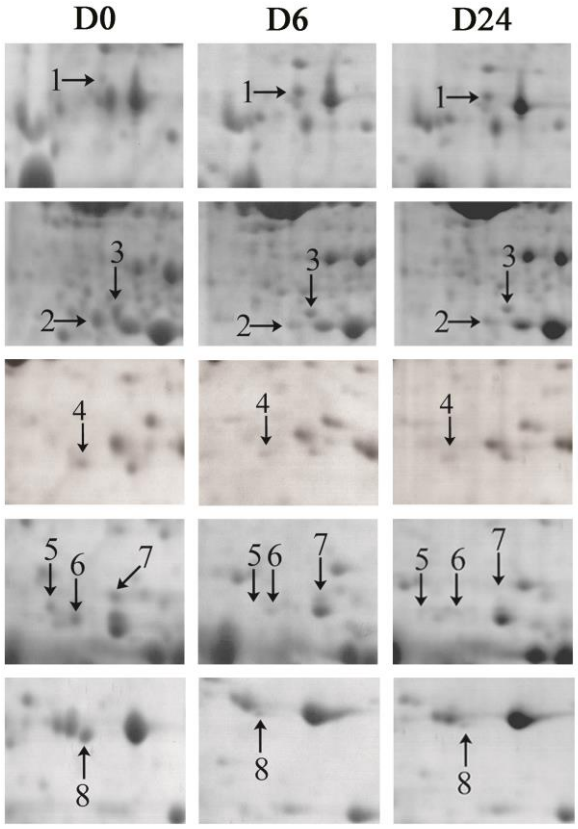

Fig 3. Enlarged protein spots of the differentially expressed antioxidant proteins identified from *N. flagelliforme*. The numbers for protein spots are the same as shown in Fig. 1.

verification. Ferritin is one of the DNA-binding proteins (Dps) found in *N. flagelliforme* under desiccation. Dps are the key factors involved in the protection of prokaryotic cells from oxidative damage, often demonstrate the ability to bind DNA nonspecifically (Emilia and Pierpaolo, 2010) and protect DNA against the harmful Fenton chemistry. Fenton chemistry generates dangerous hydroxyl free radical under conditions when H_2O_2 levels are increased in the presence of free ferrous iron (Emilia and Pierpaolo, 2010). Dps catalyze the oxidation of free ferrous iron using H_2O_2, but the reaction product is harmless water rather than toxic hydroxyl radicals (Simon, 2010). In the present study, three protein spots (spots 5, 6, and 7) were identified as ferritin, all of which decreased during desiccation. Multiple spots may be the result of isozymes or post-translational modifications of a single protein (Liu et al., 2010; Liang et al., 2012), but different types of ferritin were also possible. However, these findings suggest that ferritin may have a critical function in regulating the ROS metabolic balance and protecting DNA against the harmful Fenton chemistry in *N. flagelliforme* under desiccation.

Materials and Methods

Bacterial materials

N. flagelliforme colonies were collected from Ningxia Xiangshan Mountain, China. They were cultured under the conditions of 25 ± 2 °C, 12 h illumination with a light intensity of 200 μmol m^{-2} s^{-1}, and a relative humidity of 45% ± 5% for 30 days.

