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Heterologous expression of *Anabaena* PCC 7120 *all*3940 (a Dps family gene) protects *Escherichia coli* from nutrient limitation and abiotic stresses

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ABSTRACT

This study presents first hand data on the cloning and heterologous expression of *Anabaena* PCC 7120 *all*3940 (a dps family gene) in combating nutrients limitation and multiple abiotic stresses. The *Escherichia coli* transformed with pGEX-5X-2-*all*3940 construct when subjected to iron, carbon, nitrogen, phosphorus limitation and carbofuron, copper, UV-B, heat, salt and cadmium stress registered significant increase in growth over the cells transformed with empty vector under iron (0%), carbon (0.05%), nitrogen (3.7 mM) and phosphorus (2 mM) limitation and carbofuron (0.025 mg ml^{-1}), $CuCl_2$ (1 mM), UV-B (10 min), heat (47 °C), NaCl (6% w/v) and $CdCl_2$ (4 mM) stress. Enhanced expression of *all*3940 gene measured by semi-quantitative RT-PCR at different time points under above mentioned treatments clearly demonstrates its role in tolerance against aforesaid abiotic stresses. This study opens the gate for developing transgenic cyanobacteria capable of growing successfully under above mentioned stresses.

1. Introduction

Cyanobacteria, the photosynthetic prokaryotes, are important contributors to the nitrogen economy of soil. While growing in the natural ecosystem, they are challenged by a host of environmental stresses such as heat, pesticide, heavy metals, salinity, UV radiation and others. Nevertheless these microbes are further challenged by iron and phosphorus deficiency of soils as about 36% Indian cultivable lands suffer from iron deficiency [1] and available phosphorus [2]. Taking recourse to the fact that iron is an essential component of the enzyme nitrogenase complex and phosphorus an indispensable element for growth of cyanobacteria under trauma set in motion by aforementioned stresses, a pressing requirement is to develop transgenic cyanobacteria capable of carbon and nitrogen fixation under aforesaid environmental hardships. However, the success of development of such transgenic cyanobacteria will primarily depend on the availability of the candidate gene that can offer multiple abiotic stress tolerance in cyanobacteria without adversely affecting its normal function.

During the search for such a gene we came across the Dps (DNA binding protein under starvation) protein first reported in *Escherichia coli* [3] to detoxify ROS and protect DNA from oxidative stress. Being a member of ferretin-like superfamily, the Dps protein was found to participate in iron-regulation, mono-oxygenation and reactive radicals detoxification. Dps has also been reported in certain cyanobacteria like *Synechococcus* sp. PCC 7942 [4], *Trichodesmium erythreum* [5] and *Anabaena* sp. PCC 7120 [6]. *Anabaena* sp. PCC 7120 contains a cluster of six genes encoding DNA binding protein, and *all*3940 is a member of the above cluster of genes [7]. It is interesting to mention that no attention has ever been paid to clone and over express any of the *dps* genes to examine their hidden potential in protecting from nutrient starvation and abiotic stresses. Semi-quantitative RT-PCR of all the six *dps* genes revealed that *all*3940 was transcriptionally most active under long-term iron and phosphorus starvation (data not shown). In view of the above and also as the first step in our long-term strategy of developing *all*3940 transformed *Anabaena*, *all*3940 gene was cloned and over expressed in *E. coli* to test if it can offer tolerance against nutrients limitation and multiple abiotic stresses. This paper is the first report on the cloning of *Anabaena all*3940 into the pGEX-5X-2 to study its role in protecting *E. coli* from nutrients (iron, carbon, nitrogen and phosphorus) limitation and abiotic stresses (pesticide, heavy metals, heat, salt and UV-B radiation).

2. Materials and methods

2.1. Cyanobacterial and bacterial strains and plasmids

Anabaena sp. PCC 7120 was grown photoautotrophically in BG-11 medium [8] 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

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of 14:10 h at pH 7.5. The cultures were shaken by hand 2–3 times daily. *E. coli* strains DH5α and BL21 (DE3) (Novagen) were used as hosts 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 containing 1.4% (w/v) agar at 37 °C. Cells harboring recombinant plasmids were grown and maintained on LB media supplemented with 100 μg ml^{-1} ampicillin [9]. Plasmid pGEX-5X-2 (GE Healthcare) was used as a vector for cloning.

