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# Comparative proteomics of oxidative stress response in three cyanobacterial strains native to Indian paddy fields☆

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ABSTRACT

Three strains of photoautotrophic, heterocystous, nitrogen-fixing cyanobacterium *Anabaena*, native to Indian paddy fields, were examined for their tolerance and proteomic response to the frequently used weedicide paraquat (methyl viologen). *Anabaena* 7120 ($LD_{50}$ dose: 2 μM for 6 h) and *Anabaena* L-31 ($LD_{50}$ dose: 2 μM for 5 h) showed distinctly better tolerance than *Anabaena doliolum* ($LD_{50}$ dose: 2 μM for 3 h), to methyl viologen induced oxidative stress. The proteomic response, at respective $LD_{50}$ dose, was mapped by 2D gel protein electrophoresis followed by protein identification by MALDI-ToF mass spectrometry. About 92 and 41 oxidative stress-responsive proteins were identified from *Anabaena* L-31 and *A. doliolum*, respectively, and compared with methyl viologen responsive proteins reported from *Anabaena* 7120 earlier. Upregulation of proteins involved in oxidative stress alleviation and protein homeostasis and downregulation of photosynthesis and carbon metabolism related enzymes appeared to underlie the oxidative stress response in all three *Anabaena* strains. Reduced photosynthesis and cellular reserves of molecular energy [ATP + NAD(P)H] seemed to overwhelm the cellular machinery to combat oxidative stress and protein denaturation, in preference to other adaptations, while the strain specific differences observed in proteome response appeared to determine the methyl viologen tolerance of individual cyanobacterial strains.
This article is part of a Special Issue entitled: Proteomics in India.



## 1. Introduction

The photosynthetic, nitrogen-fixing cyanobacteria are often used as bio-fertilizer and for nitrogen supplements in tropical paddy fields [1] where they are frequently exposed to multiple abiotic stresses, such as salinity, desiccation, heat-shock, pesticides and heavy metals [2]. Oxidative stress is central to most of the aforesaid stresses and manifests as super oxide, peroxide and hydroxyl radicals, singlet oxygen and other reactive oxygen species (ROS) or free radicals. The ROS damage several cellular components such as DNA, protein, lipids and membranes [2]. The herbicide paraquat (methyl viologen), commonly used for weed control in paddy fields, also generates superoxide radicals through Mehler reaction and inhibits photosynthesis and diazotrophic growth [3].

Among the three strains of cyanobacteria used in the present study, *Anabaena* sp. strain PCC 7120 (hereafter referred to as *Anabaena* 7120) is a sequenced strain (http://genomekazusa.or.jp/Cyanobase/anabaena), while *Anabaena doliolum* and *Anabaena* sp. strain L-31 (hereafter referred to as *Anabaena* L-31) are isolates from Indian paddy fields. In paddy fields, they are frequently exposed to abiotic stresses like heat, heavy metal, salinity, etc. [4–7]. The effects of methyl viologen on *Anabaena* 7120 have been recently investigated by our laboratory [8]. *Anabaena* 7120 responded to MV by enhancement of levels of proteins involved in oxidative stress alleviation, translation and protein folding [2,8] while proteins involved in light and dark reactions of photosynthesis were significantly reduced. However, whether this is a strain-specific unique response of *Anabaena* 7120, or a generic response common to photodiazotrophic *Anabaena* strains is unknown since similar studies have not been conducted with other native cyanobacterial inhabitants of paddy fields. It is equally of interest to understand what determines the individual tolerance of different *Anabaena* strains to oxidative stress. Proteomic modifications in response to environmental stress provide an excellent tool for such analyses. Comparative proteomic approach has been successfully used to compare butachlor tolerance and salt stress response; among these the three strains of cyanobacteria [9,10].

In the present study, the methyl viologen-triggered proteomic response was compared in *Anabaena* L-31 ($LD_{50}$ dose: 2 μM MV for 5 h) and *A. doliolum* ($LD_{50}$ dose: 2 μM MV for 3 h) and *Anabaena* 7120 ($LD_{50}$ dose: 2 μM MV for 6 h). At respective $LD_{50}$ dose, proteins were isolated from each strain, resolved by 2D gel electrophoresis and 92 and 41 proteins of interest were respectively identified from *Anabaena* L-31 and *A. doliolum*, by MALDI-ToF mass spectrometry and the MV responsive proteomic modulations were compared among the three *Anabaena* strains. Although differences were observed in the number or type of proteins differentially regulated following MV exposure, in general proteins involved in oxidative stress alleviation and protein homeostasis were upregulated while crucial enzymes involved in photosynthesis

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and carbon metabolism were downregulated in all the three strains. Further, the strain specific differences in the ability to maintain cellular homeostasis appeared to determine differential MV sensitivity of individual *Anabaena* strains.

## 2. Materials and methods

### 2.1. Cyanobacterial strains and growth condition and oxidative stress

*A. doliolum* and *Anabaena* L-31 were grown in combined nitrogen free BG-11 medium (pH 7.2) [11], under continuous illumination (30 μE $m^{-2}$ $s^{-1}$) at 27 ± 2 °C and with aeration (3 $Lm^{-1}$). Three day old culture of *Anabaena* L-31 and *A. doliolum* with cell density of 10 μg chl *a* per 1 ml culture were exposed to different concentrations of MV for specified duration. Cell survival was assessed at each MV concentration and at appropriate time points by plating 100 μl culture on BG 11N$^-$ agar plate and estimating the number of colony forming units (CFU) after 10 days of incubation under continuous illumination at 27 ± 2 °C. Each experiment was performed with three replicate sets and the observed variation was less than 10%. The $LD_{50}$ dose which reduced growth by 50% was determined for each strain.

### 2.2. Estimation of ROS

MV mediated ROS generated in both the strains of *Anabaena*, exposed to $LD_{50}$ doses of MV, were estimated using 2′, 7′-dichlorofluorescin diacetate (DCFDA) and compared with ROS levels in respective untreated controls, as described earlier [8].

