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Cloning expression and analysis of phytochelatin synthase (*pcs*) gene from *Anabaena* sp. PCC 7120 offering multiple stress tolerance in *Escherichia coli*

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ABSTRACT

Phytochelatin synthase (PCS) is involved in the synthesis of phytochelatins (PCs), plays role in heavy metal detoxification. The present study describes for first time the functional expression and characterization of *pcs* gene of *Anabaena* sp. PCC 7120 in *Escherichia coli* in terms of offering protection against heat, salt, carbofuron (pesticide), cadmium, copper, and UV-B stress. The involvement of *pcs* gene in tolerance to above abiotic stresses was investigated by cloning of *pcs* gene in expression vector pGEX-5X-2 and its transformation in *E. coli* BL21 (DE3). The *E. coli* cells transformed with pGEX-5X-*pcs* showed better growth than control cells (pGEX-5X-2) under temperature (47 °C), NaCl (6% w/v), carbofuron (0.025 mg ml^{-1}), $CdCl_2$ (4 mM), $CuCl_2$ (1 mM), and UV-B (10 min) exposure. The enhanced expression of *pcs* gene revealed by RT-PCR analysis under above stresses at different time intervals further advocates its role in tolerance against above abiotic stresses.

Exposure to abiotic stresses such as UV-B, heavy metals, heat, salinity, drought, has been found to induce excess accumulation of reactive oxygen species (ROS) in the organisms [1]. ROS are potentially harmful to the cell, as they can raise the level of oxidative damage hence loss of cellular structure and functions. An important aspect of survival under stress condition is development of multiple stress tolerance. Several approaches used to develop stress tolerance in organisms include insertion of foreign genes encoding membrane-modifying enzymes, radical-scavenging enzymes, and stress-induced proteins.

Phytochelatins (PCs) are heavy metal binding thiolate peptides enzymatically synthesized from glutathione [2] that play a major role in offering protection against heavy metals. In addition, other important functions of PCs include its strong scavenging ability of ROS than the glutathione [3] and alleviation of UV-B toxicity in *Anabaena doliolum* [4]. The genes involved in the formation of phytochelatins (PCs) are phytochelatin synthases (*pcs*) i.e., γ-glutamylcysteine transpeptidase. PC synthases catalyze the net synthesis of PCs from GSH. Since 1999, many genes encoding *pcs* have been cloned from different organisms for example, *OsPCS1*, *TaPCS1*, *AtPCS1*, and *CePCS1* from rice, wheat, *Arabidopsis*, and *Caenorhabditis elegans* [5–7] to enhance tolerance against heavy metals.

Although much work has been done on the role of phytochelatin synthase by transformed organisms in offering tolerance to various metals, there still exists a gap regarding its involvement in multiple stress tolerance in any organism.

In the light of the reports of Mallik and Rai [8] that cadmium induced low-molecular weight protein (probably phytochelatin) in *A. doliolum* offers not only co-tolerance to different heavy metals but also provides protection against heat shock, cold shock, anaerobiosis, and UV-B as well as the known role of PCs as scavenger of ROS [4], we hypothesized that enhanced expression of *Anabaena* sp. PCC 7120 PCS protein in *E. coli* might offer protection against abiotic stresses like heat, UV-B, pesticide, salt because all these stresses directly or indirectly enhance ROS production in the organisms. This speculation finds support from the reports that heat shock and gamma radiation induced the production of PCs in barley and garlic seedlings, respectively [9,10].

Materials and methods

Cyanobacterial and bacterial strains and plasmids. *Anabaena* sp. PCC 7120 was grown photoautotrophically in BG-11 medium [11] buffered with Tris/HCl at 24 ± 2 °C under day light fluorescent tubes emitting 72 μmol photon m^{-2} s^{-1} PAR (photosynthetically active radiation) light intensity with a photoperiod of 14:10 h at pH 7.5. The cultures were shaken by hand 2–3 times daily. *E. coli* strain DH5α and *E. coli* BL21 (DE3) (Novagen) were used as host for cloning and expression, respectively. *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 100 μg/ml ampicillin [12]. Plasmid pGEX-5X-2 (GE Healthcare, USA) was used as a vector for cloning.

