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ORIGINAL ARTICLE

Overexpression of phytochelatin synthase (*pcs*) enhances abiotic stress tolerance by altering the proteome of transformed *Anabaena* sp. PCC 7120

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Abstract The present study provides data on the insertion of an extra copy of phytochelatin synthase (*alr0975*) in *Anabaena* sp. PCC 7120. The recombinant strain (AnFPN-*pcs*) compared to wild type showed approximately 22.3% increase in growth rate under UV-B, NaCl, heat, $CuCl_2$, carbofuran, and $CdCl_2$. It also registered 2.25-fold enhanced nitrogenase activity and 5-fold higher phytochelatin production. A comparison of the protein profile of wild type with the recombinant strain revealed that recombinant strain accumulated proteins belonging to the following categories: (i) detoxification (nutrient stress induced DNA binding protein, Mn-SOD, Alr0946 (CalA)), (ii) protein folding and modification (molecular chaperone DnaK, FKBP-type peptidyl-prolyl cis-trans isomerase), (iii) nucleotide and amino acid biosynthesis (dihydroorotase and Ketol-acid reductoisomerase), (iv) photosynthesis and respiration (coproporphyrinogen III oxidase, phycocyanin alpha chain, ferredoxin-$NADP^+$ reductase), and (v) transport (sugar transport ATP-binding protein). Thus, it can be concluded that, above category proteins with their respective role in scavenging reactive oxygen species, proper folding of unfolded proteins, and protection of protein from degradation, sustained carbon fixation and energy pool and active transport of sugar together conceivably help the recombinant cyanobacterium (AnFPN-*pcs*) to cope with abiotic stress employed in the present study. Such recombinant strains have potential for future use as biofertilizer.

Keywords Phytochelatins · Phytochelatin synthase · Transformation · Overexpression · Proteome

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Introduction

Cyanobacteria, an extremely diverse group of photosynthetic prokaryotes, were the sole photosynthesizers for eons and contributed oxygen to the primitive reducing and anaerobic atmosphere (Seckbach and Oren, 2007). Diazotrophic cyanobacteria endowed with the exclusive ability of photosynthesis and nitrogen fixation are an integral component of paddy fields. However, they are inevitably exposed to various stresses such as salinity, drought, heat, heavy metals, and pesticides and display enormous plasticity to adapt under aforesaid stresses (Pandey et al., 2012; Rai et al., 2014; Agrawal et al., 2014; Shrivastava et al., 2015; Singh et al., 2015). Moreover, existence of cyanobacteria under extreme environments (harsh conditions of temperature, pH, harmful radiation, and hypersalinity) makes them an excellent source for mining stress-tolerant genes.

Various reports witnessed the above statement and stress tolerant transgenics in cyanobacteria as well as in plants have been raised utilizing cyanobacterial genes. For example, overexpression of groESL operon (Chaurasia and Apte, 2009), AhpC (Shrivastava et al., 2016), and *dps* (Narayan et al., 2016) in *Anabaena* PCC7120 offered tolerance to multiple abiotic stresses. Moreover, the expression of

phytoene desaturase (*Pds*) from *Synechococcus* sp. PCC7942 and *isiB* (flavodoxin) from *Anabaena* sp. in tobacco offered tolerance to various abiotic stresses (Wagner et al., 2002; Tognetti et al. 2006, 2007).

Phytochelatins (PCs) are small cysteine-rich heavy metal-binding peptides, functionally analogous to metallothioneins (Grill et al., 1987, 1989) and display up-accumulation in response to heavy metal stress (Mallick et al., 1994; Tsuji et al., 2004). Metal tolerance through increase in PCs can be accredited to the presence of a thiol group which coordinates with metals (Hall, 2002; Cobbett and Goldsbrough, 2002). However, their role is not constrained to metal-associated stress; various reports suggest their significant role under a variety of stresses such as salinity, heat, UV-B, and drought (Zhang et al. 2005; Bhargava et al. 2005; Seki et al. 2003). PC-mediated tolerance to various abiotic stresses other than heavy metals can be attributed to shielding of thiols through conformational changes of PCs, thus protecting thiols from the damaging effects of stress as thiols are well known for their central role in abiotic stress tolerance (Bhargava et al., 2005).

Extensive work has been done on PCs from organisms other than cyanobacteria. In green alga *Stigeoclonium*, PCs displayed abundance following heavy metal stress (Pawlik-Skowrońska 2001). Similar results were found in *Arabidopsis thaliana* (Vatamaniuk et al. 1999), wheat (Ha et al. 1999), marine and fresh water algae (Gekeler et al. 1989; Pawlik-Skowrońska et al. 2007), fungi (Mehra et al. 1988; Kneer et al. 1992; Bolchi et al., 2011), and lichens (Pawlik-Skowrońska et al. 2002). However, the functional importance of cyanobacterial PCs under abiotic stresses has remained confined to only few reports (Bhargava et al., 2005; Chaurasia et al., 2008a, b). Heterologous expression of phytochelatin encoding gene (*pcs*) of *Anabaena* PCC7120 in *Escherichia coli* alleviated damaging effects of high temperature, salinity, carbofuran, cadmium, copper, and UV-B (Chaurasia et al., 2008a, b). In view of the above, to determine the role of PCs in abiotic stresses, an extra copy of phytochelatin-encoding gene phytochelatin synthase (*pcs*) was constitutively expressed in *Anabaena* PCC7120. An integrative expression vector pFPN, used for incorporating a cassette of genes for genomic integration and expression into *Anabaena* sp. PCC 7120 (Chaurasia et al., 2008a, b; Chaurasia and Apte 2009), was employed. Moreover, to address the question as how far the overexpression of *pcs* (*alr0975*) affects the performance of other metabolic proteins which might collectively help in the stress tolerance, comparative proteomic studies of the wild-type (*Anabaena* sp. PCC 7120) and the transformed *Anabaena* (hereafter AnFPN-*pcs*) by using 2-DE and MALDI-TOF/MS has also been carried out.