### 2.3. Sample preparation and protein resolution by 2D gel electrophoresis

Protein samples were prepared from MV treated (2 μM MV exposure for 5 h for *Anabaena* L31 or 2 μM MV exposure for 3 h for *A. doliolum*) as well as from corresponding untreated control cells (equivalent of ~150 μg chl *a*), as described earlier [8]. The protein content was estimated using a Lowry protein estimation kit (Sigma, India). Iso-electric focussing was carried out on the immobilized pH gradient strips, nonlinear pH 3–10, 17 cm (Bio-Rad, India) by cup-loading method following the protocol described earlier [8]. Typically ~1 mg protein was solubilized in 80 μL of rehydration buffer [8 M urea, 1 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 150 mM Dithiothreitol (DTT), 2% IPG buffer, traces of Bromophenol Blue] for 1 h at room temperature and loaded onto the cup (100 μl) that was placed near the cathode. IEF was performed with the Protean Isoelectric Focusing Cell (Bio-Rad, India) at 20 °C and the 2nd dimensional resolution was performed by 14% SDS-PAGE. Each experiment was repeated three times resulting in 3 biological replicates, for each strain.

### 2.4. Gel imaging and spot analysis

Gels were digitized using Dyversity-6 gel imager [Syngene, UK] and GeneSnap software [Syngene, UK]. First level match set was generated from three biological replicates of 2D gels with a minimum correlation coefficient value of 0.6., for each strain, using PDQuest 2D gel analysis software (version 8.1.0, Bio-Rad). Between replicate gels, spots were detected and matched in automatic detection mode, followed by manual editing. The spots that were not present in all the replicate gels were excluded. Local regression method was applied to normalize spot densities. Student's *t*-test was used to determine statistically significant (*p*-values less than 0.05) differentially expressed proteins (DEPs) in MV treated sample of each strain as compared to their respective controls. Protein spots of interest, from *Anabaena* L-31 and *A. doliolum* 2D gel profiles were excised manually from the gel and were processed exactly as described earlier [8]. Eluted peptides were vacuum concentrated to a final volume of 5 μL and stored at −70 °C, until further use.

### 2.5. Mass spectrometry and protein identification

Protein gel plugs were destained, reduced, alkylated, digested with trypsin (in-gel) and the oligopeptides were eluted exactly as described earlier [8]. Mass spectrometry was carried out using UltraFlex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany). Further steps such as, co-crystallization of eluted oligopeptides with matrix (CHCA, 5 mg/ml in 0.1% TFA and 30% ACN) on MALDI target plate (384-well stainless steel plate, Bruker Daltonics, Germany), external calibration of the machine (Peptide calibration mix I, Bruker Daltonics, Germany or with the trypsin autodigest peptides), acquisition of mass spectra (mass range of 600–4500 Da using standard ToF-MS protocol in positive ion reflection mode), generation of peak list (using flexAnalysis software 3.0, Bruker Daltonics, Germany) and import of mass spectra into the database search engine (BioTools v3.1 connected to Mascot, Version 2.2.04, Matrix Science) were carried out as described earlier [8]. Mascot searches were conducted using from NCBI nonredundant database (released Jan 2012 or later, at least 17910093 entries actually searched). The settings chosen for identification were as follows: number of missed cleavages permitted 1 (or 2 for DnaK and Fda-1 in *A. doliolum*); fixed modifications such as carbamidomethyl on cysteine; variable modification of oxidation on methionine residue; peptide tolerance 100 ppm (or 150 for HrcA-2 and Tkt-2, and 200 ppm for Mpe1 in *Anabaena* L-31; 50 for Ef-Tu-1, RbcL-1, Pgk, Tkt-1 and GlnA; and 150 for Tkt-2 and Prk in *A. doliolum*), enzyme used as trypsin and a peptide charge setting as +1. A match with *Anabaena* 7120 protein with the best score in each Mascot search was accepted as successful identification. Protein identification was considered to be significant if minimum 2 of the following 4 criteria were fulfilled: a Mascot score of > 60 ($p < 0.05$), a minimum of 5 peptide matches, sequence coverage ≥ 15% and consistent location of the protein spot on 2D gel, among 3 strains.

## 3. Results and discussion

### 3.1. Survival of Anabaena strains in the presence of different concentrations of methyl viologen

Exposure to MV at >1 μM concentration for 24 h was lethal for *A. doliolum* and *Anabaena* L-31, as was also observed earlier for *Anabaena* PCC 7120 [8]. About 50% survival was observed upon exposure to either 7.5 μM MV for 30–60 min or 5 μM MV for 3–5 h, or 2 μM MV for 3–6 h (Fig. 1). $LD_{50}$ dose of MV for *A. doliolum* and *Anabaena* L-31 was calculated as 2 μM MV with exposure time of 3 h and 5 h, respectively (Fig. 1). All further studies were carried out at the respective $LD_{50}$ doses, for both the strains. MV treated cells of *Anabaena* 7120 and *Anabaena* L-31 displayed 2.5 and 2.4 fold increases in ROS levels, respectively, as compared to their controls while ROS level increased by 2.0 fold in *A. doliolum*. *A. doliolum* was found to be less tolerant to MV than *Anabaena* 7120 ($LD_{50}$: 2 μM MV for 6 h), while the tolerance of *Anabaena* L-31 was comparable to that of *Anabaena* 7120 [8]. The herbicide paraquat (methyl viologen) is commonly used at 0.07–0.14% (2–4 mM of Gramoxone SL) field concentration (or more, depending on weed height and density), in paddy fields [12]. The $LD_{50}$ dose of MV for the cyanobacteria under study was found to be much lower than the agronomically beneficial field application dose of paraquat.

### 3.2. MV responsive proteome modulation in Anabaena L-31 and A. doliolum

Proteomic response of *Anabaena* L-31 and *A. doliolum* to MV induced oxidative stress was analyzed and compared with that of *Anabaena* 7120. The representative 2D protein profiles of *Anabaena* L-31 and *A. doliolum* are shown in Figs. 2 and 3, respectively. On an average, PDQuest 2D analysis software detected about 300 and 325 reproducible spots from biological triplicates of 2D gels of control and MV-treated samples of *Anabaena* L-31 while in *A. doliolum*, 348 and 334 spots were identified from control and MV-treated sample, respectively.