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Cloning of the pcs gene from Anabaena sp. PCC 7120. Genomic DNA from *Anabaena* sp. PCC 7120 was isolated as previously described [13]. An open reading frame *alr0975*, putatively encoding phytochelatin synthase was amplified by polymerase chain reaction using genomic DNA as the template with a pair of primers, Pf (5′GCGAATTCCGATAGTTATGAAACTCTTTATC3′) and Pr (5′GCATGCGGCCGCCTAATCTTGTGTTTTACTTACG3′). The underlined bases are *Eco*RI and *Not*I 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 standard freeze–thaw method for cloning.

Construction of expression vector. The purified PCR product was digested with *Eco*RI and *Not*I (NEB), and the resultant DNA fragment was cloned into the high expression vector pGEX-5X-2 that had been digested with the same restriction enzymes. To construct the recombinant plasmid pGEX-5X-2-*pcs* was introduced into *E. coli* BL21, and the later was then grown in LB medium. The plasmid was then isolated and the DNA sequence of *alr0975* was confirmed by sequencing.

Expression analysis of pcs gene using RT-PCR and SDS–PAGE. Escherichia coli BL21 (DE3) harboring the pGEX5X-2-*pcs* plasmid was grown in LB medium supplemented with 100 μg/ml ampicillin in an orbital shaker (200 rpm) at 37 °C. When A600 of the culture reached optical density 0.5, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM and the culture was grown for another 6 h at 37 °C. RNA was isolated from mid-exponential phase cells harboring the pGEX-5X-2 and pGEX-5X-2-*pcs* plasmid before and after IPTG induction using the TRIzol reagent as per the instructions given in the manufacturer's protocol. All RNA samples were diluted to a concentration of 3 μg/mL prior to RT. In a clear nuclease free 0.2 mL microcentrifuge tube, 1 μL of 10 mM dNTP mix, 1 μL RNA template and 6 μL of nuclease free water was mixed gently and incubated at 70 °C for 10 min to remove any secondary structure of RNA. This was now placed on ice. M-MLV 200 U/μL reverse transcriptase (SIGMA chemical company, USA) was added along with the RT buffer. The final volume was adjusted to 20 μL with nuclease free water. The reaction mixture was incubated at 25 °C for 5 min followed by 37 °C for 50 min. To test the purity of the cDNA, control reaction mixtures were prepared in the same way as mentioned above except that M-MLV RT was not added. To ascertain the equal concentration of RNA in the different samples, RT-PCR of 16S rDNA was also performed and used as an internal control. The primer pair 8F (5′AGAGTTTGATCCTGGCTCAG3′) and 518R (5′ATTACCGCGGCTGCTGG3′) was used to amplify 16S rDNA by using the cycle as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of incubation each consisting of 1 min denaturation at 94 °C, 1.5 min annealing at 58 °C, 2 min extension at 72 °C and a final extension of 10 min at 72 °C. To allow relative quantitation of the *pcs* gene preliminary experiments were carried out with stepwise reduction of the number of PCR cycles to determine the maximum cycle number where samples do not reach amplification plateau. For amplification of the *pcs* genes, 1 μL of the RT reaction product was used in subsequent PCR. PCR was performed in 25 μl final volume of reaction mixture containing 100 ng of cDNA, 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 (Bangalore Genei, India) in an Icycler (Bio-Rad, USA). The Icycler profile was as follows: initial denaturation for 5 min at 94 °C followed by 40 incubation cycles each consisting of 1.5 min denaturation at 94 °C, 1 min annealing at 56 °C, 2 min elongation at 72 °C and a final 10 min elongation at 72 °C. The intensities of the RT-PCR products on agarose gels were quantified with the Gel Doc 2000 system using the Quantity one software (Bio-Rad, USA).

For preparation of cell-free extracts (for SDS–PAGE) bacteria were harvested by centrifugation at 5000 rpm for 10 min in SIGMA 3K-30 laboratory centrifuge, Germany and washed twice with extraction buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM KCl, pH 7.5). The pellet was resuspended in 5 ml extraction buffer and subjected to grinding under liquid nitrogen to break the cells. The extract was centrifuged at 10,000 rpm for 60 min. The supernatant was subjected to acetone/TCA precipitation overnight at −20 °C followed by centrifugation at 10,000 rpm for 15 min. The pellet was resuspended in 500 μl Tris buffer (10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 7.5). The sample was stored in small aliquots at −20 °C until further characterization.