Materials and methods

Conserved domain analysis

The amino acid sequence of Alr0975 was retrieved from cyanobase (http://genome.microbedb.jp/cyanobase/Anabaena). Conserved domain analysis was performed with conserved domain database of NCBI (Marchler-Bauer et al. 2015).

Organism and growth conditions

Anabaena sp. PCC 7120 was grown photoautotrophically in BG-11 medium (Rippka et al. 1979) without nitrogen, buffered with Tris/HCl at 24 ± 2 °C under day light fluorescent tubes emitting 72 μmol photon m^{-2} s^{-1} photosynthetically active radiation (PAR) light intensity with a photoperiod of 14:10 h at pH 7.5. The cultures were shaken by hand two to three times daily. *E. coli* strain DH5α was used as a host for cloning. *E. coli* cultures were stored as 10% (*v*/*v*) glycerol stocks at −80 °C and maintained on Luria-Bertani (LB) plates at 37 °C containing 1.4% (*w*/*v*) agar. Cells harboring recombinant plasmids were grown and maintained on LB medium supplemented with neomycin (Sambrook and Russell, 2001).

DNA isolation, PCR amplification, and cloning of *alr0975* gene into the pFPN vector

Genomic DNA from An7120 was isolated as described by Srivastava et al. 2007. An open-reading frame *alr0975*, putatively encoding phytochelatin synthase (*pcs*) (Fig. S1), was amplified by polymerase chain reaction using genomic DNA as the template with a pair of primers: Pf (5′-GAATTCCATATGGAATTCATGGCTCTCCGTCTTGGT-3′) and Pr (5′-′CGGGATCCCGTTACTTGTTAGGTTGAG GAGT-3′). The underlined bases are *Nde*I and *Bam*HI recognition sites, respectively. The PCR was done in a reaction mixture of 25 μl for 30 cycles at 94 °C for 1.5 min, 56 °C for 1 min, and 72 °C for 2 min using standard PCR conditions (100 ng of DNA, 2.5 μl of 10× PCR buffer with 15 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer, and 0.2 U Taq DNA polymerase in an Icycler (Bio-Rad, USA)). The amplified product was purified using a Qiagen gel extraction kit.

Generation of recombinant DNA construct for overexpression of *alr0975*

The purified PCR product was digested with *Nde*I and *BamHI*(NEB), and the resultant DNA fragment was cloned into an integrative expression vector pFPN that had been digested with the same restriction enzymes. To construct the recombinant plasmid, pFPN-*alr0975* was introduced into *E. coli* DH5α and transformed colonies were grown in LB medium. The plasmid was then isolated and the sequence of *alr0975* was confirmed by

sequencing and double digestion of recombinant plasmid to confirm that no mutations had occurred during PCR amplification.

Electroporation of recombinant plasmid into *Anabaena* sp. PCC 7120

The *pcs* gene *alr0975* amplicon was cloned into plasmid vector pFPN, which allows integration at a defined undisruptive site in the *Anabaena* sp. PCC 7120 genome and expression from a strong cyanobacterial *PpsbA1* promoter. The recombinant plasmids, pFPN-*alr0975*, were individually introduced into *Anabaena* by electroporation as demonstrated by Chaurasia et al., 2008a, b. The electrotransformants were selected on BG11, N^+ neomycin (25 μg ml^{-1}) plates by repeated subculturing for at least 24 weeks to achieve complete segregation and were designated as AnFPN-*pcs*.

Expression analysis using semiquantitative RT-PCR, western blotting, and enzymatic assay

Anabaena strain harboring the pFPN-*pcs* plasmid was grown in BG-11 medium supplemented with 100 μg ml^{-1}neomycin in an orbital shaker (200 rpm) at 37 °C. Mid-exponential phase of this culture was used to isolate RNA using TRIzol reagent as per the manufacturer's protocol (Invitrogen, USA). Reverse transcriptase (RT)-PCR was performed as per Mishra et al., 2009 using a Bio-Rad cDNA synthesis kit (Bio-Rad, Singapore), and gene-specific primers sets were designed using primer3 software. For RT-PCR of alr0975, forward primer 5′TAATCGCGCCCGAAGTAGTA3′ and reverse primer 5′TCTTGCCCAATTTCCTTACG3′ were used while for 16SrDNA (as an internal control), Pf 5′CACACTGG GACTGAGACAC3′ and Pr 5′CTGCTGGCACGGAG TTAG3′ were used. First-strand cDNA synthesis was performed using 1 μg of DNase I treated RNA in a 20-μl reaction volume according to the manufacturer's protocol.