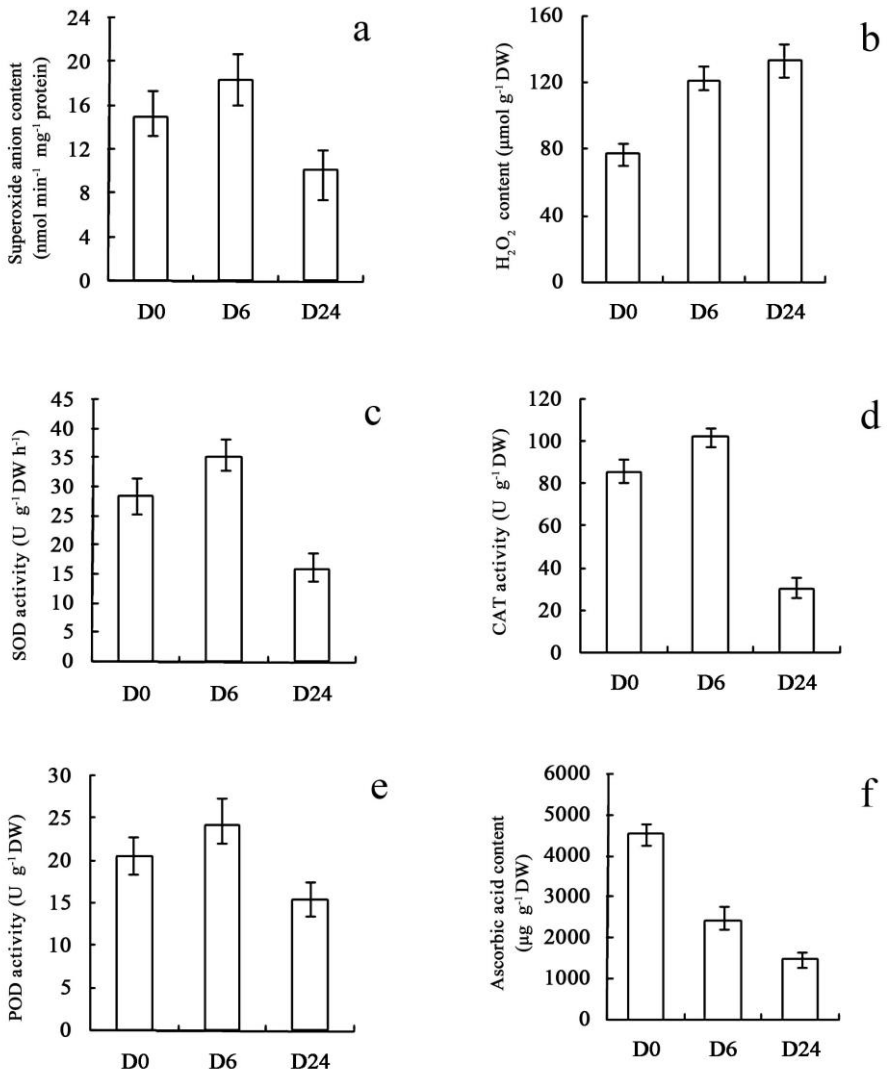

Fig 4. Changes of superoxide anion and H_2O_2 contents, activities of antioxidant enzymes (SOD, CAT and POD) and ascorbic acid content of *N. flagelliforme* subjected to desiccation. (a) superoxide anion level; (b) H_2O_2 level; (c) SOD activity; (d) CAT activity; (e) POD activity; (f) ascorbic acid content. Values (means ± SD) were determined with 3 independent experiments (n = 3).

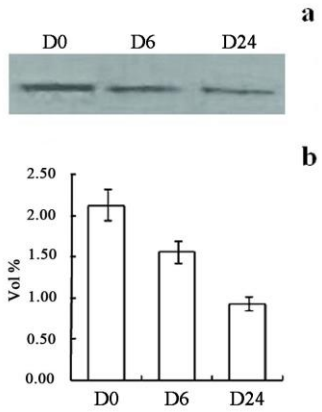

Fig 5. Western blot analysis of Mn-CAT in *N. flagelliforme* during the process of desiccation. (a) Western blot result of Mn-CAT; (b) Relatively quantity of the Western blot analysis of Mn-CAT. Data are average of three independent experiments and shown as mean ± SD.

Colonies were rehydrated 4 hours and subsequently subjected to desiccation (Table 2). Then, the colonies were harvested for physiological and proteomic analyses.

Preparation of total protein extraction and 2DE

Total protein extraction was performed as per the protocol of Liang et al. (2012). Protein quantification was determined using Bradford (Bradford, 1976). To ensure data reliability, three biological triplicates were performed for each sample.