Enzyme activity assay. Activity of phytochelatin synthase (PCS) was determined by the level of phytochelatins inside the cells by following methods of Bhargava et al. [4].Total cell protein was estimated by the method of Bradford [14].

Western blot analysis. In order to confirm the expression of *pcs* gene in *E. coli* cells, SDS–PAGE was carried out as per the method of Sambrook and Russell [12]. 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 or overnight at 15 V at 4 °C. Membrane was blocked for 4 h in TTBS (Tris buffer saline containing 0.1% Tween-20) and 5% (w/v) non-fat dried milk. The primary antibody was diluted 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 HRP (horseradish peroxidases) conjugated secondary antibody (Genei, India) for 4 h. Following four consecutive 5 min wash 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 paper, which considerably reduced the background staining. Polyclonal antibodies against used for the detection of PCS by immunoblotting were obtained as generous gift from Dr. Stephan Cuine (CEA Cadarache, France).

Assay for abiotic stress tolerance in transformed E. coli cells. In order to confirm the role of phytochelatin synthase, the effects of above abiotic stresses on the growth of transformed *E. coli* BL21 (DE3) cells with pGEX-5X-2 (empty vector) and the recombinant plasmid pGEX-5X-2-*pcs* were examined.

Mode of stress application. NaCl autoclaved separately was added directly into the sterilized medium to achieve the appropriate concentration. Stock solution of $CdCl_2.2H_2O$ (100 mM), $CuCl_2.2H_2O$ (1 M) and carbofuron (120 mg ml^{-1}) was prepared in glass-distilled water and sterilized by passing through a Millipore membrane filter (0.22 μm). Temperature treatment to culture suspensions was given in a temperature controlled incubator. Two milliliters bacterial culture suspensions (OD 0.5) were transferred into quartz cuvettes (Pye Unicam B538751 A, thickness 1 mm, capacity 4 ml) exposed to artificial UV-B radiation (from UV-B lamp, CAT No. 34408, fotodyne, Inc. USA giving its maximum output at 310 nm).

Measurement of survival and growth. To measure the survival of *E. coli* cells transformed with pGEX-5X-2 (empty vector) against, salt, carbofuron, cadmium, and temperature cells were treated with different concentrations of NaCl (2%, 4%, 6%, 8%, and 10%), carbofuron (0.01, 0.02, 0.03, 0.04, and 0.05 mg ml^{-1}), $CdCl_2$ (2, 4, 6, and 8 mM), temperature (42, 47, 52, and 57 °C), $CuCl_2$ (0.5, 1, 2, 4, and 6 mM) and UV-B (5, 10, 20, 30, 40, 50, and 60 min), respectively. The LC_{50} for NaCl, carbofuron, cadmium ($CdCl_2$), temperature, copper ($CuCl_2$), and UV-B was determined by the plate colony count method. Approximately 50% survival of *E. coli* was ob-

served at NaCl (6%), carbofuron (0.025 mg ml^{-1}), $CdCl_2$ (4 mM), temperature 47 °C, $CuCl_2$ (1 mM) and UV-B (10 min), respectively. The growth of *E. coli* cells transformed with pGEX-5X-2 (empty vector) was not inhibited on the plates as well as in liquid culture at lower doses of above stresses (data not shown).

Overnight cultures of *E. coli* BL21 (DE3) cells transformed with either pGEX-5X-2 (empty vector) or pGEX-5X-2-*pcs* (recombinant plasmid) were grown by inoculating fresh LB medium with a single *E. coli* colony containing 100 μg ml^{-1} ampicillin under continuous shaking condition at 37 °C for overnight on a rotary shaker. On the following day the cultures were diluted to 0.05 OD with a fresh LB medium (25 ml in a 100 ml conical flask) and incubated at 37 °C on a rotary shaker (200 rpm) until optical density (OD) value become approximately 0.5. For abiotic stress treatments, 50 μl of *E. coli* culture (OD 0.5) was inoculated into a fresh 50 ml of liquid medium containing NaCl (6% w/v); carbofuron (0.025 mg ml^{-1}); $CdCl_2$ (4 mM) $CuCl_2$ (1 mM) and then cultured at 37 °C by shaking. Further, for UV-B stress 2 ml of *E. coli* culture (OD 0.5) was subjected to 10 min of UV-B stress and 50 μl of such UV-B treated cells were inoculated into 50 ml of liquid medium and allowed to grow at 37 °C on a rotary shaker in dark. For heat shock treatment, 50 ml of liquid medium inoculated by 50 μl of *E. coli* cells (OD 0.5) was subjected to 47 °C in orbital shaker. The bacterial suspension was harvested at every 30 min and optical density measured by spectrophotometer (GE Healthcare, USA). Specific growth rate was calculated by using the equation: $\mu = [\ln(n_2/n_1)]/[t_2 - t_1]$ where μ stands for specific growth rate and n_1, n_2 are absorbance of culture suspension at the beginning (t_1) and end (t_2) of selected time interval. Three independent measurements were carried out and the average value was used for making the final data.

