Proline biosynthesizing enzymes (glutamate 5-kinase and pyrroline-5-carboxylate reductase) from a model cyanobacterium for desiccation tolerance

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Abstract Drought is the most important abiotic stress, challenging sustainable agriculture globally. For desiccation being the multigenic trait, a combination of identified genes from the appropriate organism may render crop tolerant to the water stress. Among the compatible solutes, proline plays multifaceted role in counteracting such stress. The genes encoding proline biosynthesizing enzymes, glutamate 5-kinase (G5K), and pyrroline-5-carboxylate reductase (P5CR) from the low-desiccation-tolerant cyanobacterium *Anabaena* sp. PCC 7120, were cloned and overexpressed in *Escherichia coli* BL21(DE3) individually. The recombinant *E. coli* cells harboring G5K, failed to exhibit enhanced desiccation tolerance relative to those with P5CR that showed increased growth/survival over the wild type. This may be ascribed to the overexpression of the reductase gene. Multiple sequence alignment showed P5CR to be conserved in all the organisms. We hypothesize that P5CR gene from high-desiccation-tolerant cyanobacteria may be adopted as the candidate for making transgenic N₂-fixing cyanobacterium for paddy fields and/or crop development in future.

Keywords Cyanobacteria · Glutamate 5-kinase · Overexpression · Pyrroline-5-carboxylate reductase · Recombinant

Introduction

Frequent drought worldwide (Lobell et al. 2008) poses problem in sustainable agriculture. The loss of intracellular water becomes fatal to most of the cells. Extreme desiccation for longer period can be tolerated by many prokaryotes (Potts 1994). Drought and salt stress induced trehalose and sucrose accumulation in cyanobacteria (Hershkowitz et al. 1991; Sakamoto et al. 2009); in addition to sucrose, trehalose, antioxidative enzymes (catalase, peroxidase, and SOD), and proline level also fluctuated in *Anabaena* 7120 under desiccation (Singh et al. 2013). Intracellular concentrations of total amino acids including proline varied in desiccated cells of *Tolypothrix scytonemoides* (Rajendran et al. 2007). Billi et al. (2000) transfected desiccation sensitive *Escherichia coli* by *sps* (sucrose-6-phosphate synthase), and the transformants were desiccation tolerant.

Proline is a multifunctional amino acid (Szabados and Savoure 2010). It serves as reductant (Hare and Cress 1997), ATP source (Atkinson 1977), carbon skeleton (Köcher et al. 2011), and even in quenching the ROS (Matysik et al. 2002). The regulation and function of proline accumulation are not yet completely understood. Little is known of the molecular biology of amino acid biosynthesis and its regulation in cyanobacteria (Riccardi et al. 1989). Biosynthesis of proline in eukaryotes takes place by twin enzymes viz. P5CS (pyrroline 5 carboxylate synthetases) and P5CR (pyrroline 5 carboxylate reductase). P5CS is a bifunctional enzyme that exhibits glutamyl kinase and γ glutamyl phosphate reduction (GPR) activities. It is suggested to have evolved from two bacterial polypeptides encoded by a single operon (Perez-Arellano et al. 2010). However in prokaryotes, the two separate enzymes exist that are γ -glutamyl kinase (GK) and GPR, are homologous to both the moieties of P5CS (Delauney and Verma 1993). P5CR catalyzes the final step in conversion

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from Δ -pyrroline-5 carboxylate to proline with the simultaneous oxidation of NAD(P)H to NAD(P)⁺ in almost all the organisms (Adams and Frank 1980). As the regulation of proline accumulation is little known, the engineering of proline metabolism may improve plant tolerance of environmental stress (Szabados and Savoure 2010).

In the present communication, we used the desiccation sensitive *E. coli* to address the problem by transfecting it with G5K and P5CR from the cyanobacterium *Anabaena* 7120 and monitor survival under desiccation.

Materials and methods

Organism and culture conditions

Anabaena sp. PCC 7120, a gift from M. Ohmori, Tokyo University, Japan was grown in BG-11 medium (Rippka et al. 1979) free from combined nitrogen source under continuous tungsten plus fluorescent illumination ($27.17 \mu \text{mol}^{-2} \text{s}^{-1}$) at $28 \pm 1^\circ \text{C}$.

Bacterial strains and plasmids

E. coli NM was used as host for vectors, *E. coli* DH5 α for transformation, and *E. coli* BL21 (DE3) for heterologous gene expression. Plasmids pGEM-T Easy (Promega) was used for DNA cloning and sequencing, and pET-19b (Novagen) for the expression of glutamate 5-kinase (G5K) and pyrroline-5-carboxylate reductase (P5CR) genes.