Western blot analysis was done as described previously (Chaurasia et al., 2008a, b). Briefly, samples containing 30 μg of protein both from wild-type and overexpression strain were mixed in 1:1 (v/v) ratio with the gel loading buffer and run at a constant current of 15 mA until the samples entered the resolving gels followed by increasing the current to 25 mA for 4–5 h SDS-PAGE. The gels were transferred to a PVDF membrane (Millipore Immobilon-P), using a dual mini-electroblot system (Precision Instruments, Varanasi, India). The gel cassette was kept in transfer buffer (3.03 g/l Tris base, 14.4 g/l glycine, and 200 ml/l methanol (99% v/v pure) for 12 h at 50 V and 4°C. The membrane was blocked for 4 h in Tris buffer saline containing 0.1% Tween 20 (TTBS) and 5% (w/v) non-fat dried milk. The primary antibody was diluted (1: 2000 times) as per the instructions of the donors. The membrane incubated overnight at 4 °C in the diluted solution of the primary antibody was washed five times for 5 min each in TTBS. This was then incubated in a goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) (Genei, India) for 4 h. Following four consecutive 5-min washes in TTBS, the membrane was developed with DAB/$NiCl_2$ visualization solution. The reaction was terminated by washing the PVDF membrane with deionized distilled water. The blots were dried between filter papers, which considerably reduced the background staining. Polyclonal antibodies against phytochelatin synthase for detection by immunoblotting were obtained as generous gift from Dr. Stephan Cuine (CEA Cadarache, France). SDS-PAGE was performed with the same concentration of protein used in western blotting both for wild-type and recombinant strain which was taken as internal control.

The activity of gene in terms of enzyme phytochelatin synthase was determined by the level of phytochelatin inside the cells as per the methods of Bhargava et al. (2005). Protein concentration was estimated following the method of Bradford (1976).

Growth behavior analysis under different abiotic stresses

In order to investigate the role of *alr0957* in abiotic stress tolerance, a comparative growth behavior of An7120 and AnFPN-*pcs* was performed under different abiotic stresses. Both wild-type and overexpressed strains at their exponential phase were treated with LC_{50} dozes of Cd^{2+} (10 μM) (Singh et al. 2015), Cu^{2+} (20 μM) (Bhargava et al., 2008), temperature (42 °C) (Rajaram and Apte 2008), UV-B (g 2.34 × 106 μE·cm − 2) (Shrivastava et al. 2015), NaCl (100 mM) (Rai et al. 2014), and carbofuran (452 μM) (Padhy and Mohapatra 2001). Growth was estimated by measuring the optical density of the cyanobacterial cultures at 663 nm in a UV-Vis spectrophotometer (CECIL, England) on every 2nd day up to the 11th day by using a reference blank of basal culture medium. Specific growth rate was calculated by using the following equation: $m = [\ln (n_2/n_1)]/[t_2 - t_1]$, where m stands for specific growth rate and n_1 and n_2 are absorbance of culture suspension at the beginning (t_1) and the end (t_2) of the selected time interval.

Estimation of nitrogenase activity

The nitrogenase activity of *Anabaena* was determined by acetylene reduction assay (Stewart et al. 1968). For acetylene reduction, both the cultures of An7120 and AnFPN-*pcs* in the exponential phase were transferred to 13-mL glass vials and sealed with Suba seals. The sealed vials were evacuated and flushed with argon twice and then sparged with argon for10–15 min. For the nitrogenase assay, acetylene was injected at 10% concentration by volume and incubated for 2 h under continuous irradiance of 72 μmol photon m^{-2} s^{-1} PAR. The reaction was terminated by injecting 0.8 mL of 15% TCA. The ethylene formed was measured in a Varian (Palo Alto, CA, USA) CP-3800 gas chromatograph equipped with a Porapak R column (Varian) and

a flame ionization detector. Activity was expressed in terms of nmol C_2H_2 formed mg^{-1} protein h^{-1}.

Total protein extraction and two-dimensional gel electrophoresis

Protein isolation and two-dimensional gel electrophoresis were done according to a modified protocol of Wagner et al. (2002). Cultures of An7120 and AnFPN-*pcs* in the exponential phase were harvested by centrifugation (10,000×*g*, 10 min.), washed with Tris buffer (pH 8.0), and suspended in 2-ml extraction buffer containing 10 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, and 10 mM KCl. Cell pellet was ground using liquid nitrogen followed by centrifugation at 9200 rpm for 1 h. Furthermore, the supernatant was precipitated with 10% TCA in acetone, left overnight for complete precipitation, and centrifuged at 6,000×*g* for 15 min to recover protein pellet. Protein pellet was washed three to four times with ice-cold acetone to remove traces of TCA. Then, the pellet was air dried and dissolved in solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and 1.0% IPG buffer (4–7). Protein content was estimated according to Bradford using BSA as standard. Before loading for 2DE, traces of bromophenol blue was added and centrifuged at 19,000×*g* for 10 min. Sample entry was made through in-gel rehydration. A total of 300 μl of solubilization buffer containing 250 μg protein sample was incubated with the dry immobilized pH gradient (IPG) gel strips (pH 4–7) linear gradients (13 cm; GE healthcare) at 200 °C for 16 h. The first dimension separation was conducted at 20 °C with an EttanIPGphor system (GE Healthcare BioSciences). Focusing was performed in seven steps: linear 30 V for 00.30 h, 150 for 2:00 h, 300 V for 00:40 h, 500 V for 4 h, gradient 1000 V for 1 h, gradient 8000 V for 2 h, and finally 8000 V for 13:00 h. Focused IPG strips were then equilibrated by first incubating them in an equilibration solution (6 M urea, 30% *v*/*v* glycerol, 2% *w*/*v* SDS, 50 mM Tris–HCl, pH 8.8, and 1% *w*/*v* DTT and a trace amount of bromophenol blue) for 15 min, followed by incubation in 2.5% *w*/*v* iodoacetamide in the same equilibration solution instead of 1% DTT for 15 min. The strip was placed on the top of 12% SDS-polyacrylamide gels and electrophoresis was performed at 15 mA for 30 min followed by 25 mA for 5 h using a Hoefer SE 600 apparatus (Amersham Biosciences). The gel was stained with Coomassie Brilliant Blue R-250.