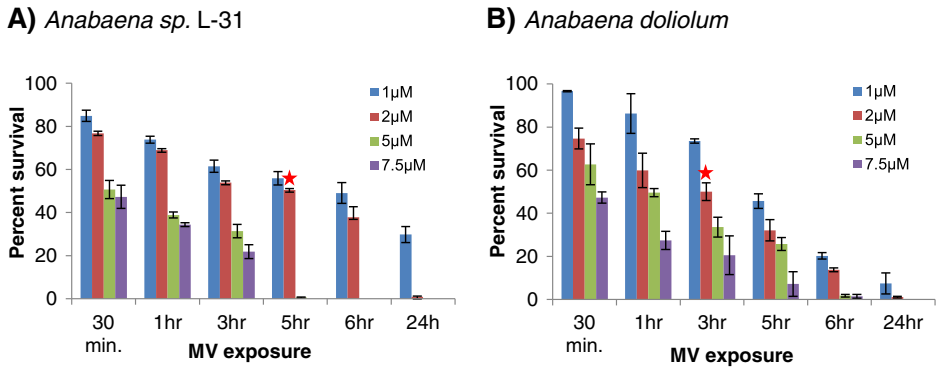


**Fig. 1.** Percent survival following different doses of methyl viologen (MV), in two strains of cyanobacteria (A) *Anabaena* L-31 and (B) *Anabaena doliolum*. Three day old cultures of these two strains (cell density 10 μg $ml^{-1}$) were exposed to different concentrations of MV (1–7.5 μM) for different time periods (30 min–24 h). Survival was observed in terms of colony forming units (CFU) in 100 μl culture. CFUs have been expressed in terms of percentage survival of MV exposed cells as compared to control cells (100% survival), at each time point. $LD_{50}$ doses for *Anabaena* L-31 (2 μM for 5 h) and *Anabaena doliolum* (2 μM for 3 h) are indicated by star mark and were chosen for all further experiments.

Among these, 42 and 28 proteins showed significant variation in their abundance (Supplementary Table 1). A total of 92 spots from *Anabaena* L-31 and 41 spots from *A. doliolum* were identified by mass spectrometry (Supplementary Table 2, Supplementary MS data). As has been shown earlier [9,10], all the proteins identified, in this study, from *Anabaena* L-31 and *A. doliolum* showed 100% match with *Anabaena* 7120, with the exception of 2 proteins. In *Anabaena* L-31, 33 identified proteins were differentially expressed ($p \leq 0.05$). The level of 11 proteins (present in 14 spots) increased while that of 12 proteins (present in 19 spots) decreased in MV treated cells (Supplementary Table 1). In *A. doliolum*, 16 identified proteins were differentially expressed ($p \leq 0.05$). The abundance of 9 proteins (present in 9 protein spots) displayed increase and that of 6 proteins (present in 7 protein spots) decreased in MV treated cells (Supplementary Table 1). These proteins belonged to the major functional categories of oxidative stress alleviation, protein transcription/translation/folding, photosynthesis, carbon metabolism (carbon fixation, glycolysis/gluconeogenesis and oxidative pentose phosphate pathway) and to other miscellaneous categories, such as amino acid metabolism, purine metabolism, TCA cycle, porphyrin-containing compound metabolism and hypothetical proteins.

### 3.3. Comparison of MV responsive oxidative stress alleviation in three cyanobacterial strains

In *Anabaena* 7120, MV elicited a strong oxidative stress alleviation response by enhancing ROS detoxifying enzymes, namely NADPH dependent thioredoxin reductase (NTR), DNA-binding ferritin-like protein (Dps), Ferredoxin–$NADP^+$ reductase (FNR), FMN reductase (FMNr), Phosphoadenosine phosphosulphate reductase (PAPR), 1-cys Prx, and a hypothetical protein (alr7524) with predicted Prx activity, but abundance of Super-oxide dismutase (Fe-SOD) decreased (−1.47 fold) [8]. In *Anabaena* L-31, MV exposure led to increased expression of Prx (1.9 fold), Fe-SOD (1.84 fold) and FNR (3.60 fold) while levels of thioredoxin (Trx), glutathione reductase (Gor), and FMN-dependent NADH-azoreductase (AzoR) did not change significantly (Fig. 4A and Supplementary Table 1). In *A. doliolum*, Prx level increased (2.9 fold) while that of Trx and Fe-SOD remained unaltered (Fig. 4B and Supplementary