Multiple sequence alignment and phylogenetic analysis

The amino acid sequence of P5CR of *Anabaena* 7120 was compared with that of P5CR enzymes from different organisms. Protein sequence database (SWISS_PROT, PIR, and Genbank) was searched using the standard search algorithm BLASTP (NCBI, NIH). The sequences were aligned and phylogenetic tree generated based on the neighbor-joining (NJ) algorithm using Clustal X2.

DNA isolation, primer design, and PCR amplification

Genomic DNA from *Anabaena* 7120 was isolated following method of Marmur (1961), and primers designed to amplify the ORFs *alr3103* (G5K), and *alr0488* (P5CR). *Nde*I and *Bam*HI restriction sites (indicated as underlined in the sequences) were incorporated in the forward and reverse primer, respectively, to facilitate cloning in pET-19b expression vector.

Primer for G5K:

Forward primer: 5' GCGCATATGGTGATTTTAGTTT CCTCTGG 3'

Reverse primer: 5' GCGGGATCCTTAAGTCAATACT AAGTTATCTC 3'

Primer for P5CR:

Forward primer: 5' GCGCATATGACTATAAAATTTG GTTTAATTG 3'

Reverse primer: 5' GCGGGATCCCTATTTTCCTAGT TCTTGCG 3'

The PCR reaction mixture (25 μl) contained 80 ng/ μl of genomic DNA, 0.2 μl of 50 pmol of each primer, 2 μl of 2 mM dNTP mix, 2.5 μl of 10X *Taq* buffer, 2.5 μl of 1.5 mM, and 1 U of *Taq* polymerase. The reaction was performed in a thermocycler (Eppendorf) using the program one cycle of pre-heat at 94°C (2 min) followed by 30 consecutive cycles of denaturation at 94°C (1 min), annealing at 52°C (1 min), extension at 72°C (1 min), and final extension at 72°C (7 min). The desired PCR products were purified using DNA gel extraction kit (Axygen).

Cloning and formation of expression vector

Each of the amplified DNA fragments was cloned separately in pGEM-T Easy vector (Promega) and transformed into competent *E. coli* DH5 α (Sambrook and Russell 2001). Positive clones containing the recombinant plasmid were selected and ascertained by DNA sequencing. The recombinant plasmids were isolated from clones and digested with *Nde*I and *Bam*HI restriction enzymes followed by ligation of ORFs with separate pET-19b bacterial overexpression vector containing His tag.

Colony PCR and expression of G5K and P5CR

Colony PCR was performed to detect the presence of desired DNA fragments followed by overexpression of the two enzymes in *E. coli* BL21 (DE3). The overnight grown culture (2 ml) was added to fresh LB medium containing ampicillin (100 $\mu\text{g}/\text{ml}$) and grown until OD₆₀₀ reached 0.6–0.8. The culture then was induced by adding IPTG (0–2 mM) for different durations (12–16 h) at different incubation temperatures (18 – 25°C). The cells were pelleted by centrifugation (10,000 rpm, 15 min, 4°C) and suspended in sodium phosphate buffer (20 mM, pH 7.4), broken in liquid nitrogen and concentrated (10,000 rpm, 20 min, 4°C) to remove the insoluble fraction. The supernatant of induced and uninduced cells with the recombinant vectors was subjected to 12 % SDS-PAGE.

Water stress and bacterial growth

Desiccation was imposed as described by Billi et al. (2000) with certain modifications by us. Recombinant *E. coli* BL21 (DE3) cells containing G5K or P5CR gene and the wild type (100 μl) were subjected to sterile air drying at room temperature (50 h)

with relative humidity (35 %). We have monitored the RH and temperature by LICOR 6400 (USA). Desiccated cells were rehydrated in 1 ml LB medium induced with 1 mM IPTG and O. D. monitored at 600 nm every 30 min for cell growth.