Data analysis

PDQuest™ version 7.1 (Bio-Rad) was used for 2D gel image analysis. Protein spot detection, quantification, background subtraction, and spotmatching between multiple gels were performed. K-nearest neighbor (KNN) algorithm [MATLAB] was used for performing missing value imputation. Normalization of spot intensities (% of individual spot intensity/Σ% spot intensity of each gel) was done with total spot intensity per gel in order to reimburse variations between the replicates. Protein spots with *p* values less than 0.05 were noted as statistically significant, and protein spots greater than minimum abundance ratio of 1.5-fold were selected and subjected to MALDI-TOF LCMS analysis followed by homology search using MASCOT.

In addition, a heat map visualizing proteomics responses of the An7120 and AnFPN-*pcs* was prepared with TIGR Multiple Experiment Viewer (MEV) 4.2 software (Saeed et al. 2003).

Results

The extra copy of *alr0975* in An7120 induced phytochelatin synthesis, enhanced nitrogen fixation, and conferred better growth against the wild type under different abiotic stresses

Transfer of pFPN-*pcs* to An7120 integrated an extra copy of *pcs* (*PpsbA1*:*pcs*) was confirmed and selected on BG-11, N^+, Neo_{25} agar plates which restricted growth of wild type (Fig. 1a). The presence of an extra copy of *pcs* gene in An7120 was further confirmed by RT-PCR and western blotting approaches. Differential expression of RNA isolated from overexpressed and wild-type strains using RT-PCR revealed increment in the transcript levels of *pcs* in transformed *Anabaena* over wild-type strain using 16S as internal control (Fig. 1b). In addition, western blotting and phytochelatin production indicated higher level of protein and its activity respectively, in AnFPN-*pcs* over An7120 under normal growth conditions (Fig. 1c, d). The overexpressed strain of *Anabaena* (AnFPN-*pcs*) showed similar growth behavior as An7120 under ambient growth condition (Fig. 2a). Nitrogenase activity in terms of acetylene reduction was found higher in recombinant strain as compared to wild type. AnFPN-*pcs* registered 2.25-fold enhanced nitrogenase activity as compared to wild type, suggesting development of elevated nitrogen fixation capacity in the transformed cyanobacterium (Fig. 2b). The enzymatic activity reveals 5-fold higher phytochelatin synthase activity in recombinant strain as compared to wild type. Comparative growth behavior of wild type and overexpressed strain under LC_{50} doses of UV-B, salt, heat, copper, carbofuran, and cadmium resulted in decrement in specific growth rate by 26, 21, 27, 13, 30, and 17%, respectively, in An7120 compared to AnFPN-*pcs* thereby suggesting that the transformed cyanobacterium AnFPN-*pcs* has developed tolerance and hence showed better growth performances under different abiotic stresses (Fig. 3a–f).

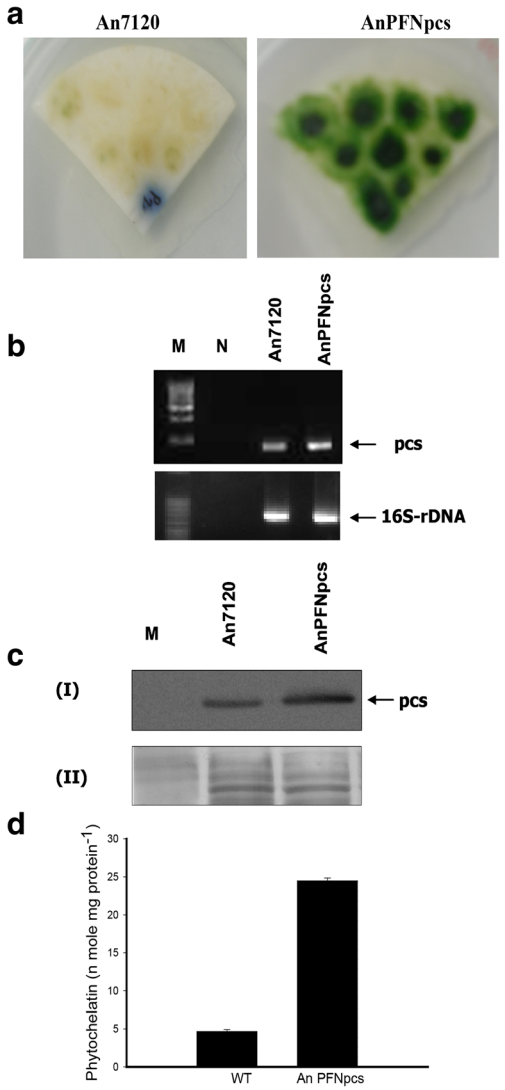

Fig. 1 **a** Colonies corresponding to wild-type *Anabaena* 7120 (An7120) and recombinant *Anabaena* AnFPN-*pcs* strains. **b** Reverse transcriptase (RT)-PCR of transcript fromAn7120 and AnFPN-*pcs* cells. Lane 1 (M) shows marker, lane 2 (N) negative control, Lane 3 transcript from An7120, and lane 4 transcript from AnFPN-*pcs*. **c** *I* Western blot image using antibody against phytochelatin synthase. Lane 1 marker, Lane 2 An7120, and Lane 3 AnFPN-*pcs*. **c** *II* SDS PAGE image with equal loading of protein in wild type as well as recombinant strain. **d** Phytochelatin synthase activity measurements in terms of phytochelatin production in n mole mg protein^{-1}