2.2. Cloning of the all3940 gene from Anabaena sp. PCC 7120

Genomic DNA from *Anabaena* was isolated as described previously [10]. An open reading frame of *all*3940 encoding DNA binding protein in starvation was amplified by polymerase chain reaction using genomic DNA as template with a pair of primers, Pf (5′CGCGGATCCATGCAAGAGCTTAAGCAA3′) and Pr (5′GCATGCGGCCGCCTAGATAAAATCTC3′). The underlined bases represent BamHI and NotI 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 45 s using standard PCR conditions (100 ng 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.

2.3. Construction of expression vector

The purified PCR product was digested with BamHI and NotI (NEB) and the resultant DNA fragment was cloned into the expression vector pGEX-5X-2 digested with the same restriction enzymes. The recombinant plasmid construct, pGEX-5X-2-*all*3940, was introduced into *E. coli*, the latter was then grown in LB medium containing 100 μg ml^{-1} ampicillin. The plasmid was then isolated and the DNA sequence of *all*3940 was confirmed by sequencing.

2.4. Expression analysis of all3940 gene using RT-PCR

The expression analysis of *all*3940 was carried out in the same way as described previously [11] except the *all*3940 gene primers in place of *ahp*C gene primers. The Icycler profile for *all*3940 gene was as follows: initial denaturation for 5 min at 94 °C followed by 40 incubation cycles each consisting of 1 min denaturation at 94 °C, 1 min annealing at 56 °C, 45 s extension at 72 °C and a final 10 min extension at 72 °C. 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.

2.5. Enzyme activity assay

For enzymatic assay of *all*3940 protein (i.e., Dps), *E. coli* BL21 harboring the pGEX-5X-2-*all*3940 and pGEX-5X-2 plasmid were grown as described previously in expression analysis of *all*3940 gene using RT-PCR. Cells harvested by centrifugation were suspended in 20 mM sodium phosphate buffer (pH 7.4) and disrupted by ultrasonication. The crude extract was centrifuged at 15,000 rpm for 1 h at 4 °C and the clear supernatant was used for enzymatic assay through ferroxidation. The kinetics of iron oxidation by Dps was measured at 310 nm using Ultraspec 2100*pro* spectrophotometer (GE Healthcare USA) as per the method of Bhattacharyya and Grove [12].

2.6. SDS–PAGE and Western blot analysis

SDS–PAGE and Western blot analysis were carried out as mentioned earlier [11] except the antibody used for the Dps protein. Polyclonal antibodies used for the detection of Dps were obtained as a generous gift from Prof. George. S. Bullerjahn, USA.

2.7. Assay for nutrients limitation and abiotic stress tolerance in transformed E. coli cells

In order to assess the role of *Anabaena all*3940 Dps in nutrient limitation and multiple stress tolerance, effects of nutrients such as iron, carbon, nitrogen and phosphorus limitation and abiotic stresses such as carbofuron, copper, UV-B, heat, salt and cadmium treatment on the growth of transformed *E. coli* BL21 cells with pGEX-5X-2 (empty vector) and pGEX-5X-2-*all*3940 (recombinant plasmid) were examined.

2.8. Mode of stress application

The nutrient limitation experiments were carried out by limiting the concentration of carbon to 0.05%, nitrogen to 3.7 mM and phosphorus to 2 mM [13] in the standard M9 medium [14] whereas iron limitation was performed following the complete iron removal from the medium. Carbofuron (0.025 mg ml^{-1}), $CuCl_2$ (1 mM), UV-B (10 min), temperature (47 °C), NaCl (6%) and $CdCl_2$ (4 mM) were applied as described earlier [11].