Expressional analysis of pcs in response to different abiotic stress. RNA was isolated using TRIzol reagent for expressional characterization of *pcs* gene using RT-PCR as described above and subjected to different abiotic stress such as UV-B, copper, salt, carbofuron, cadmium, and temperature, respectively. To ascertain the equal concentration of RNA in the different samples RT-PCR of 16S rDNA was also performed and used as an internal control.

Results

Molecular cloning of pcs gene

The PCR amplified product was about 754 bp (Fig. 1A), which conforms with the theoretical length of the *pcs* gene (729 bp). It was cut with *Eco*RI and *Not*I, ligated to the pGEX-5X-2 *Not*I and *Eco*RI digested backbone fragment and transformed into *E. coli* strain BL21 (DE3). The constructed plasmid was verified by DNA sequencing. The open reading frame of 729 bp encodes 242 amino acids with a calculated molecular weight of 27.17 kDa.

Expressional analysis of pcs gene in E. coli using RT-PCR and SDS–PAGE

A 5–6 h exposure of cells transformed with recombinant plasmid to 0.5 mM IPTG was found to produce an increase of 4.0-fold in the transcript level compared to non-induced cells (Fig. 1C). The induction of fusion protein (on the SDS–PAGE) was also observed after IPTG induction (Fig. 2A). The molecular mass of fusion protein GST-PCS as estimated by comparison with protein molecular weight marker was ~55 kDa.

Western blotting and enzymatic activity assay

Western blot analysis with anti-PCS produced a single band of ~55 kDa that was in good agreement with the molecular weight (54.6) deduced from the nucleotide sequence of *pcs* gene (Fig. 2B). This confirmed that *E. coli* cells containing pGEX-5X-2-*pcs* produced a PCS protein. The activity of phytochelatin synthase was assayed by monitoring production of phytochelatin. The cells containing (pGEX-5X-2-*pcs*) plasmid showed phytochelatin production over cells containing empty vector. This amount was further increased