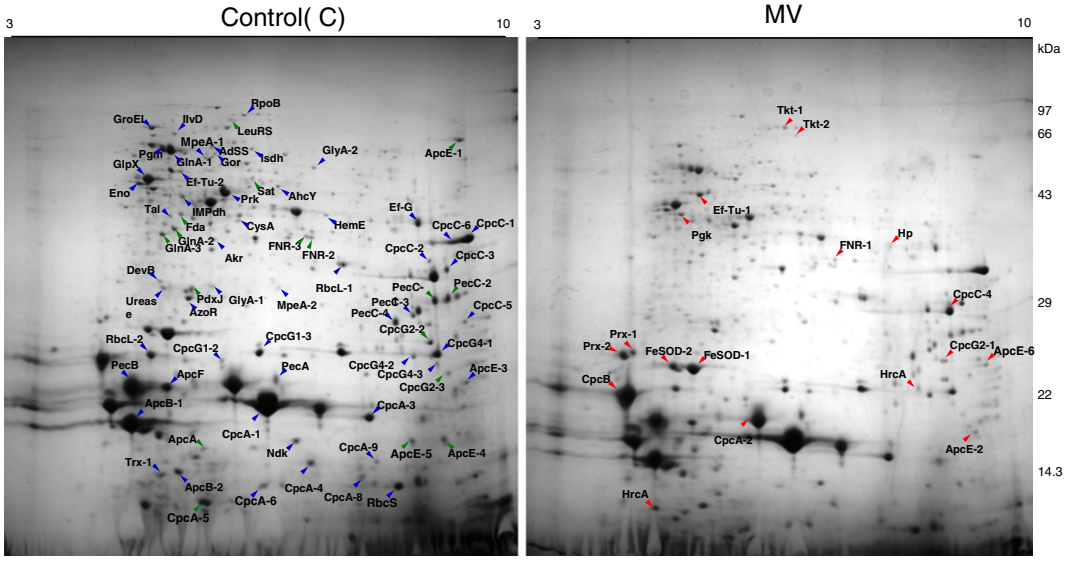


**Fig. 2.** Representative proteome profile of *Anabaena* L-31 cells exposed to 2 μM MV for 5 h (MV) compared with unstressed (control) culture. Cellular proteins (1 mg) were resolved on IPG strips (pH 3-10NL, 17 cm) by Iso-electric Focusing (IEF), followed by 14% SDS-PAGE. Significantly increased proteins are marked by red arrow heads, significantly decreased proteins are marked by green arrow heads and proteins with no significant change are marked by blue arrow heads. Molecular masses of marker proteins and pI are mentioned on right hand side and at the top, respectively.

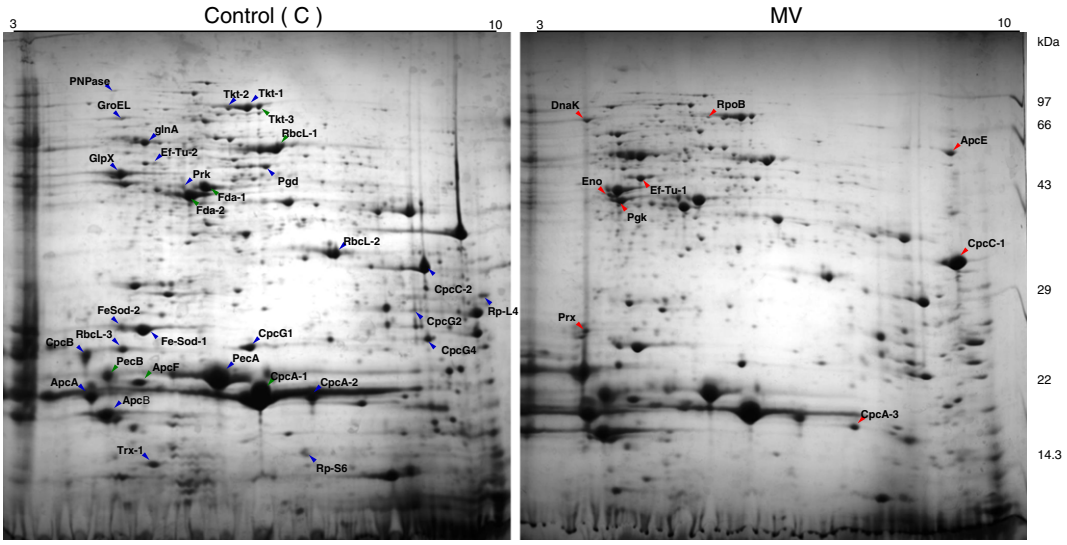


**Fig. 3.** Representative proteome profile of *Anabaena doliolum* cells exposed to 2 μM MV for 3 h (MV) compared with unstressed (control) culture. Other details were as described in the legend of Fig. 2.

Table 1). Fe-SOD proteins displayed a differential pattern in the three strains of *Anabaena* in response to MV. Both reduced as well as oxidized forms of Prx increased in abundance following MV exposure, in *Anabaena* L-31 indicating active involvement of Prx in oxidative stress alleviation. In *A. doliolum*, only reduced form of Prx was identified with increased abundance. The level of FNR, which protects the cells from oxidative stress, was increased in *Anabaena* L-31 as was also observed in *Anabaena* 7120 [8]. The abundance of Trx did not change significantly in both, *A. doliolum* and *Anabaena* L-31, following MV exposure. Trx catalyzes dithiol-disulfide exchange in light-dependent regulation of many enzymes and converts disulfides to dithiols in their respective target enzymes in the chloroplasts of higher plants and algae. Upregulated levels of Trx proteins in response to several abiotic stresses in different cyanobacteria have been reported [13–17]. Enzymes involved in Calvin cycle in chloroplast and glycogen synthesis are considered as major targets of Trx [18]. Trx would detoxify the

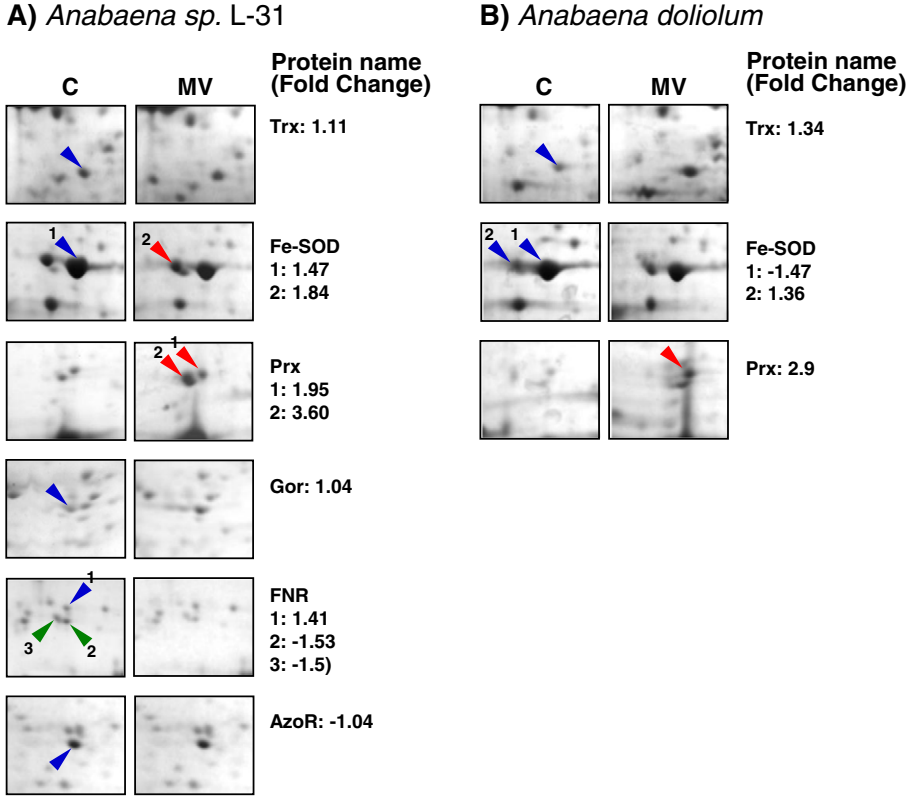


**Fig. 4.** MV responsive changes in the abundance of oxidative stress alleviation proteins in (A) *Anabaena* L-31 and (B) *Anabaena doliolum*. Protein abbreviations are as mentioned in Figs. 2, 3 and Supplementary Table 2. Differentially expressed proteins with increased or decreased abundance are shown by red or green arrow heads in the MV or control gel, respectively. Proteins with no significant change in abundance are shown with blue arrow heads.