Purification of enzymes

Purification of His-tagged enzyme was done by affinity chromatography using Bio-Rad Economopac column (poly-Prep)

with Ni^{+2} -NTA His-trap resin (Novagen). Cells were suspended in 100 μl of Ni^{+2} -NTA Bind Buffer (50 mM NaH_2PO_4 , pH 8; 300 mM NaCl; 10 mM imidazole) and incubated (30 min) on ice after adding lysozyme (1 mg/ml). The lysate was centrifuged (15,000 rpm, 10 min), supernatant mixed with 20 μl of 50 % Ni^{+2} -NTA His Bind resin followed by incubation at 4 °C (30 min). Resin was pelleted by centrifugation (15,000 rpm, 10 s), supernatant transferred to fresh tube followed by washing with 1 $\times$ Ni^{+2} -NTA wash buffer

Fig. 1 Multiple sequence alignment of amino acids in glutamate 5-kinase (G5K) of *Anabaena* sp. PCC 7120 with the same enzyme of selected prokaryotes. Asterisks indicates conserved amino acids in toto, colon, high and dots low conservation. ana *Anabaena* (Nostoc PCC 7120), nos *Nostoc punctiforme* PCC 73102, art *Arthrobacter chlorophenolicus* A6, myc

Mycobacterium smegmatis MC2 155, bac *Bacillus halodurans* C-125, xan *Xanthomonas oryzae* pv. *oryzae* PXO99A, rhi *Rhizobium leguminosarum* bv. trifolii WSM1325, E. coli *Escherichia coli*, sulf *Sulfolobus solfataricus* 98/2

(50 mM NaH₂PO₄, pH 8; 300 mM NaCl; 20 mM imidazole). Finally, the protein was eluted with 1X Ni²⁺-NTA elution

buffer (50 mM NaH₂PO₄, pH 8; 300 mM NaCl; 250 mM imidazole) and eluates analyzed on 12 % SDS-PAGE.

Fig. 2 Multiple sequence alignment of amino acids in pyrroline-5-carboxylate reductase (P5CR) of *Anabaena* sp. PCC 7120 with the same enzyme of selected prokaryotes and eukaryotes. Asterisks indicates conserved amino acids in toto, colon, high and dots low conservation, ana *Anabaena* (Nostoc PCC 7120), nos *Nostoc punctiforme* PCC 73102, art *Arthrobacter chlorophenolicus* A6, myc *Mycobacterium smegmatis* MC2

155, bac *Bacillus halodurans* C-125, xan *Xanthomonas oryzae* bv. *oryzae* PXO99A, rhi *Rhizobium leguminosarum* bv. *trifolii* WSM1325, E. coli *Escherichia coli*, sulf *Sulfolobus solfataricus* 98/2, trt *Triticum aestivum*, ara *Arabidopsis thaliana*, pea *Pisum sativum*, dro *Drosophila melanogaster*

P5CR enzyme activity determination

Desiccated (50 h) recombinant cells of *E. coli* both induced and uninduced were taken. The P5CR activity was determined following Cheng and Lee (1999). The reaction mixture contained 50 mM potassium phosphate (pH 8.0), 1 mM DTT, 0.125 mM P5C, 200 μ M β -NADH, and 50 μ L of desalted extract in a final volume of 1 mL. One unit of P5CR is 1 nmol β -NAD⁺ min⁻¹ and molar extinction coefficient of NADH is 6.2 mM cm⁻¹. The P5CR activity was determined by recording the decrease of A₃₄₀.

Results

Multiple sequence alignment and phylogenetic analysis

The mature peptide G5K consisted of 326 amino acids with the theoretical molecular weight of 40.79 kDa while P5CR of 270 amino acids, corresponded to 28.14 kDa (cyanobase). Amino acid homology of the G5K from *Anabaena* 7120 with G5K from *Nostoc punctiforme* PCC 73102 was maximum (88 %), followed by *E. coli* (44 %), *Xanthomonas oryzae* bv. *oryzae* PX099A (43 %), *Arthrobacter chlorophenolicus* A6 (43 %), *Bacillus halodurans* C-125 (41 %), *Rhizobium leguminosarum* bv. *Trifolii* WSM 1325 (41 %), *Mycobacterium smegmatis* str. MC2 155 (40 %), and *Sulfolobus solfataricus* 98/2 (14 %; Fig. 1). Also, P5CR from *Anabaena* 7120 had 82 % amino acid homology with those from *N. punctiforme* PCC 73102, *Drosophila melanogaster* (43 %), *M. smegmatis* MC2 155

(42 %), *Arabidopsis thaliana* (42 %), *Triticum aestivum* (41 %), *Pisum sativum* (41 %), *E. coli* (40 %), *X. oryzae* bv. *oryzae* PX099A (39 %), *A. chlorophenolicus* A6 (38 %), *B. halodurans* C-125 (33 %), *S. solfataricus* 98/2 (33 %), and *R. leguminosarum* bv. *Trifolii* WSM 1325 (30 %; Fig. 2). The percent identities are indicated in brackets. Multiple sequence alignments show the phylogenetic relatedness among the selected organisms. Among all, the amino acid sequences of G5K and P5CR, from *N. punctiforme* was most identical to its counterpart from *Anabaena* sp. PCC 7120 thus placing the two in the