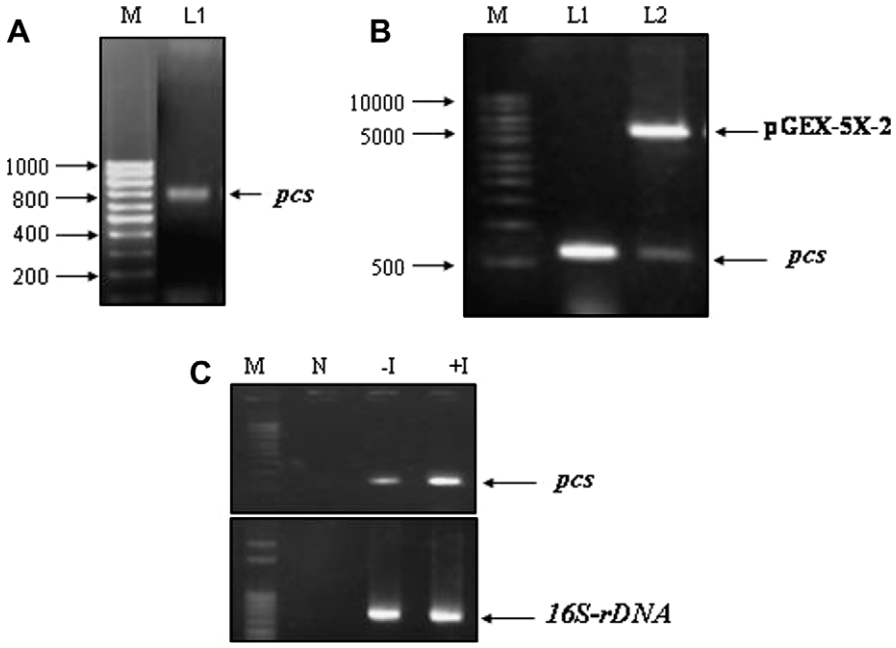

Fig. 1. (A) PCR amplification of the *Anabaena* sp. PCC 7120 ORF *alr0975*. Genomic DNA from *Anabaena* sp. PCC 7120 was isolated and amplified as described in materials and methods. DNA samples were run on 1.2% agarose gel and visualized by ethidium bromide staining. Lane M DNA ladder (100 bp, NEB), Lane 1 (L1) PCR product *alr0975* of (~729 bp) (B) Agarose gel showing double digested recombinant clones with *EcoR*1 and *Not*1 showing the presence of ~729 bp fragment and 4.9 kb pGEX-5X-2 vector. Lane M DNA ladder (100 bp), lane 1 (L1) PCR product of alr0975 (~729 bp), lane (L2) double digestion of pGEX-5X-2-*pcs* with *EcoR*I and *Not*I showing release of (~729 bp) fragment. (C) Expression pattern of *pcs* gene showing in lane1 (M) as DNA ladder (100 bp), lane 2 (N) as negative control, lane 3 (−I) without IPTG induction, lane 4 (+I) with IPTG induction.