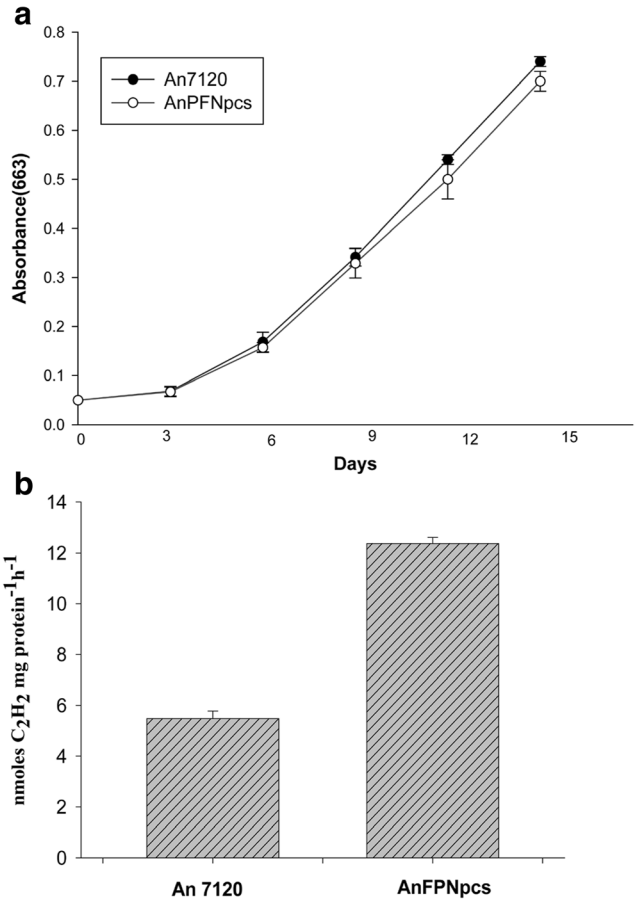

Fig. 2 **a** Comparison of growth behavior of An7120 and AnFPN-*pcs* under ambient condition. **b** Nitrogenase activity assessment in nanomoles C_2H_2 mg^{-1} protein^{-1} h from An7120 and AnFPN-*pcs* cells under ambient condition

Comparison of differentially expressed proteins in An7120 and AnFPN-*pcs*

To investigate the effect of *pcs* gene on cytosolic protein of An7120, 2-DE was performed. On analyzing the electrophoretic profiles of transformed and wild-type strain (as revealed by PDQuest), 389 and 431 spots were found respectively (Fig. 4). A total of 17 proteins depicted significant and reproducible changes in all the gels of An7120 and AnFPN-*pcs* (Fig. 4). Further, the analysis of the proteins using MALDI-TOF MS and MASCOT showed close homology to molecular chaperone DnaK (spot1), coproporphyrinogen III oxidase (spot2), hypothetical protein Alr4979 (spot3) with conserved domain belonging to DUF3386 superfamily (Fig. S2), manganese superoxide dismutase (spot4), sugar transport ATP-binding protein (spot5), phycocyanin alpha chain (spot6), FKBP-type peptidyl-prolyl cis-trans isomerase (spot7), hypothetical protein All0457(spot8) with conserved domain belonging to ChaB superfamily (Fig. S3), dihydroorotase (spot9), carboxyl-terminal processing protease (spot10), hypothetical protein Alr0946 (spot11) with conserved domain as AbrB superfamily (Fig. S4) and asl0060 (spot12) with conserved domain as DUF427 superfamily (Fig. S5), elongation factor Ts (spot13), acyl carrier protein phosphodiesterase (spot14), ferredoxin-NADP$^+$ reductase (spot15), nutrient stress-induced DNA-binding protein (spot16), and ketol-acid reductoisomerase (spot17) (Table 1).

Dynamics of differentially expressed proteins in An7120 and AnFPN-*pcs*

The analysis of the MALDI-TOF MS results characterized 17 protein spots in the form of heat map (Fig. 5).

