Overexpression of glutaredoxin-2 from Cyanobacterium *Synechocystis* PCC 6803 in *Escherichia coli* conferring enhanced salt stress tolerance

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ABSTRACT

Glutaredoxin (Grx), widely found in bacteria, plants, and mammalian cells, is an electron carrier for ribonucleotide reductase and a general glutathione-disulfide reductase of importance for redox regulation. Cyanobacterium Synechocystis strain PCC 6803 contains two genes (slr1562 and ssr2061) encoding two glutaredoxins (Grx1 and Grx2, respectively). The amino acid sequences deduced from both proteins share high identity with those of Grxs from other organisms. In the present study, we found that the steady-state transcript levels of ssr2061 were increased in the wild-type of Cyanobacterium cells under oxidative stress conditions imposed by high salinity (NaCl), chilling or application of H_2O_2, methylviologen or t-butyl hydroperoxide. Moreover, the protein Grx2 encoded by ssr2061 was successfully overexpressed as a soluble fraction in Escherichia coli JM109. The transformed Escherichia coli cells showed high tolerance to NaCl (over than 700 mM) mediating growth inhibition compared to cells transformed with the vector alone.

Key words: *Synechocystis PCC 6803, Glutaredoxin, recombinant enzyme, salt stress.*

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INTRODUCTION

Glutaredoxins (Grxs) are small heat-stable oxidoreductases, first discovered in *E. coli* as GSH-dependent hydrogen donors for ribonucleotide reductase. The substrate specificity of glutaredoxin is either protein disulfides, like in ribonucleotide reductase or glutathione mixed disulfides of proteins or low molecular compounds like cysteine.

The first function assigned to Grx was as an alternative hydrogen donor in the reduction of intramolecular disulfide in ribonucleotide reductase; the essential enzyme for DNA synthesis (Holmgren, 1976). Grxs roles in diverse cellular processes including the regulation of transcription factors (for example, NF-jB), reduction of ascorbate and dehydroascorbate, protein folding, and sulfur metabolism (Wells *et al.*, 1990; Wells *et al.*, 1993; Jung and Thomas, 1996; Hirota *et al.*, 2000; Fernandes and Holmgren, 2004). Three major groups of Grxs have been classified (Vlamis-Gardikas and Holmgren, 2002; Fernandes and Holmgren, 2004). Classical Grxs are 10-kDa proteins with a CPYC active site (Grx1 and Grx3 in *E. coli* and Grx1 and Grx2 in yeast). A second group, with a CGFS active site, corresponds to yeast Grx3, Grx4, and Grx5 (Rodriguez-Manzaneque *et al.*, 1999). The third type, represented by *E. coli,* Grx2 is structurally related to the glutathione S-transferase (Xia *et al.*, 2001). Grxs have been

identified and isolated from various organisms such as *E. coli* (Holmgren, 1976; Rouhier *et al.*, 2002), yeast (Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999), rice (Sha *et al.*, 1997), spinach (Morell *et al.*, 1995), bovine (Hatakeyama *et al.*, 1984), rabbit (Hopper *et al.*, 1989) and human (Holmgren and Äslund, 1995; Lundberg *et al.*, 2001).

Previous studies have shown that the yeast glutaredoxins are active as antioxidants and are required for protection against reactive oxygen species (ROS). In addition, overexpression of Grx1 or Grx2 increased resistance to H_2O_2. Furthermore, the expression of Grx1 and Grx2 is up-regulated in response to various stress conditions, including exposure to oxidants, with both genes regulated by stressresponsive elements (STRE). Also, glutaredoxin, found in rice, acting as glutathione-dependent peroxidase and the maximal catalytic efficiency (V_{max}/K_m) is obtained with cumene hydroperoxide rather than H_2O_2 or *t*-BuOOH. Thiol/disulfide interchange catalyzed by Grx is crucial for intracellular redox homeostasis, especially under oxidative stress (Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999). Moreover, Grxs play important roles in protection against reactive oxygen species and regulation of the DNA binding activity of nuclear factors (Bandyopadhyay *et al.*, 1998; Chrestensen *et al.*, 1995; Holmgren, 1989).

Most of Grxs were structurally and functionally characterized in great details. However, little information was described previously about cyanobacterium Grx. According to GenBank database search, there are two candidate Grx genes in *Synechocystis* sp. PCC 6803. The present manuscript describes, for the first time, the transcription, expression and identification of cyanobacterium *ssr2061*. The involvement of Grx2 in the tolerance to salt stress was also investigated by overexpressing the gene in *E. coli* JM109 strain and subjecting the strain with high levels of Grx2 to salt stress.

MATERIALS AND METHODS

Materials

Restriction enzymes and ligase were obtained from Takara Biotech. (Japan). H_2O_2 and *t*-BuOOH were obtained from Sigma (St. Louis, USA). All other chemicals were of the commercially available highest grade.