2.9. Measurement of survival and growth

The growth curve of *E. coli* BL21 cells grown in standard M9 medium was prepared by measuring the absorbance at 600 nm for 12 h. Single colony of *E. coli* BL21 cells transformed either with empty vector or recombinant plasmid was inoculated in tubes containing fresh M9 medium supplemented with 100 μg ml^{-1} ampicillin and grown overnight. On the next day the cultures were diluted to 0.05 optical density (OD) with fresh M9 medium (25 ml in 100 ml conical flask) and incubated until OD value reached around 0.5. For nutrients limitation, 200 μl of *E. coli* culture (OD 0.5) was inoculated into separate flasks containing 100 ml M9 medium having no iron (0%), carbon (0.05%), nitrogen (3.7 mM) and phosphorus (2 mM), respectively. Thus survival of *E. coli* cells transformed with empty vector and recombinant plasmid was measured under nutrient limitation by measuring OD at 600 nm and comparing it with the control cells. For all the nutrients limitation experiments, M9 medium supplemented with 100 μg ml^{-1} ampicillin was used. Survival of the *E. coli* cells transformed with empty vector and pGEX-5X-2-*all*3940 against carbofuron (0.025 mg ml^{-1}), $CuCl_2$ (1 mM), UV-B (10 min), temperature (47 °C), NaCl (6%) and $CdCl_2$ (4 mM) was measured as explained previously [11]. 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. All the growth experiments were conducted on a rotary shaker (200 rpm) at 37 °C. Three independent measurements were taken and the average value was used for making the final data.

2.10. Expressional analysis of all3940 in response to different abiotic stress

Escherichia coli cells subjected to iron, carbon, nitrogen and phosphorus limitation and treated with carbofuron, copper, UV-B, temperature, salt and cadmium were withdrawn at 0, 3, 6 and 9 h, their RNA was isolated using TRIzol reagent and expressional characterization of *all*3940 gene was done using RT-PCR as described above. To ascertain the equal loading of RNA in the different samples RT-PCR of 16S rDNA was also performed and used as an internal control.

3. Results

3.1. Molecular cloning of all3940 gene

The PCR amplified product of about 435 bp (Fig. 1A) conforming to the theoretical length of the *all*3940 gene (435 bp) was cut with BamHI and NotI, ligated to pGEX-5X-2 BamHI and NotI digested backbone fragment and transformed into *E. coli* strain BL21. The construct was verified by DNA sequencing and double digestion (Fig. 1B). The open reading frame of 435 bp encodes 145 amino acids with a calculated molecular weight of 15.95 kDa.

3.2. Expressional analysis of all3940 gene in E. coli using RT-PCR and SDS–PAGE

A 3–4 h exposure of cells transformed with recombinant plasmid to 0.5 mM IPTG (Isopropyl-β-D-1thiogalactopyranoside) was found to produce 2.0-fold increase in the transcript level compared to non-induced cells (Fig. 1C). Likewise, the fusion protein (on the SDS–PAGE) also showed induction after IPTG treatment (Fig. 1D). The molecular weight of GST-Dps was found to be 42 kDa.

3.3. Western blotting and enzymatic activity assay

Western blot analysis with anti-Dps produced a single band of 42 kDa showing good agreement with the molecular weight of *all*3940 gene deduced from the nucleotide sequence. This confirmed that *E. coli* cells containing pGEX-5X-2-*all*3940 produced Dps protein (Fig. 1E). The activity of Dps was assayed by ferroxidation in the presence of ferrous ammonium sulphate as substrate and measured by monitoring the change in OD at 310 nm due to formation of the ferric ions. The cells containing pGEX-5X-2-*all*3940 plasmid showed increased activity over those containing empty vector. This level was further increased after IPTG treatment. Control cells containing empty plasmid pGEX-5X-2 did not depict any enzymatic activity (Fig. 2A).

3.4. Effect of nutrients limitation and various abiotic stresses on growth of transformed E. coli cells

The cells transformed with recombinant plasmid (pGEX-5X-2-*all*3940) showed better growth (approximately 15–29% more) than those transformed with empty vector (Fig. 2B–E and 3A–F). When nutrients such as iron, carbon, nitrogen and phosphorus were lim-