oxidative stress and help regulate Calvin cycle and redox regulation in chloroplast. Gor protein, observed in *Anabaena* L-31, is crucial for maintaining high levels of reduced glutathione in cytosol which is essential for maintaining cellular redox homeostasis. Thus, elevation in the abundance of oxidative stress alleviation proteins seems to be a mechanism common to all the three cyanobacterial strains.

### *3.4. Comparison of MV responsive modulations in transcription, translation and protein folding in three cyanobacterial strains*

MV mediated oxidative stress damages important cellular biomolecules such as proteins and DNA [4]. In defense, *Anabaena* 7120 increased the abundance of DnaK, GroEL, RP-L9, polynucleotide phosphorylase (PNPase), ArgS, EF-Tu, EF-G and EF-Ts [8]. In *Anabaena* L-31, abundance of HrcA (2.34 fold) and EF-Tu (1.84 fold) increased, while that of GroEL decreased (−1.40 fold) significantly, in response to MV exposure (Fig. 5A and Supplementary Table 2). Abundance of Ndk, RpoB, or EF-G did not change. In *A. doliolum*, levels of RpoB (2.20 fold), DnaK (1.85 fold) and EF-Tu (1.75 fold), while that of GroEL, PNPase, RP-L4 and RP-L6 did not change, in response to MV exposure (Fig. 5B and Supplementary Table 2). HrcA is a negative regulator of heat shock proteins (DnaK, DnaJ, GrpE and GroESL) which are necessary for protein folding. Enhanced levels of HrcA could be the reason for observed low abundance of heat shock proteins in *Anabaena* L-31. Upregulation of RpoB was the major change observed in MV response in *A. doliolum*.

Elevated level of EF-Tu was also observed in *Anabaena* 7120 and *Synechocystis* sp. PCC6803 under different abiotic stress conditions [8,19,16] and postulated to be the major alleviator of stress-induced protein damage. EF-Tu exhibits chaperone properties by interacting with denatured proteins [16,20]. The sustained/enhanced levels of chaperones and elongation factors would secure the maintenance of cellular homeostasis.

### *3.5. Comparison of MV responsive modulations in photosynthesis in three cyanobacterial strains*

The photooxidative effect of MV affects photosystem II (PSII) by diverting electrons from PSII to oxygen and enhancing generation of superoxides that further degrade the PSII activity in chloroplast [4,5]. In cyanobacteria, phycobiliproteins are the antenna proteins of phycobilisomes. They are comprised of the subunits of allophycocyanin (ApcA, ApcB, ApcE and ApcF), phycocyanin (CpcA, CpcB, CpcC, CpcG1 and CpcG4), and phycoerythrin (PecA, PecB and PecC) and are directly involved in photolysis of water [21]. Phycobiliproteins of *Anabaena* L-31 and *A. doliolum* were observed as multiple protein spots, as was the case in *Anabaena* 7120 and other cyanobacteria [8,22]. In the proteome of *Anabaena* L-31 and *A. doliolum*, 43 protein species (belonging to 14 proteins) and 15 protein species (belonging to 12 proteins) were identified as phycobiliproteins, respectively. Their modulation has been interpreted as described earlier [8]. In *Anabaena* L-31 (i) levels of CpcB and CpcG2 increased; (ii) levels of ApcA, ApcE, CpcA and PecC decreased; (iii) levels of ApcB, ApcF, CpcC, CpcG1, CpcG4, PecA, PecB and C-phycoerythrin class 2 subunit alpha (MpeA) remained unaltered; (iv) degradation of ApcE (ApcE-2) and CpcC (CpcC-4) increased; (v) degradation of ApcE (ApcE-4 and −5), CpcA (CpcA-5) and CpcG2 (CpcG2-2 and −3) decreased (Fig. 6A and Supplementary Table 2). In *A. doliolum* (i) abundance of ApcE and CpcC increased; (ii) that of CpcA and PecB decreased; (iii) while levels of ApcA, ApcB, ApcF, CpcB, CpcG1, CpcG2, CpcG4 and PecA remained unaltered; and (iv) degradation of CpcA (CpcA-3) increased (Fig. 6B and Supplementary Table 2). MV mediated ROS caused visual bleaching of PSII and thereby, severely affected photosynthesis and generation of ATP and NADPH. Disruption in stoichiometry of PSII due to disproportionately enhanced or reduced levels of phycobiliproteins would further affect dark reaction of photosynthesis.