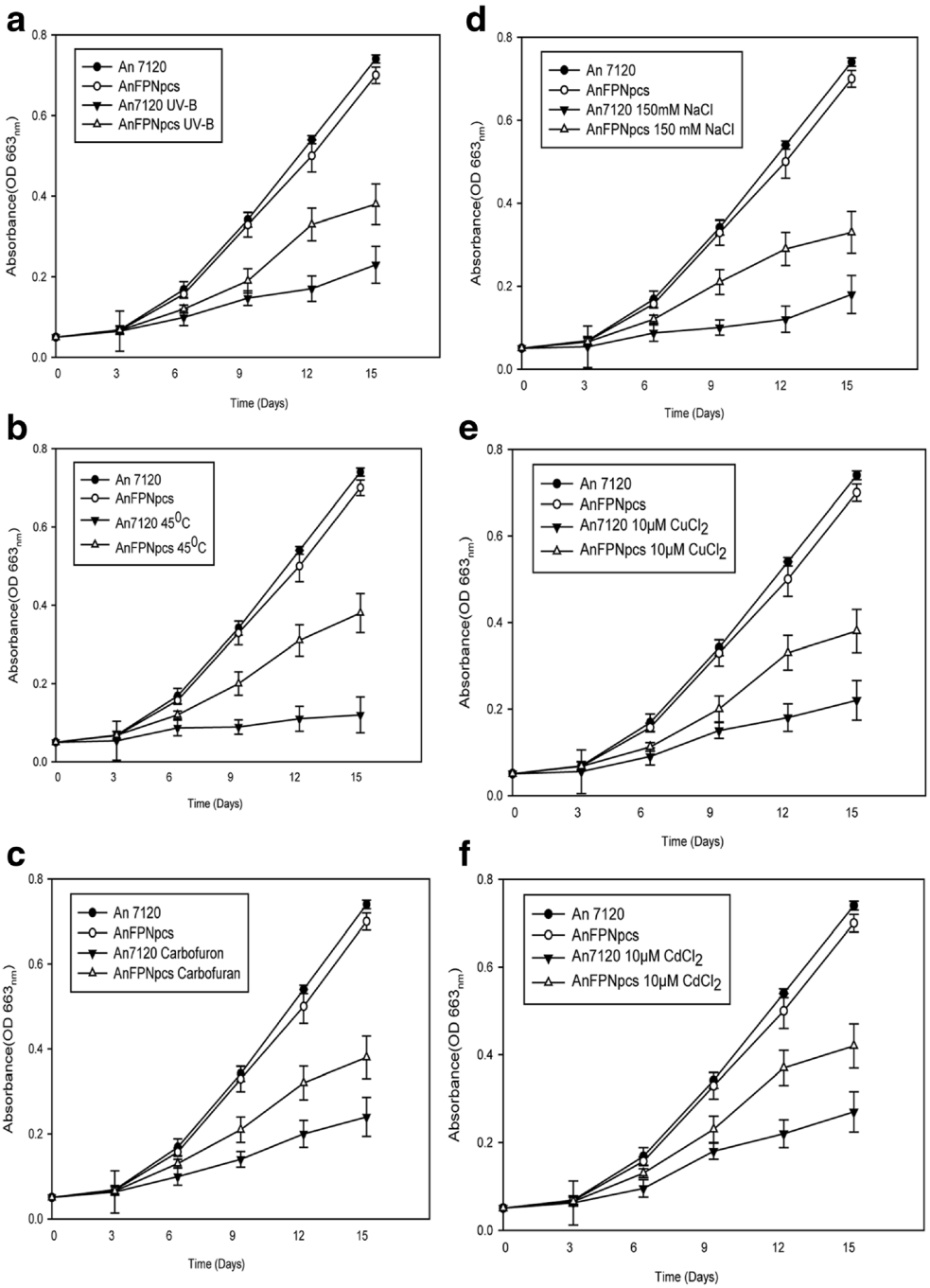

Fig. 3 **a**–**f** Effect of different abiotic stresses on growth behavior of An7120 and AnFPN-*pcs*. **a** UV-B, **b** heat (45 °C), **b** carbofuran (pesticide), **d** salt(NaCl), **e** copper ($CuCl_2$), and **f** cadmium ($CdCl_2$) on the growth of An7120 and AnFPN-*pcs*. The mean of three independent experiments are plotted with *error bars* indicating standard deviations

When the altered proteomic responses were analyzed, wild-type and transformed cyanobacteria formed two separate clusters. The significantly affected proteins also showed two clear major patterns, one group majorly contains up-accumulated proteins comprising molecular chaperone DnaK, coproporphyrinogen III oxidase, hypothetical protein Alr4979, manganese superoxide dismutase, sugar transport ATP-binding protein, phycocyanin alpha chain, FKBP-type peptidyl-prolyl cis-trans isomerase, dihydroorotase, acyl carrier protein phosphodiesterase, ferredoxin-$NADP^+$ reductase, nutrient stress-induced DNA-binding protein, and ketol-acid reductoisomerase in AnFPN-*pcs* (at the top of the heat map) while a second group showed the up-accumulated proteins in An7120 including elongation factor Ts, carboxy terminal processing protease, hypothetical protein All0457, and Asl0060 at the bottom of the heat map. The first group represents the overexpression-dependent changes in the proteome of the transformed cyanobacterium (Fig. 5).

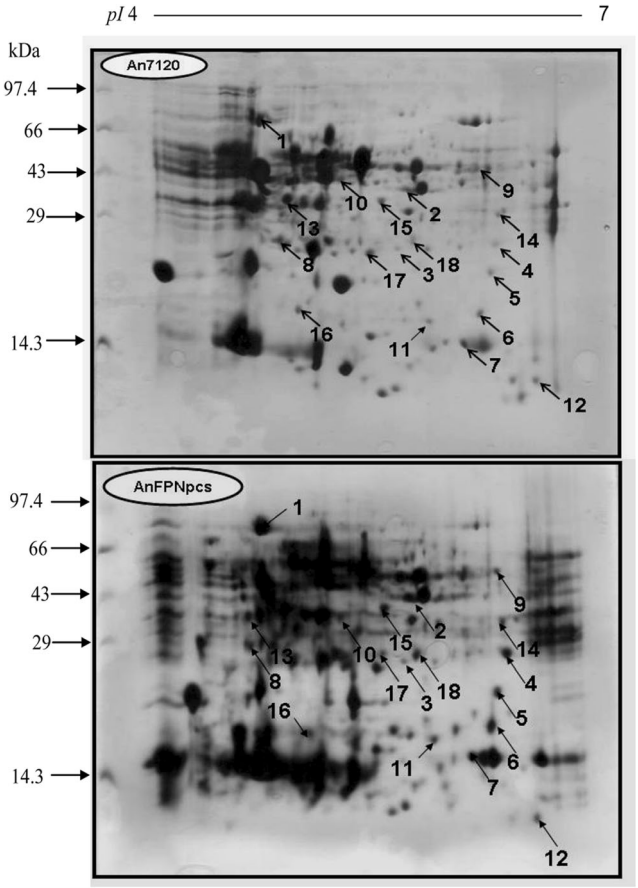

Fig. 4 Two-dimensional gel electrophoretic images of total cytosolic protein extracts from An7120 and AnFPN-*pcs*. All gels were run in triplicate. The gels were visualized by CBB staining. The total 18 protein spots differentially expressed were analyzed by MALDI-TOF MS