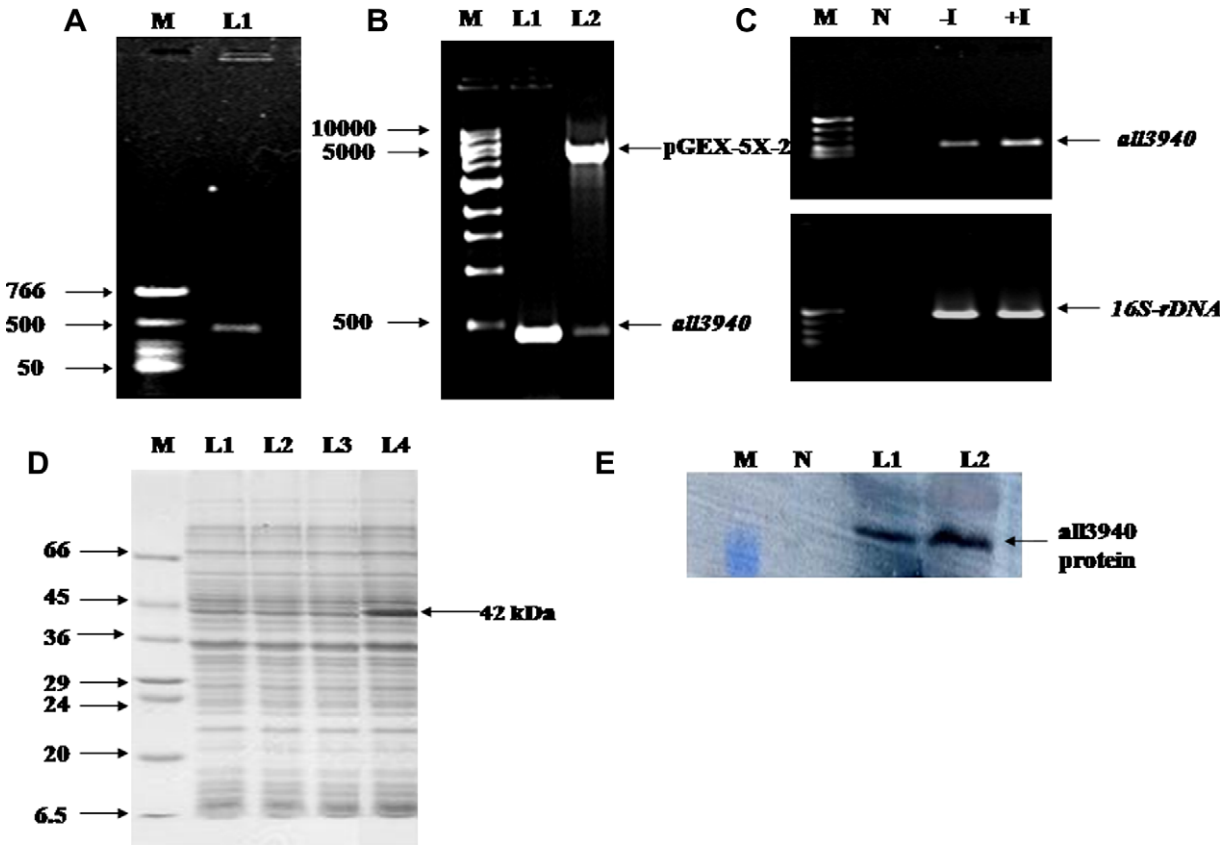

Fig. 1. (A) PCR amplification of *Anabaena* sp. PCC 7120 ORF *all*3940. Lane M DNA ladder (PCR marker), Lane 1 (L1) PCR product of *all*3940 (435 bp). (B) Agarose gel showing double digested recombinant clones with BamHI and NotI showing the presence of 435 bp fragment and 4.9 kb pGEX-5X-2 vector. Lane M DNA ladder (1 kb), lane 1 (L1) PCR product of *all*3940 (435 bp), lane (L2) double digestion of pGEX-5X-2-*all*3940 with BamHI and NotI showing release of 435 bp fragment. (C) Expression of *all*3940 gene and 16S rDNA as an internal control, lane M DNA ladder (PCR marker), lane N negative control, lane −I without IPTG, lane +I with IPTG (D) SDS–PAGE (12%) analysis of *all*3940 protein expression in *E. coli* BL21 (Coomassie blue staining). Lane 1 (M) protein marker; lane 2 (L1), whole cell lysate of *E. coli* BL21 cells containing the empty vector pGEX-5X-2 without IPTG induction; lane 3 (L2) the empty vector pGEX-5X-2 obtained after 4 h post-induction with 0.5 mM IPTG; lane 4 (L3) plasmid pGEX-5X-2-*all*3940, uninduced; lane 5 (L4), plasmid pGEX-5X-2-*all*3940 obtained after 4 h induction with 0.5 mM IPTG. The number on the right side is the apparent molecular weight of the recombinant *all*3940 protein (42 kDa). (E) Immunoblot detection of *all*3940 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); lanes 3 and 4 (L1 and L2) sample from *E. coli* cells containing (pGEX- 5X-2-*all*3940) incubated without (−I) and with (+I) IPTG, respectively.