Fig. 3 Phylogenetic tree showing evolutionary relatedness of G5K (a) and b of P5CR among selected prokaryotes and eukaryotes. Branch length represents phylogenetic distances

Fig. 4 a PCR amplified *g5k* (lanes 1–4) and 1 kb DNA ladder (lane M); b SDS-PAGE of uninduced *E. coli* BL21 (DE3) cells containing recombinant vector (pET 19b-G5K) (lane 1); and IPTG-induced cells at varying concentrations; 0.5 mM (lane 2), 1 mM (lane 3), 2 mM (lane 4) along with molecular weight marker (lane M); and c SDS-PAGE showing recombinant G5K after Ni²⁺-NTA affinity chromatography (lane 1) and molecular marker (lane M)

cyanobacterial phylum (Fig. 3a and b). Phylogenetic tree was constructed using ClustalW to reveal the evolutionary relationship among the organisms. It is evident that *N. punctiforme* PCC 73102 was closely related to *Anabaena* while to *E. coli* and *D. melanogaster* distantly.

Cloning and overexpression of G5K and P5CR genes and purification of their enzymes

PCR amplified sequences of *alr3103* (Fig. 4a) was cloned into TA cloning vector (pGEM-T Easy) followed by subcloning in pET-19b and positive overexpression of the target genes monitored in *E. coli* BL-21 (DE-3; Fig. 4b). The optimization of

overexpression was carried out by varying incubation time, temperature, and IPTG concentration of the bacterial culture. The target enzyme was maximally expressed after 16 h at 18 °C with 1 mM IPTG. The G5K enzymes were expressed maximally in 1 mM IPTG followed by 2 and 0.5 mM, respectively. The expression of P5CR is also in line with G5K, and 1 mM IPTG was found to be optimum for its overproduction. Since *E. coli* BL21(DE3) possessed the proline biosynthesizing enzymes, the bands of G5K and P5CR in uninduced cells were less intense than in the induced ones indicating the overproduction of such enzymes following overexpression of the respective genes.

The recombinant G5K purified from Ni^{+2} -NTA affinity chromatography, showed a single band of G5K on SDS-PAGE (Fig. 4c). The molecular weights of the target proteins G5K (40.79 kDa) corresponded well with the respective enzymes from *Anabaena* 7120 (cyanobase). Likewise, *alr0488* was PCR amplified (Fig. 5a) and cloned into TA cloning vector (pGEM-T Easy) followed by subcloning in pET-19b, and positive overexpression of the target genes monitored in *E. coli* BL-21 (DE-3; Fig. 5b). The recombinant P5CR purified from Ni^{+2} -NTA affinity chromatography showed a single band of P5CR along with some other bands of low visibility on SDS-PAGE (Fig. 5c).

Fig. 5 a PCR amplified *p5cr* (lanes 1 and 2) and 1 kb DNA ladder (lane M), b SDS-PAGE of uninduced *E. coli* BL21 (DE3) cells containing recombinant vector (pET 19b-P5CR) (lane 1); IPTG (1 mM) induced cells (lane 2) and molecular weight marker (lane M); and c SDS-PAGE showing recombinant P5CR after Ni^{+2} -NTA affinity chromatography (lane 2), IPTG (1 mM) induced cells (lane 1) and molecular marker (lane M)

Desiccation tolerance of transformed cells

Desiccated cells (OD_{600} 0.07) got rehydrated by adding the liquid growth medium, and tolerance monitored in terms of growth (OD_{600}). It was observed that cells transformed with recombinant P5CR grew rapidly compared to the wild-type and G5K transformants (Fig. 6). P5CR specific activity of *E. coli* BL 21 (DE3) in nonrecombinant and recombinant cells after desiccation (50 h) have been observed as 0.18 and 0.4 U mg^{-1} protein, respectively. However, after Ni-NTA column purification, the specific activity was 18 U mg^{-1} .