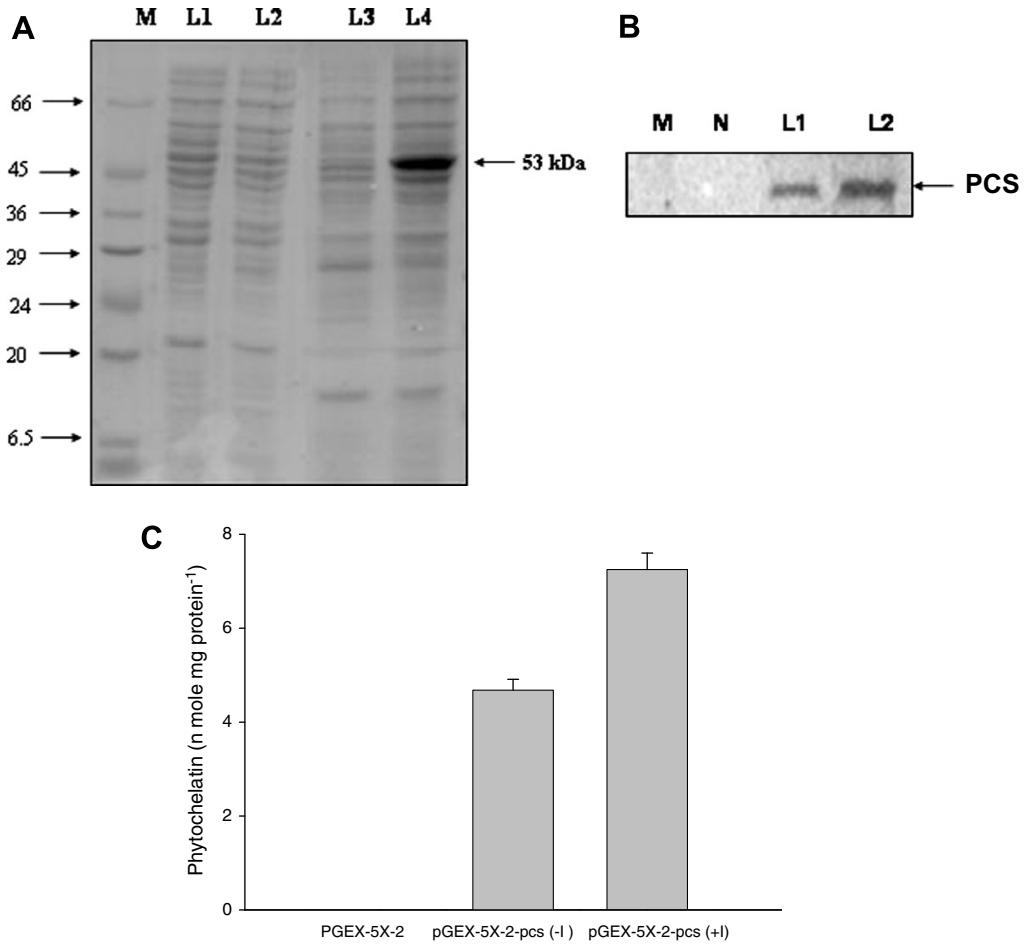

Fig. 2. (A) SDS–PAGE (12%) analysis of PCS protein expression in *E. coli* BL21 (coomassie blue staining). Lane 1 (M), Protein marker; lane 2 (L1), whole cell lysate of BL21 *E. coli* cells containing the empty vector pGEX-5X-2 without IPTG induction; lane 3 (L2) whole cell lysate of BL21 *E. coli* cells containing the empty vector pGEX-5X-2 obtained at 4 h post-induction with 0.5 mM IPTG; lane 4 (L3), whole cell lysate of non-induced BL21 *E. coli* cells containing the plasmid pGEX-5X-2-*pcs*; lane 4 (L4), whole cell lysate of the same cells obtained at 4 h post-induction with 0.5 mM IPTG. The number on the right is the apparent molecular mass of the recombinant PCS protein (~55 kDa) (B) Immunoblot detection of PCS protein before and after IPTG induction. Lane 1 (M) molecular weight maker; lane 2 (N) negative control i.e., protein sample from *E. coli* cells containing empty vector (pGEX-5X-2-*pcs*); lane 3–4 (L1 and L2) sample from *E. coli* cells containing (pGEX-5X-*pcs*) incubated without (−I) and with (+I) IPTG, respectively. (C) Activity assay of phytochelatin synthase (PCS) in terms of phytochelatin production from *E. coli* cells containing empty vector (pGEX-5X-2) and (pGEX-5X-*pcs*) with and without IPTG induction.

to 1.56-fold after IPTG induction. Control cells containing empty plasmid did not demonstrate enzymatic activity. (Fig. 2C).

Effect of various abiotic stresses on growth of transformed E. coli cells

Fig. 3A–F showed impact of heat, salt, carbofuron and cadmium on *E. coli* cells transformed with pGEX-5X-2-*pcs* and vector (pGEX-5X-2) as a control. The transformed cells with recombinant plasmid (pGEX-5X-2-*pcs*) showed better growth than those transformed with empty vector. When LC_{50} dose for cells transformed with empty vector was applied on pGEX-5X-2-*pcs* transformed cells, the decrease in specific growth rate was only 30%, 22%, 20%, 5%, 23%, and 20% under heat, salt, carbofuron, cadmium, copper, and UV-B stress, respectively.

Expression of pcs in response to various abiotic stresses

All the selected stresses triggered a significant increase in the transcript level of *pcs* gene at various time points (Fig. 4A–F).This increase being 1.93-, 2.46-, 3.2-fold in heat; 1.78-, 2.17-, and 2.5-fold in salt; 1.96-, 2.51-, and 3.26-fold in carbofuron; 2.17-, 2.50-, and 4.19-fold in cadmium; 1.4-, 1.61-, and 1.89-fold in copper and 1.74-, 2.08-, and 2.62-fold in UV-B over control after 3, 6, and 9 h of treatment, respectively.

Discussion

Data compiled in the result clearly demonstrated *Anabaena* sp. PCC 7120 phytochelatin synthase (*pcs*) gene cloning into pGEX-5X-2 and expression in *E. coli* BL21(DE3). The expression of gene was confirmed by accumulation of transcripts of *pcs* gene by RT-PCR, 55 kDa fusion protein (GST-PCS) by SDS–PAGE in *E. coli* cells transformed with pGEX-5X-2-*pcs* with and without IPTG induction. The appearance of enzymatic activity in *E. coli* cells transformed with pGEX-5X-2-*pcs* with and without IPTG induction reconfirmed the functional expression of this gene in *E. coli*.

A better growth rate of pGEX-5X-2-*pcs* transformed *E. coli* cells than those transformed with pGEX-5X-2 subjected to heat, salt, carbofuron, cadmium, copper, and UV-B stress (Fig. 4A–F), finds support from those of Zhang et al. [10] where heat shock and gamma radiation induced the accumulation of phytochelatins in garlic seedlings. The enhanced tolerance of *E. coli* to above abiotic stresses may be due to (i) enhanced expression of *pcs* gene and (ii) PCS protein induced activation of the transcriptional activity of stress responsive gene in *E. coli* cells.