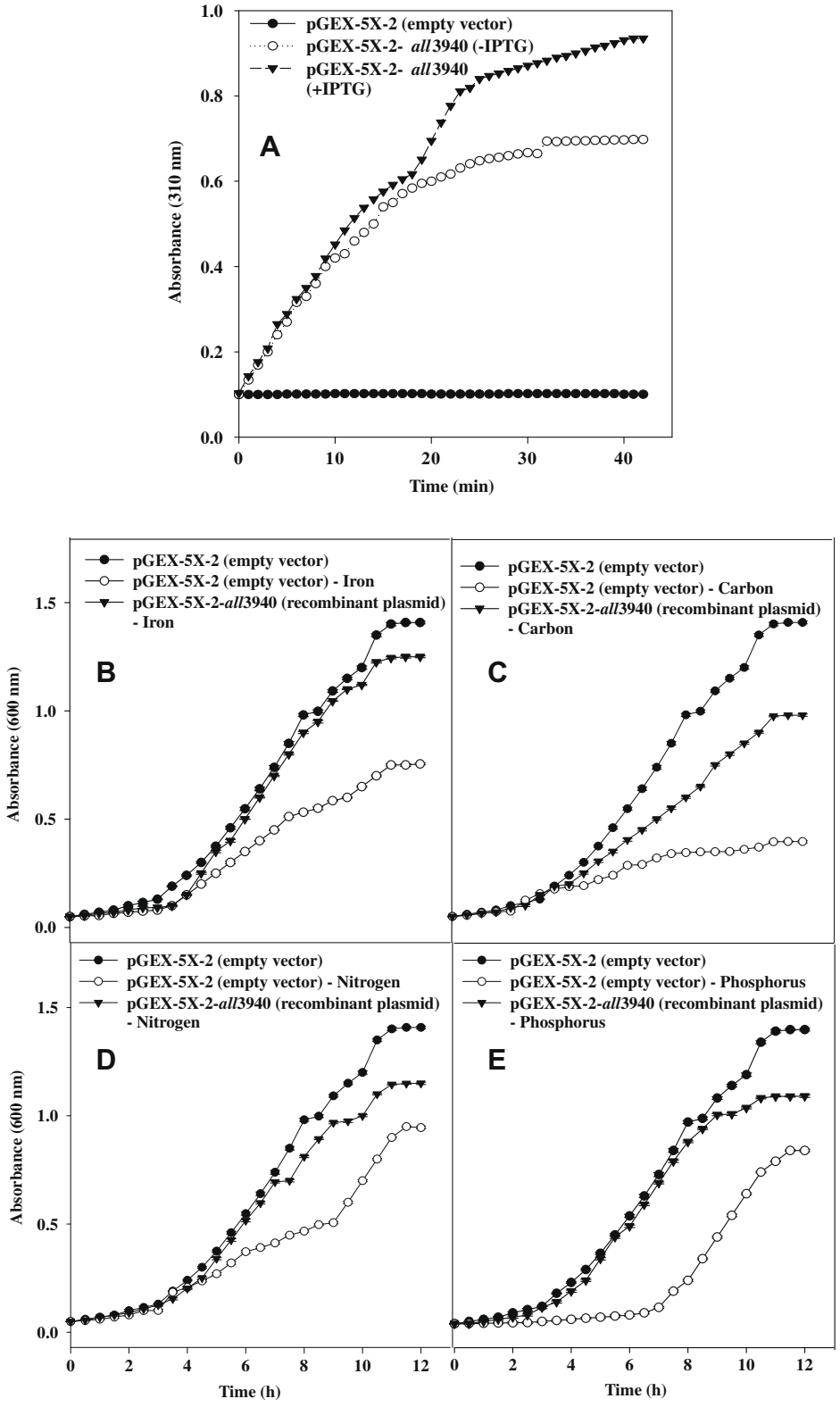

Fig. 2. (A) Ferroxidase activity assay of Dps following oxidation of Fe (II) to Fe (III) from *E. coli* cells containing empty vector (pGEX-5X-2) and recombinant plasmid (pGEX-5X-2-*all*3940) with and without IPTG induction. Effect of (B) iron, (C) carbon, (D) nitrogen, (E) phosphorus, limitation on the growth of transformed *E. coli* cells containing recombinant plasmid (pGEX-5X-2-*all*3940) and empty vector (pGEX-5X-2). The mean of three independent replicates were plotted with error bars indicating standard deviations. (B–E) Represents the growth curves of *E. coli* cells in M9 medium (supplemented with 100 μg ml^{-1} ampicillin) exposed to iron, carbon, nitrogen and phosphorus limitation, respectively.