Fig. 6 Survival of the desiccated wild-type and transformed *E. coli* BL21(DE3) cells following different rehydration durations

Discussion

Desiccation of cyanobacteria resulted in alterations of gene expression affecting nucleic acid, proteins, and the accumulation of trehalose sucrose along with other targets Potts (1994). Cyanobacteria are also high desiccation tolerant as *Chroococcidiopsis*, *Microcoleus*, and *Lyngbya* can withstand the marked removal of cellular water and can be revived after prolonged stress (Potts 1999). However, *Anabaena* 7120 desiccated for 22 h (R.H. 30 % at 30–40 $\mu\text{E}/\text{m}^2/\text{s}$ desiccation), remained only 1 % viable (Kato et al. 2004) that marked it to be low desiccation tolerant. Skirvyicz et al. (2011) emphasized the identification of newer genes that may render crop plants survive drought extremes. Thus cyanobacterial species if screened for candidate genes for transferring in plants for desiccation tolerance will help improving crop productivity.

Multiple sequence alignment of G5K from *Anabaena* 7120 showed significant similarity to the orthologous proteins of prokaryotes, while P5CR to prokaryotes and eukaryotes as well. The amino acid sequence alignments of various G5Ks showed several domains of unknown function within the protein. The functional significance of the highly conserved residues common to these proteins requires additional study with site-directed mutagenesis and functional assays. Multiple sequence alignment of amino acid sequences of P5CR from *Anabaena* 7120 showed several conserved regions as well as specific amino acids such as K221 (lysine) residue corresponding to E221 (glutamic acid) residue of *Homo sapiens* as reported by Meng et al. (2006), and thus indicating its pivotal role as the conformational switch for co-factor selectivity. The latter investigators also observed that the conserved dinucleotide-binding motif having Rossmann fold was required for structural formation as well as P5CR catalysis in humans. Kleiger and Eisenberg (2002) pointed out that a subset of protein that adopts Rossmann folds (with motifs such as GXXXA and GXXXG), also binds to nucleotide cofactors [FAD and NAD(P)] to function as oxidoreductase. Likewise, *Anabaena* 7120 has the GXXXA motif starting G235 onward representing the site for NAD(P) attachment. In line with pea, this domain also contains a GTTIAG sequence involved in NAD(P)H-ribose binding indicating that P5CR utilizes NAD(P)H as the co-factor (Williamson and Slocum 1992).

Regarding the role(s) of G5K and P5CR in desiccation tolerance, *E. coli* BL21(DE3) cells were separately transformed with these two genes. The recombinant *E. coli* cells having G5K did not show increased desiccation tolerance. The possible reason could be the feedback inhibition of G5K by proline (Smith 1985). The increased tolerance may be observed using the G5K mutant as it leads to proline overproduction with markedly altered site for feedback inhibition during osmotic stress in *Salmonella typhimurium* (Csonka 1981), *E. coli* (Smith 1985), and freeze-tolerant *Saccharomyces cerevisiae* (Terao et al. 2003; Sekine et al. 2007).

In contrast to G5K, *E. coli* cells fortified with P5CR gene showed increased growth (~10 %) over the wild type. P5CR activity was also enhanced in *Mesembryanthemum nodiflorum* facing salt stress (Treichel 1986), *Chlorella autotrophica* (Laliberte and Hellebust 1989), water-stressed barley (Argandona and Pahllich 1991), osmotically stressed soybean (Delauney and Verma 1990), and *A. thaliana* (Verbruggen et al. 1993). The conversion of P5C to proline is not the rate-limiting step in proline biosynthesis, yet the control of P5CR activity implies the complex transcriptional control under developmental and osmotic regulations (Verbruggen et al. 1993). In the present case, *E. coli* cells transformed with P5CR, showed ~10 % increment in growth. Increased P5C production against salinity and proline accumulation was attributed to enhanced P5CR activity (Ma et al. 2008).

P5CR transcripts increased in abundance in response to osmotic stress in plants indicating that P5CR gene transcription is under osmotic stress control (Delauney and Verma 1990; Verbruggen et al. 1993). The P5CR cDNA from soybean, when overexpressed in tobacco, did not significantly increase proline level in the transgenic plants despite a 100-fold P5CR activity over the wild type (Szoke 1992). However, we did not measure the proline level in P5CR recombinants except specific activity that was 0.4 U mg^{-1} protein in transformed *E. coli* cells relative to control (0.18 U mg^{-1}). However, for the Ni-NTA purified P5CR activity (specific), increased 45-fold over the crude enzyme derived from 1mM IPTG induced recombinant cells. Therefore targeting proline biosynthetic pathway related genes seems to be an important determinant for stress adaptation (Szabados and Savoure 2010). To sum up, the recombinant *E. coli* having G5K and P5CR showed varied response towards desiccation, and transformants having P5CR were efficient in combating desiccation over their G5K counterpart. Thus cyanobacteria with high desiccation tolerance can be adopted for candidate genes for future transgenics.

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