The strongly nucleophilic sulfhydryl groups of the Cys groups of PCs react with a broad spectrum of agents ranging from free radicals, active oxygen species, and cytotoxic electrophilic organic xenobiotics to heavy metals [15].

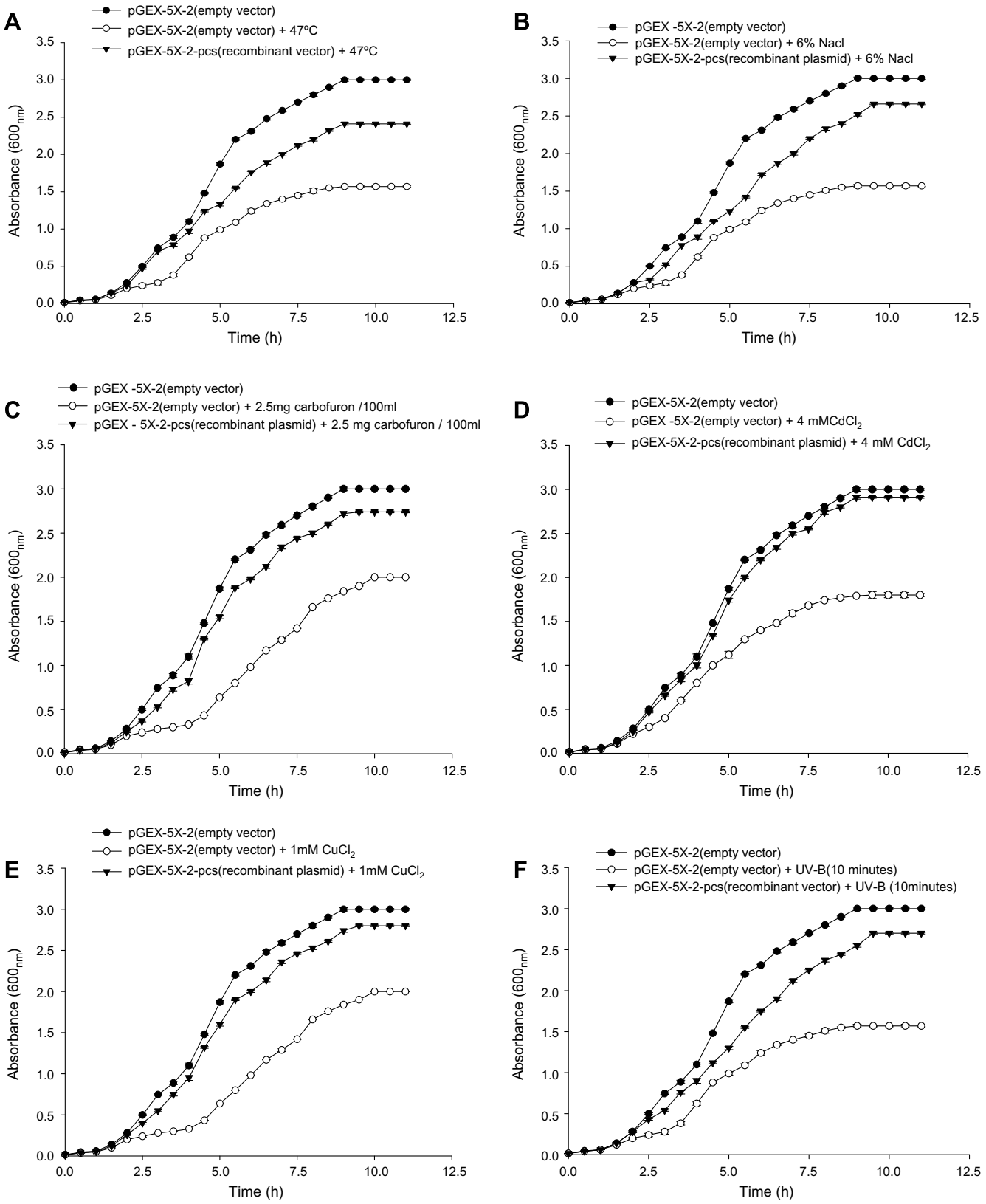

Fig. 3. Effect of (A) heat (B) salt (C) carbofuron (pesticide) (D) cadmium (E) copper, and (F) UV-B on the growth (heat, salt, carbofuron cadmium, copper, and UV-B) of transformed *E. coli* cells with recombinant plasmid (pGEX-5X-2-*pcs*) and empty vector (pGEX-5X-2). The mean of three independent experiments are plotted with error bars indicating standard deviations. (A–F) represents the growth curves of *E. coli* cells in liquid medium on exposure to heat, salt, carbofuron, cadmium, copper, and UV-B, respectively.