Discussion

The present study evaluates the possible role of expression of an extra copy of *pcs (alr0975)* in abiotic stress tolerance and its impact on cytosolic proteome of transformed strain AnFPN-*pcs*. The AnFPN-*pcs* strain showed 5.0-fold higher expression of *pcs* and enhanced PCS activity adjudged by increased production of phytochelatin over An7120 (Fig. 1c (i), d), thereby offering functional validation of this gene in AnFPN-*pcs*. Interestingly, no major differences were noticed in the growth of AnFPN-*pcs* and An7120 under ambient condition (Fig. 2a). However, the AnFPN-*pcs* exhibited better nitrogen fixation ability as compared to An7120. Such findings suggested that the integration of an extra copy of *pcs* improved the nitrogen fixation ability in An7120 (Fig. 2b). The better growth performance of AnFPN-*pcs* over wild type following exposure to different abiotic stresses (Fig. 3a–f) attested the role of *pcs* in multiple stress tolerance of *Anabaena* sp. PCC 7120. This may be attributed to the protection of thiols from the damaging effects of physical stresses such as heat and UV-B (Bhargava et al., 2005) through conformational changes of PCs.

To understand how *pcs* overexpression modulates the proteome of the transformed strain, the proteome of An7120 and AnFPN-*pcs* were compared (Fig. 4). Qualitative and quantitative proteome profiling of wild-type as well as overexpressed strain revealed 17 differentially expressed proteins. Out of 17, 13 proteins were found to be up-accumulated in AnFPN-*pcs* compared to An7120 as depicted in the heat map (Fig. 5). The up-accumulated proteins in wild-type compared to recombinant strain belong to different functional categories including stress-inducible proteins, photosynthesis and respiration, protein folding and modification, and nucleotide and amino acid biosynthesis and transport. These proteins appear to play a crucial role in providing tolerance against abiotic stress and maintaining metabolism.

Among the up-accumulated proteins in AnFPN-*pcs*, one of the important proteins involved in chlorophyll biosynthesis is coproporphyrinogen III oxidase (CPO) which catalyzes oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX in the porphyrin metabolism of cyanobacteria (Cornah and Smith, 2009). The upregulation of this protein probably helped in continuation of chlorophyll synthesis under stressful conditions. Ferredoxin-$NADP^+$ reductase (FNR) belongs to a family of flavoproteins catalyzing the electron flow from ferredoxin to $NADP^+$ and ATP synthase (ATP-B) for ATP synthesis (Carrillo and Vallejos 1987). Its upregulation in AnFPN-*pcs* was not only involved in the maintenance of photosynthesis and ATP pool under stressful conditions but also in the maintenance of nitrogen fixation by transfer of electrons to the nitrogenase (Sehrautemeier et al. 1984). Thus, the up-accumulation of FNR supported the enhanced nitrogen fixation ability of AnFPN-*pcs* compared to An7120 (Fig. 2b).

Dihydroorotase involved in the catalysis of reversible interconversion of carbamoyl aspartate and dihydroorotate in the pathway for the synthesis of pyrimidine nucleotides by catalyzing the reversible interconversion of carbamoyl aspartate and dihydrorotate. Liu et al. (2009) reported the importance of dihydroorotase in stress tolerance strategies of rice against salinity and drought. Hence, upregulation of this enzyme helped AnFPN-*pcs* to have more tolerance against the damaging effects of different stresses.

Up-accumulation of an important HSP, DnaK, in AnFPN-*pcs* might be involved in folding of unfolded proteins which otherwise is detrimental for regular cellular functioning under stress. It not only ensures proper folding of the polypeptide chains but prevents misfolding and aggregation of proteins generated during cellular stress (Boston et al. 1996). A high level of Mn-SOD in AnFPN-*pcs* compared to An7120 suggested that the transformed organism has developed better antioxidative defense system to combat oxidative stress. Such findings support the better growth responses as reflected by growth curves (Fig. 3a–f) of the overexpressed strain compared to wild type. In addition, Mn-SOD has been found to be involved in binding with metals ions (Ciriolo et al. 1994).

Table 1 Number of spots with corresponding proteins, their homologous proteins identified, mowse score, molecular weight, pI, accession number, and matched peptides