Gel staining and image analysis

The 2DE gels were stained with Coomassie Brilliant Blue (CBB)-R250, then scanned with an EPSON Perfection 2480 photo scanner (Seiko EPSON CORP, Japan) at 300 dpi resolution. Protein abundances were analyzed with PDQuest version 7.3 (Bio-Rad, Hercules, CA, USA). Relative comparison of the intensity abundance among three states colonies (3 replicate samples for each group) were performed with the ANOVA. Expression intensity larger than 2.0 ($P \leq 0.05$) or smaller than -2.0 ($P \leq 0.05$) were set as a threshold indicating significant changes.

MS identification of proteins

Protein identification was performed according to the protocol of Liang et al. (2012). Protein spots were excised from the 2DE gels then each spot was destained with destaining buffer and digested by trypsin. The peptide mixtures were anlyzed by 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Combined MS and MS/MS spectra were submitted to MASCOT (V2.1, Matrix Science, London, U.K.) by GPS Explorer software (V3.6, Applied Biosystems) and searched with the following parameters: NCBInr database (release date: 2012.08.02), taxonomy of cyanobacteria, trypsin digest with one missing cleavage, none fixed modifications, MS tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 75 were considered statistically significant ($P \leq 0.05$). The individual MS/MS spectra with statistically significant (confidence interval > 95%) best ion score (based on MS/MS spectra) was accepted.

Analysis of superoxide anion and hydrogen peroxide level

The determination of superoxide anion content is followed the procedures described by Wang and Luo (1990). Concentration of H_2O_2 was determined as described by Patterson et al. (1984).

Assay methods for antioxidant enzymes

For antioxidant enzymes assay, 0.5 g of *N. flagelliforme* colonies was ground in 5 mL extraction buffer containing 0.1 M potassium phosphate (pH 7.5), 0.5 M NaCl and 2 % PVPP for 10 min at 4 °C. The homogenate was centrifuged at 20,000 g for 20 min at 4 °C. The supernatant was assayed for enzyme activity as follows: SOD activity was determined by p-Nitro-Blue tetrazolium chloride (NBT) photoreduction at 560 nm according to the method as described (Beyer and Fridovich, 1987). Catalase (CAT) activity was assayed according to the method described by Aebi (1984). Peroxidase (POD) activity assay was based on the method as described (Angelini et al., 1990). Ascorbic acid content was measured according to Law et al. (1983)

Western blot analysis

Western blot analysis of CAT was performed according to the protocol of Liang et al. (2012). The proteins of *N. flagelliforme* colonies were prepared and separated with 12.5% SDS-PAGE and then transferred to the nitrocellulose membrane that was incubated for 2 h with rabbit polyclonal antibody raised against CAT (Agrisera, Sweden) at 1: 5000 dilutions at 4 °C for 12 h. The immunodetection image was scanned, and the intensities were quantified using Quantity
software (Bio-Rad, USA).

Data analysis

Three independent biological replicates were performed in all experiments. Data were analyzed using one-way analysis of variance (ANOVA) with significance being defined as $P \leq 0.05$, and values represent the mean of three biological replicates.

Conclusions

To our knowledge, this is the first report about the changes in expression level of antioxidant proteins in *N. flagelliforme* under drought stress. In the present study, we found that antioxidant proteins Mn-CAT, Fe-CAT, SOD and ferritin of *N. flagelliforme* were moderately decreased in abundance during 6 h desiccation treatment (223% water content), but sharply decreased, except for Prx that was up-regulated, when the colonies were subjected to 24-h desiccation (15% water content). The desiccated colonies become dormant with very low physiological activities in order to tolerate xeric environments. We suggest that Prx, Mn-CAT, ferritin, SOD and Fe-SOD might serve as biochemical markers of *N. flagelliforme* in response to drought stress because of their remarkable dynamics changes in expression level. These findings provide a new insight into the characteristics of antioxidative defense system of *N. flagelliforme* in dry habitats.

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