RT-PCR analysis of the protein gene subjected to the abiotic stresses examined in the present study provides ample evidence of their differential expression vis-à-vis tolerance under abiotic stresses employed (Fig. 4A–F). Particularly as part of its adaptive repertoire to oxidative stress, there can be variation in intracellular enzyme activity of *E. coli* in response to different ROS generated

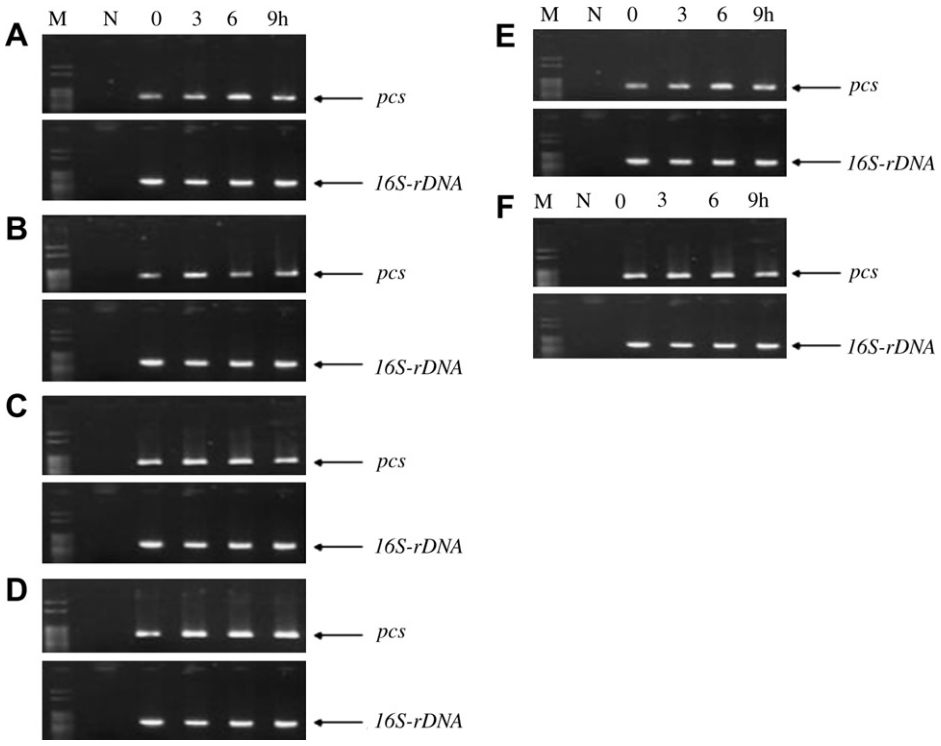

Fig. 4. RT-PCR analysis of the expression of *pcs* gene during exposure to (A) heat (B) salt (C) carbofuron (pesticide) (D) cadmium (E) copper (F) UV-B at various time points (heat, salt, carbofuron, cadmium, copper, and UV-B) as described in materials and methods.

abiotic stresses. The differential level of tolerance provided by PCS supports the hypothesis of defense against oxidative stress generated by above abiotic stressors. The addition of IPTG, however did not affect the magnitude of tolerance to above stresses by cells expressing cyanobacterial *pcs* gene (data not shown).This is probably because a sufficient amount of PCS protein for acquisition of stress tolerance was produced in the cells even in the medium without IPTG.

These results suggest that *pcs* gene from photoautotrophic cyanobacterium *Anabaena* sp. PCC 7120, a heterologous source can confer multiple stress tolerance to the non-photosynthetic *E. coli*. This study suggests that *pcs* gene can be used to develop a genetically modified cyanobacterium against all those stresses which create oxidative stress in the organisms. However, a comprehensive understanding of mechanisms of multiple stress tolerance towards environmental stresses has yet to be achieved.

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