ited in the M9 medium the pGEX-5X-2-*all*3940 transformed cells showed only 3%, 18%, 5%, 1% decline in the specific growth rate as compared to control. When LC_{50} dose of abiotic stresses e.g., carbofuron, copper, UV-B, heat, salt and cadmium for cells transformed with empty vector was applied on pGEX-5X-2-*all*3940 transformed cells, the decrease in specific growth rate was only 8.23%, 15.54%, 10%, 11.34%, 11.34% and 5%, respectively than the control.

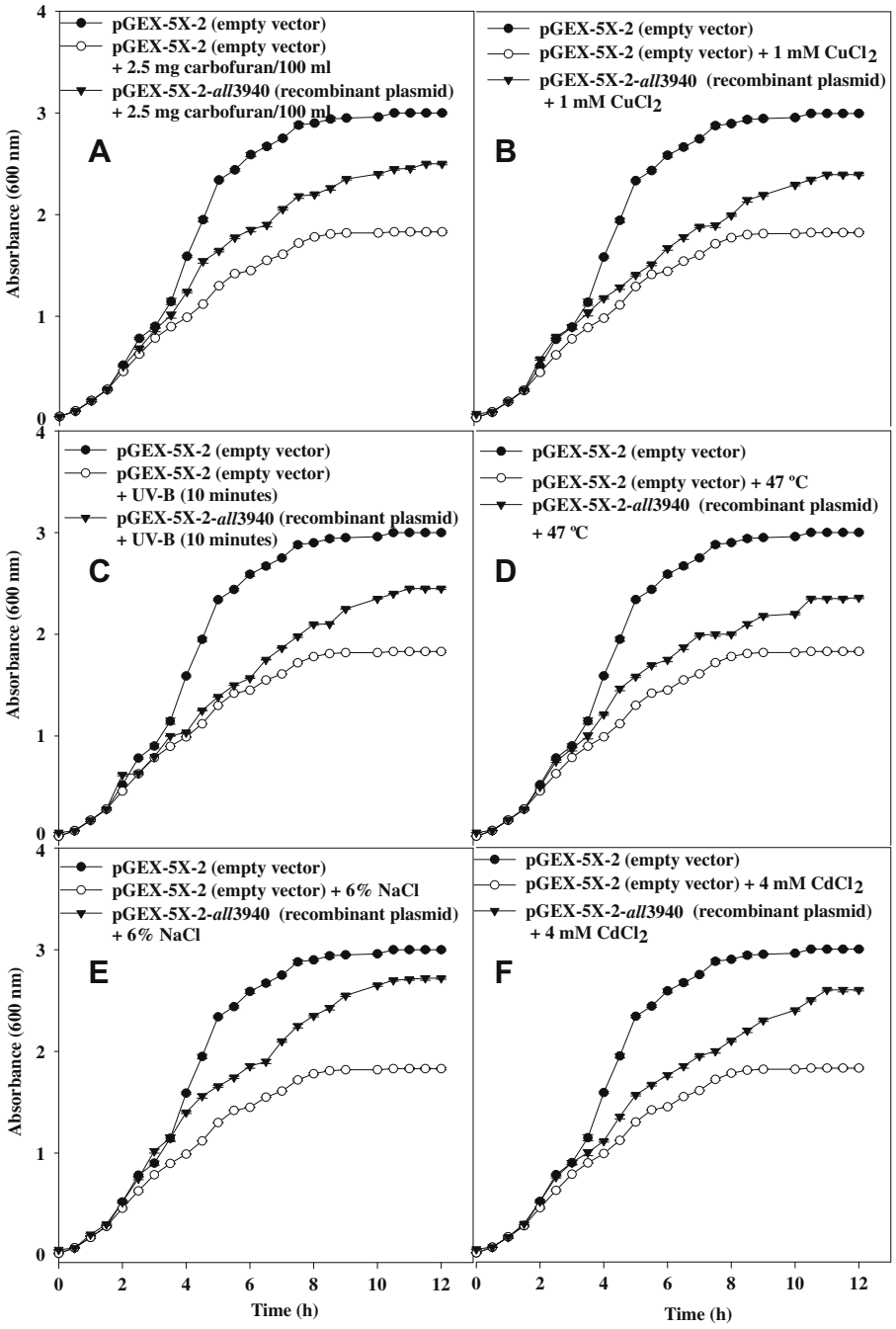

Fig. 3. Effect of (A) carbofuron (pesticide), (B) copper, (C) UV-B, (D) heat, (E) salt and (F) cadmium on the growth of transformed *E. coli* cells containing recombinant plasmid (pGEX-5X-2-*all*3940) and empty vector (pGEX-5X-2). The mean of three independent replicates are plotted with error bars indicating standard deviations. (A–F) Represents the growth curves of *E. coli* cells in LB medium (supplemented with 100 μg ml^{-1} ampicillin) exposed to carbofuron, copper, UV-B, heat, salt and cadmium, respectively.

3.5. Expression of all3940 in response to various abiotic stresses

All the selected stresses triggered significant increase in the transcript level of *all*3940 gene at various time points (Fig. 4A–J). This increase being 1.50-, 1.94-, 2.45-fold in iron; 1.25-, 1.50-, 1.54-fold in carbon; 1.60-, 1.96-, 2.50-fold in nitrogen and 1.25-, 1.60-, 1.65-fold in phosphorus limitation over control after 3, 6 and 9 h, respectively. However, the transcript level registered an increase of 1.24-, 1.50-, 1.45-fold in carbofuron; 1.10-, 1.25-, 1.45-fold in copper; 1.25-, 1.35-, 1.52-fold in UV-B; 1.12-, 1.25-, 1.30-fold in heat; 1.08-, 1.15-, 1.20-fold in salt and 1.16-, 1.75-, 1.89-fold in cadmium stress over control after 3, 6 and 9 h, respectively.

4. Discussion

The data compiled in the results vividly confirmed *Anabaena all3940* gene cloning into pGEX-5X-2 and its expression in *E. coli* BL21. The expression of gene was reconfirmed by accumulation of 42 kDa Dps fusion protein and its respective transcript. Specific growth rate of pGEX-5X-2-*all*3940 transformed *E. coli* cells was about 15–30% better than cells transformed with pGEX-5X-2