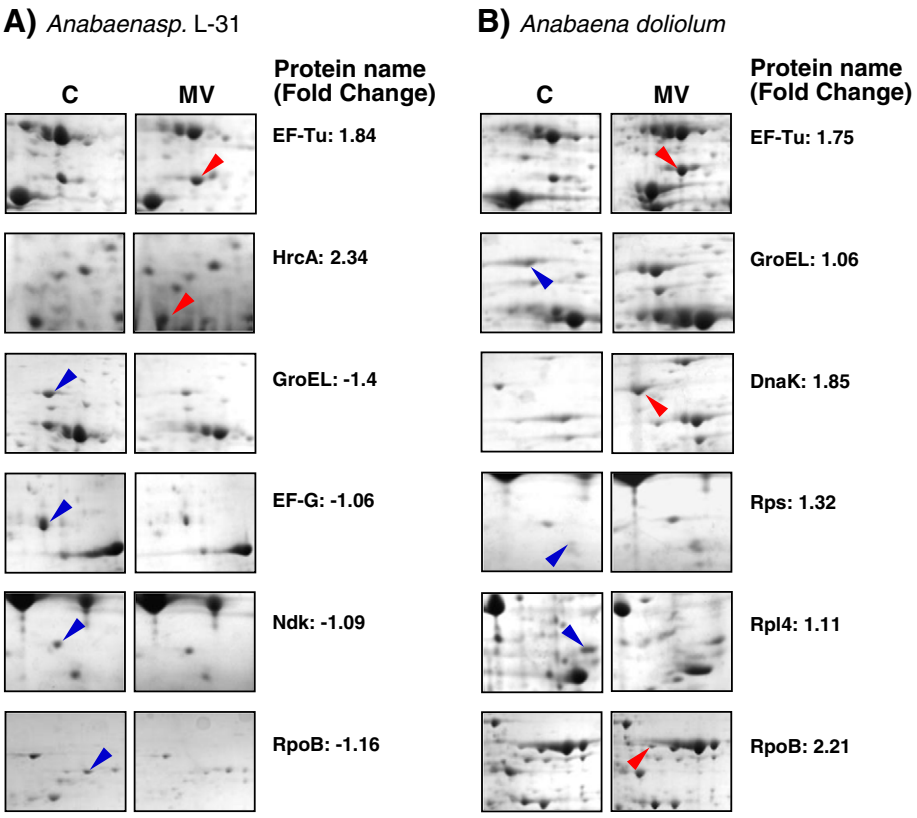


**Fig. 5.** MV responsive changes in the abundance of proteins involved in transcription, translation and protein folding in (A) *Anabaena* L-31 and (B) *Anabaena doliolum*. Other details were as described in the legend of Fig. 4.

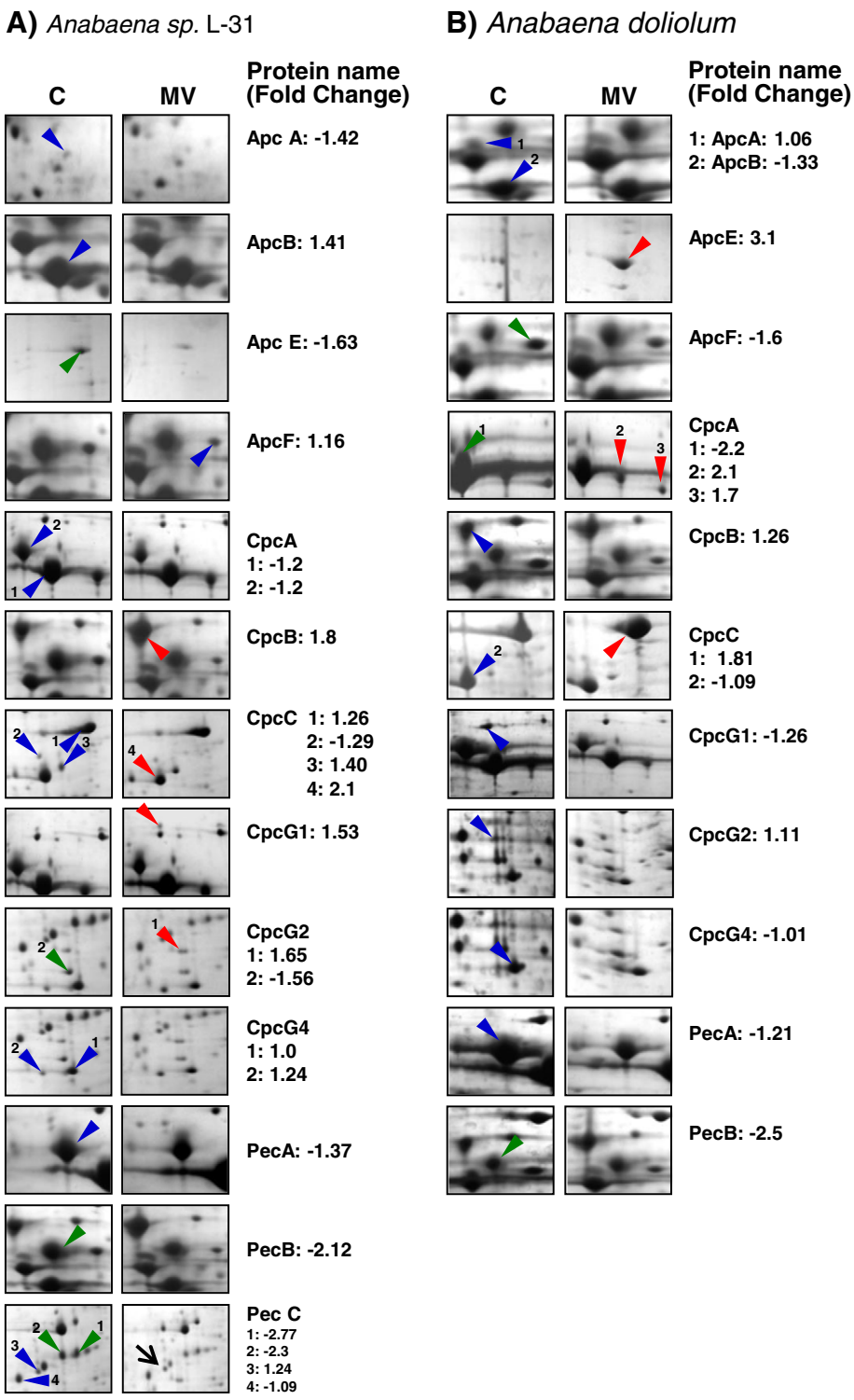


**Fig. 6.** MV responsive changes in the abundance of photosynthetic proteins in (A) *Anabaena* L-31 and (B) *Anabaena doliolum*. Other details were as described in the legend of Fig. 4.