Spot no.	Homologous protein (showing homology with)	Probability based mowse score	Mr (KDa)	pI	Accession number	Match peptide
1	Molecular chaperone DnaK [Nostoc sp. PCC 7120]	192	68,037	4.7	gi|17229234	21
2	Coproporphyrinogen III oxidase[Nostoc sp. PCC 7120]	101	36,606	5.2	gi|17228146	25
3	Hypothetical protein alr4979 [Nostoc sp. PCC 7120]	113	24,185	5.7	gi|17232471	9
4	Chain A, The 2.0 Å resolution structure of The catalytic portion of a cyanobacterial membrane-bound manganese superoxide dismutase [Nostoc sp. PCC 7120]	139	28,161	6.4	gi|23200074	10
5	Sugar transport ATP-binding protein [Fusobacterium nucleatum subsp. nucleatum ATCC 25586]	88	58,232	5.3	gi|19705203	13
6	Phycocyanin alpha chain [Nostoc sp. PCC 7120]	105	17,504	7.7	gi|17228025	8
7	FKBP-type peptidyl-prolyl cis-trans isomerase [Nostoc sp. PCC 7120]	82	17,550	5.8	gi|17228073	7
8	Hypothetical protein all0457 [Nostoc sp. PCC 7120]	116	22,732	4.6	gi|17227953	11
9	Dihydroorotase [Nostoc sp. PCC 7120]	102	48,291	5.7	gi|17229795	29
10	Carboxyl-terminal processing protease [Nostoc sp. PCC 7120]	86	45,093	5.4	gi|17230912,	12
11	Hypothetical protein alr0946 [Nostoc sp. PCC 7120]	139	16,106	5.3	gi|17228441	9
12	Hypothetical protein asl0060 [Nostoc sp. PCC 7120]	70	10,702	6.1	gi|17227556	13
13	Elongation factor Ts [*Nostoc* sp. PCC 7120]	212	34,425	4.8	gi|17232283	22
14	Acyl carrier protein phosphodiesterase [*Nostoc* sp. PCC 7120]	111	22,770	5.2	gi|17229597	11
15	Chain A, structure of ferredoxin-NADP(+) reductase with Lys 72 replaced by Glu (K72e)[*Nostoc* sp. PCC 7120]	150	34,278	6.1	gi|24987253	4
16	Nutrient stress-induced DNA binding protein [*Nostoc* sp. PCC 7120]	84	20,731	5.0	gi|17231300	9
17	Ketol-acid reductoisomerase [*Nostoc* sp. PCC 7120]	110	36,102	5.3	gi|17229807	31

The sugar transport ATP-binding proteins are is to be involved in the transport of sugar across the membrane. The up-regulation of this protein in AnFPN-*pcs* provides an important way to maintain sugar transport under stressful conditions. Phycocyanin alpha chain in cyanobacteria constitute a substantial fraction of the total soluble proteins (50%); their degradation can supply amino acids for the synthesis of essential proteins under nutrient-limited conditions (Allen and Smith 1969).

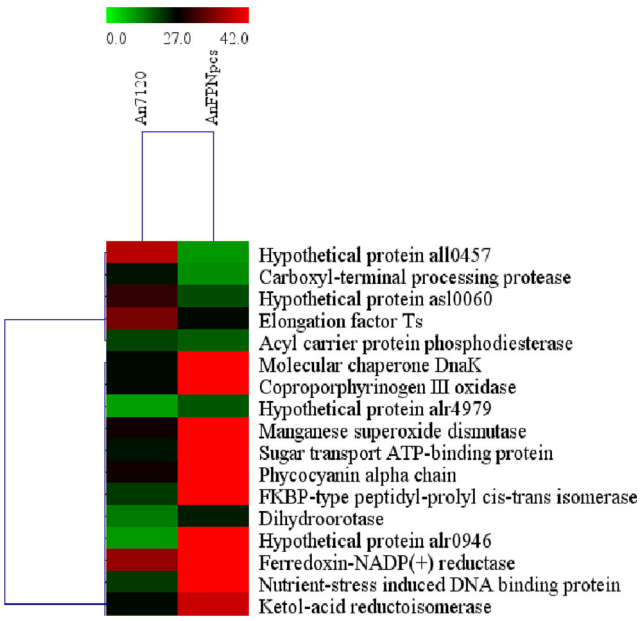

Fig. 5 Heat map and cluster tree representation of the identified proteins from An7120 and AnFPN-*pcs*. The changes in protein spots were calculated with PDQuest software. Values are means ± S.E. of protein volumes of gels from three independent experiments

The FKBP is an important signaling molecule (Vallon, 2005) involved in the repair of stress-dented proteins which showed up-accumulation in AnFPN-*pcs*. This might have been helpful in maintaining signaling events as well as proper functioning of important proteins during stress leading to better growth (Fig. 3a–f). Another interesting protein, i.e., DNA-binding protein, has been reported to protect organism under nutrient-starved condition as well as abiotic stresses (Richaud et al., 2001; Narayan et al. 2010) by binding to the DNA of the host organism. The up-accumulation of this protein in AnFPN-*pcs* might support a better growth under stressful conditions.

Out of the total proteins examined in the present study, 27% were hypothetical proteins with mostly unknown function. One of the hypothetical proteins, Alr0946 (CalA), with conserved domain belonging to an AbrB-like superfamily (Fig. S4), was up-accumulation in the AnFPN-*pcs*. Proteins with such functional domains are known to be involved in DNA binding and act as a transcriptional regulator of Fe-SOD, a key player of the antioxidative defense system during stress (Agervald et al. 2010a). In addition, Alr0946 (CalA) also interacts with the upstream regions of *hypC*, a gene

encoding a hydrogenase (Agervald et al., 2010b). Such findings argue in favor of efficient hydrogen metabolism of AnFPN-*pcs* over An7120.

Taken together, the data presented suggests that phytochelatin synthase acts as an intermediate regulator in the complex redox system of the cell by connecting photosynthesis, carbon, and nitrogen assimilation with protection from oxidative stress, maintenance of unfolded proteins, and active transport of nutrient. Owing to the importance of cyanobacteria in nitrogen fixation and adaptation to environmental vagaries and the increasing impact of abiotic stresses in the environment, it can be proposed that stress-induced protein may be used for the engineering of multiple stress-tolerant crop plants capable of growing in variable climate.

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