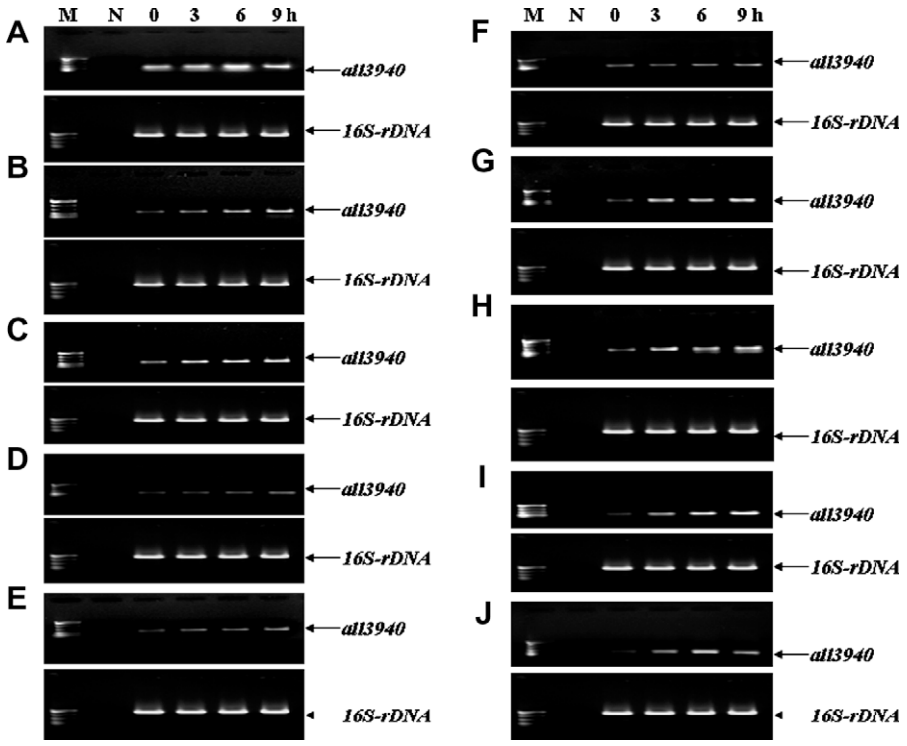

Fig. 4. RT-PCR analysis of *all*3940 gene showing effect of (A) iron, (B) carbon, (C) nitrogen, (D) phosphorus limitation and exposed to (E) carbofuron (pesticide), (F) copper, (G) UV-B, (H) heat, (I) salt and (J) cadmium at various time points.

(empty vector) under different stresses (Fig. 2B–E and 3A–F). The nutrients limitation and multiple abiotic stresses generate ROS and disrupt redox potential of the cells ultimately resulting into DNA damage. The *Anabaena all*3940 protein may scavenge ROS and protect DNA thereby alleviating the destructive impacts of these stresses. It finds support from the work of Peña et al. [15] in *Synechococcus* PCC 7942 where Dps has been described to form hexameric aggregate to protect chromosomal DNA from iron, nitrogen, and phosphorus starvation. However, nitrogen, carbon and phosphate starved *E. coli* cell's proteomic analysis has demonstrated oxidation of several proteins to their carbonyl derivatives during growth under these conditions [13]. It has been suggested that these oxidatively modified proteins are derived from the balance between the rate of protein synthesis, oxidation and degradation, which are individually regulated under a given growth condition. Thus it is speculated that these proteins may serve as alternative energy source for the starved cells. Our results also find support from the work of Nair and Finkel [16] where *dps* mutants failed to compete with wild type strains in nutritionally restricted medium and the wild type cells remained viable for more than 15 days under continuous batch culture even without nutrients supplementation. Nevertheless, it has also been reported that some cryptic growth may take place in carbon starved *E. coli* and 50% cells remain viable under such condition even after 6 days [17]. It is quite likely that over expressed Dps may scavenge the ROS produced by nutrients starvation thereby providing congenial environment for cell growth. Notwithstanding above, the enhanced tolerance of *all*3940 transformed *E. coli* may also be due to stress-induced biocrystallization as suggested by Wolf et al. [18] and formation of Dps-DNA biocrystal in *E. coli* cells thereby protecting DNA from damage under nutrient-stress. Further, Wei et al. [6] put forward that Dps1173 combats oxidative stress through its ferroxidase activity in *Anabaena* sp. PCC 7120. In view of the ferroxidase activity of *all*3940 (Fig. 2A) tolerance against identified abiotic stress appears justified. Our results can also find support from the work of Chaurasia et al. [19] and Mishra et al. [11] where *E. coli* transformed with *pcs* and *ahpC* genes from *Anabaena* PCC 7120 offered tolerance against multiple abiotic stresses, respectively.

To explore the relationship between environmental stresses and *all*3940 induction, the expression of *all3940* gene was attested by RT-PCR (Fig. 4A–F). A significant up-regulation of *all*3940 transcript as observed under different stresses at different time points suggested a stress dependent regulation of *all*3940 gene. Our results can be explained in the light of the report of Nair and Finkel [16] where *E. coli dps* null mutants were sensitive to UV-B, heat and metal stress. In view of the report where Dps has been described as stress response protein and nutrient-stress induced DNA binding protein in *Thermosynechococcus elongatus* BP-1 and *Synechococcus* sp. JA-3-3B, respectively [20], our speculations seem rational.

These results vividly demonstrated that enhanced expression of *all3940* gene from *Anabaena* sp. PCC 7120, a heterologous source, can offer protection in non-photosynthetic *E. coli* cells against nutrients limitation and abiotic stresses. Depending on the nutritional status of the cells and the environmental stress, *Anabaena all*3940 protein may also act as ferritins and protect from oxidative stress via non-specific DNA binding or from Fenton mediated DNA damage. The present study, therefore, recommends that *all*3940 over expressing *Anabaena* may be an excellent biofertilizer in paddy fields suffering from nutrients limitation and abiotic stresses. Cyanobacterial transformation with *all*3940 gene using integrative plasmid is in progress in our laboratory.

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