### *3.6. Comparison of MV responsive modulations in carbon metabolism in three cyanobacterial strains*

Carbon metabolism is mediated through Calvin cycle, glycolysis/gluconeogenesis, and oxidative pentose phosphate pathway (OPP). In *Anabaena* 7120, high abundance was observed for ribulose-1,5-bisphosphate carboxylase (RbcL-1), glucose-6-phosphateisomerase (Pgi) and phosphoglycerate kinase (Pgk), while glucokinase (Glk), phosphoribulokinase (Prk) and glyceraldehyde 3-phosphate dehydrogenase-2 (Gap2) levels decreased [8]. The abundance of other identified proteins under this category displayed minor modulations in their levels [8]. These were Ribulose-1,5-bisphosphate carboxylase/oxygenase, Small subunit (RbcS), fructose-1, 6-bisphosphate aldolase (Fda), phosphopyruvate hydratase (Eno), 6-phosphogluconolactonase (DevB), transketolase protein species (Tkt-1–3), fructose 1, 6-bisphosphatase II (GlpX-2), pyruvate

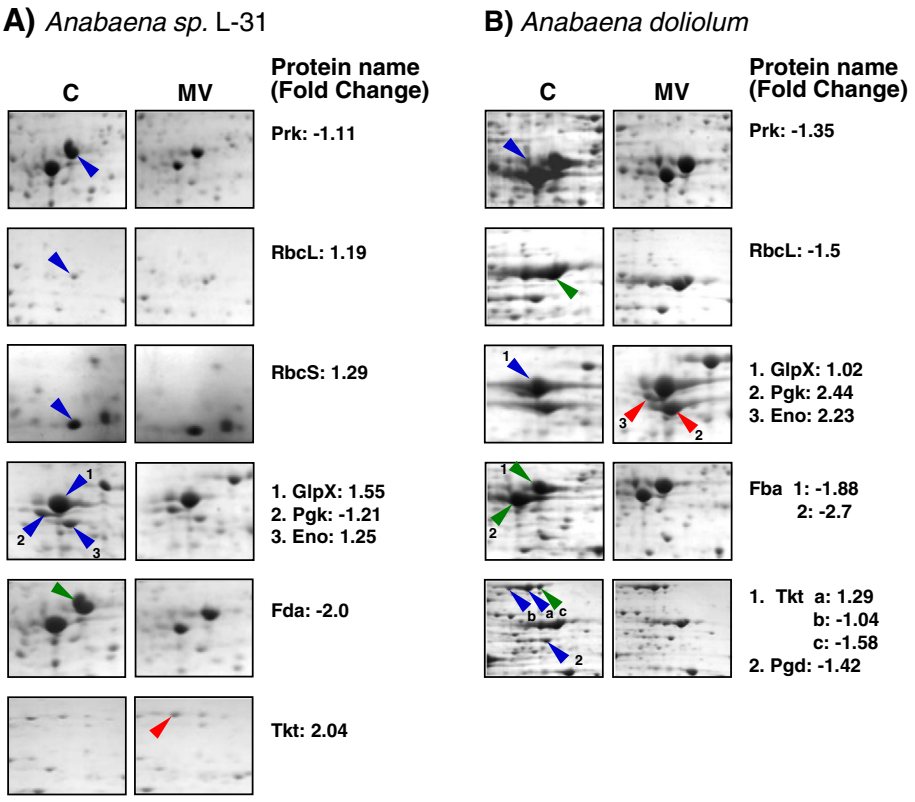


**Fig. 7.** MV responsive changes in the abundance of proteins involved in carbon metabolism in (A) *Anabaena* L-31 and (B) *Anabaena doliolum*. Other details were as described in the legend of Fig. 4.

dehydrogenase E1 subunit (Pde1), alpha glucotransferase (GlgB), galactose mutarotase (GalM) and aldo keto reductase (Akr) [8]. In *Anabaena* L-31, enhanced level was observed for Tkt protein species (2.04 & 2.13 fold) while Fda level (−2.0 fold) decreased significantly in MV exposed cells (Fig. 7A and Supplementary Table 2). The levels of RbcL, RbcS, Pgk, GlpX, Prk, DevB, phosphoglucomutase (Pgm), Eno, transaldolase (Tal) and Akr did not change significantly (Fig. 7A and Supplementary Table 2), following MV exposure. In *A. doliolum*, MV exposure significantly enhanced abundance of Eno (2.23 fold) and Pgk (2.44 fold) but decreased levels of RbcL-1 (−1.5 fold), Fda (−1.88 and −2.7 fold), and Tkt-3 (−1.58 fold) (Fig. 7B and Supplementary Table 2). Other enzymes whose levels displayed no significant change in expression were RbcL-2, RbcL-3, GlpX, Prk, Tkt-1, Tkt-2, and 6-phosphogluconate dehydrogenase (Pgd) (Fig. 7B and Supplementary Table 2).

It appears that in the 3 strains of *Anabaena*, carbon metabolism was differentially regulated. Both *Anabaena* L-31 and *A. doliolum* displayed reduced expression of Fda that would affect conversion of glyceraldehydes-3-phosphate to fructose-1,6-bisphosphate, a crucial link between Calvin cycle, glycolysis/gluconeogenesis and oxidative pentose phosphate (OPP) pathway. Glycolysis and OPP pathway are the major sources of ATP and NADPH, which is necessary for cell survival [23,24]. Their disruption would drastically reduce ATP and NAD(P)H levels and affect cell viability. In addition, reduced levels of RbcL and Tkt would further affect these 2 pathways in *A. doliolum*.

### *3.7. MV responsive modulations in expression of miscellaneous enzymes in three cyanobacterial strains*

In *Anabaena* 7120, among 15 proteins identified under this category, only 5 and 1 overlapped with *Anabaena* L-31 and *A. doliolum*, respectively. Glutamate-ammonia ligase (GlnA) was identified in all the three stains of *Anabaena*. MV exposure did not affect the abundance of GlnA significantly in either strain, but decreased its degradation in *Anabaena* L-31 and *Anabaena* 7120 (Figs. 2, 3 and Supplementary Table 2). Among others, inosine 5-monophosphate dehydrogenase (IMPdh), S-adenosyl-L-homocysteine hydrolase (AhcY) and cysteine synthase A (CysA) remained unaltered in both, *Anabaena* L-31 and *Anabaena* 7120 (Fig. 2 and Supplementary Table 2). The abundance of Pyridoxine 5′-phosphate synthase (PdxJ) decreased in MV exposed *Anabaena* L-31 but remained unaltered in *Anabaena* 7120.

## 4. Conclusions

This study, for the first time (i) documented $LD_{50}$ doses of commonly used weedicide methyl viologen for cyanobacteria native to Indian paddy fields and found that they were much below the agronomically relevant field application doses of paraquat, and (ii) examined and compared cellular responses induced by $LD_{50}$ dose of MV in these photoautotrophic, heterocystous, nitrogen-fixing *Anabaena* strains. A comparative proteomic approach was used to explore common mechanisms adopted by cyanobacteria to combat MV induced oxidative stress and to assess strain specific differences that would define differential sensitivity of individual strain to MV exposure. The assessment revealed that proteome modulation (increase, decrease or no change in level) pattern of (i) 8 proteins matched in all the three strains; (ii) 14 proteins matched between *Anabaena* L-31 and *Anabaena* 7120; (iii) 7 proteins matched between *A. doliolum* and *Anabaena* 7120 and (iv) 6 proteins matched between *A. doliolum* and *Anabaena* 7120 (Fig. 8). Disruption in stoichiometry of proteins corresponding to the photosynthetic machinery was evident in all 3 strains, which could be the direct effect of MV mediated Mehler reaction. It would affect photosynthesis and downstream carbon metabolism and appeared to be the root cause of MV sensitivity in all the three strains. A similar negative effect was observed for enzymes involved in glycolysis/

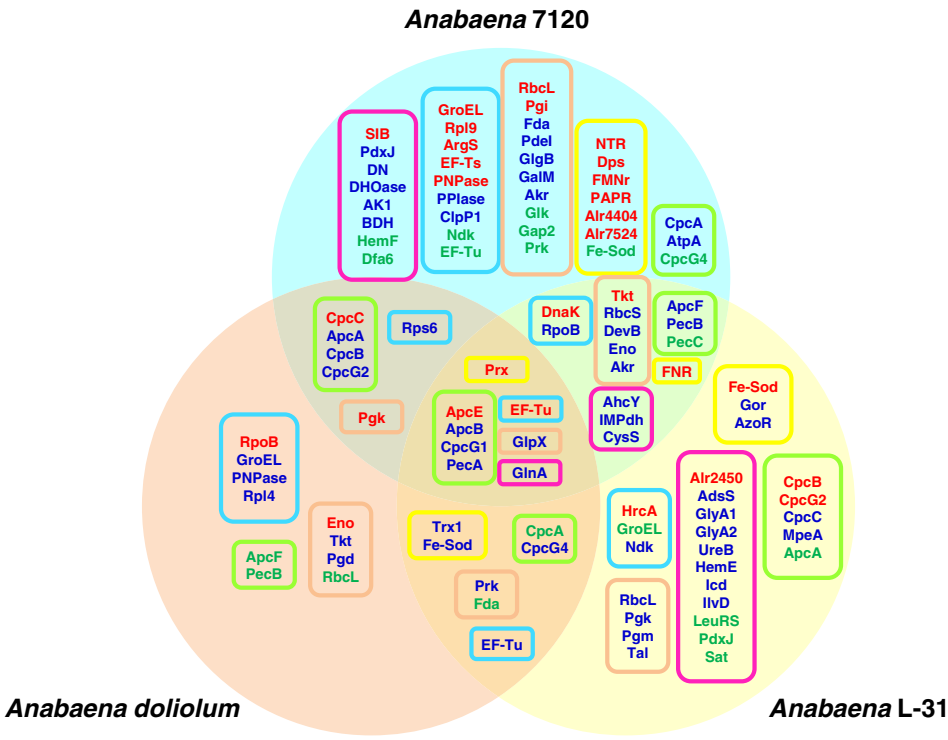


**Fig. 8.** Venn diagram of MV response in three cyanobacterial species, *Anabaena* 7120 (blue), *Anabaena* L-31 (yellow) and *Anabaena doliolum* (red). Differentially expressed proteins with increased or decreased abundance are shown in red and green, while proteins with no significant change in abundance are sown in blue. Protein abbreviations are as mentioned in Figs. 2, 3 and Supplementary Table 2 and as in reference 8. Proteins are categorized into 5 groups, Oxidative stress alleviation (yellow box), Transcription, translation and protein folding (blue box), Photosynthesis (green box), Carbon metabolism (peach box) and miscellaneous (magenta box).

gluconeogenesis and oxidative pentose phosphate pathway, important for ATP and NAD(P)H generation.

Among the three strains, *Anabaena* 7120 displayed maximum MV tolerance which correlated well with the highest number/levels of oxidative stress alleviator proteins and chaperone (especially GroEL) proteins. Neutralization of ROS and maintenance of proteins in functionally competent structures under oxidative stress conditions would help the organism cope up with stress better. In comparison, *Anabaena* L-31 also had elevated levels of Prx, FNR and Fe-SOD but could not elevate levels of chaperones due to elevated levels of heat shock repressor HrcA and showed somewhat less oxidative stress tolerance than *Anabaena* 7120. *A. doliolum* showed elevated levels of only Prx, among oxidative stress alleviators, and its protein homeostasis response did not match with that of *Anabaena* 7120, resulting in significant stress sensitivity.

Thus, although MV negatively affected photosynthesis and carbon metabolism, better management of oxidative stress and protein homeostasis by *Anabaena* 7120 makes it a more successful cyanobacterium in stressful paddy fields, followed by *Anabaena* L-31. *A. doliolum* which had the lowest MV tolerance among the three strains, predictably showed a proteomic response divergent from both *Anabaena* L-31 and *Anabaena* 7120. The study brings forth contrasting outcomes in three common paddy-inhabiting cyanobacterial strains. The results explain the differential sensitivity of individual strains to MV induced oxidative stress. The study also identifies major targets of oxidative stress in nitrogen-fixing cyanobacteria that need to be manipulated for combating oxidative and other biotic or abiotic stresses prevalent in Indian paddy fields.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.05.020.

## Conflict of interest

The authors declare no conflict of interest.

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