Culture conditions

The wild-type strain of *Synechocystis* PCC 6803 was grown photoautotrophically at 27°C in Allen's medium at 30 μE m^{-2} s^{-1} under fluorescent lamps. Log-phase cells of *Synechocystis* PCC 6803 (A_{730} = 0.6–1.0) were subjected to stress treatments.

Expression of *ssr2061* gene in *E. coli*

The chromosomal DNA was isolated from *Synechocystis* PCC 6803 by the method of Williams (1988). Two DNA fragments containing the open reading frame *ssr2061* were amplified by PCR with the following primers: 5´P- AAGCGTT**C**ATATG GCTGTCT -3´P (*ssr2061* F), and 5´P-TACGAATAAGATTAGCCAGC -3´P (*ssr2061* R). The forward primer was designed to introduce an *NdeI* site with an ATG codon for the initiation of translation (bold sequence). Amplified DNA fragments were cloned into a pT7Blue-T vector (Novagen, Madison, WI, USA) and sequenced with an automated DNA sequencer (ABI310A, Applied Biosystems, Japan). For the construction of the plasmid to express *ssr2061*, the plasmid was digested with *NdeI* and each 0.5-kb DNA fragment was cloned into a pET3a vector (Novagen, Madison, NI, USA) digested with the same restriction enzyme. The resulting constructs, designated pET/Gpx2, and was introduced into the *E. coli* strain JM109. The recombinant enzyme in *E. coli*

was produced by the method described previously by Tamoi *et al.* (1996).

Northern-Blot Analysis

Total RNAs (20 mg) were isolated from the cells (Los *et al.*, 1997) and used for northern-blot analysis with *ssr2061* DNA used a probe. RNA was quantified using a Mac BAS 1000 image scanner (Fuji Photo Film, Tokyo).

SDS-PAGE

Cell extracts were homogenized with SDS-loading buffer (150 mM Tris-HCl, pH 6.8, 4% [w/v] SDS, and 10% [v/v] 2-mercaptoethanol). The homogenates were boiled for 5 min and centrifuged at 10,000 g for 5 min at 4°C. The supernatants (40 mg) were analyzed by 15% (w/v) SDS-PAGE according to Laemmli (1970).

RESULTS AND DISCUSSION

Identification and sequence analysis of ORF *ssr2061* encoding Grx2

Analysis of the complete genome sequence of *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996) revealed two genes encoding Grx. The *slr1562* consisted of 330 bps encoding 109 amino acids with a calculated molecular mass of about 12 kDa, while the *ssr2061* contained 267 bps encoding 88 amino acids with a molecular mass of about 10 kDa. The deduced amino acid sequence of Grx1 shared 66% identity to that of Grx2. The putative protein Grx2 shared 29–42% identity with the amino acid sequence of Grxs from *E. coli* (35%), yeast (31%), rice (42%) and human (29%) (Fig.1). Like other monothiol Grxs family, three conserved regions have been identified comprising the active site (Cys-Pro-Tyr-Cys), hydrophobic surface area, and a GSH or ribonucleotide reductase binding site (Xia *et al.*, 1992; Holmgren and Äslund, 1995). All three regions were found in Grx2 (underlined in Fig. 1). However, the amino acid residues between the active cysteine residues, which are highly conserved in known *E. coli*, yeast and mammalian Grxs, were different in Grx2, where the Tyr residue was replaced by Phe residue. The same difference at an active site has also been shown in rice Grx. Interestingly, the hydrophobic surface area TVP, as a partial region around the activity site that has been proposed to play a role in interactions with other proteins, was replaced by the hydrophobic area SLP in Grx2. In summary, the sequence similarity and conserved active site motifs of the protein suggest that this candidate gene (Grx2) is a member of the Grx family.

Changes of the levels of transcript of *ssr2061* under stress conditions

Grxs are found to be involved in cellular responses to various stresses especially oxidative and osmotic stresses (Cotgreave and Gerdes, 1998; Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999; Rouhier *et al.*, 2003; Kim *et al.*, 2005). To examine the physiological role of Grx2 *in vivo*, the mRNA level of *ssr2061* was assessed by northern blot analysis. The transcript levels of *ssr2061* were induced within 15 min under oxidative stress caused by the addition of H_2O_2 (2 mM), MV (1 μM), and *t*-BuOOH (0.2 mM). By the treatment with 200 mM NaCl, the *ssr2061* mRNA level was steadily increased with time till 2 hr. interestingly, the transcript level of *ssr2061* started to increase after 1 hr from the chilling stress condition (4°C) and steadily increased with time till 3 hr (Fig. 2). Thus, Grx2 seems to contribute in the scavenging of the free radicals generated by oxidative stress caused by H_2O_2, *t*-BuOOH, MV treatment, high salinity and chilling.

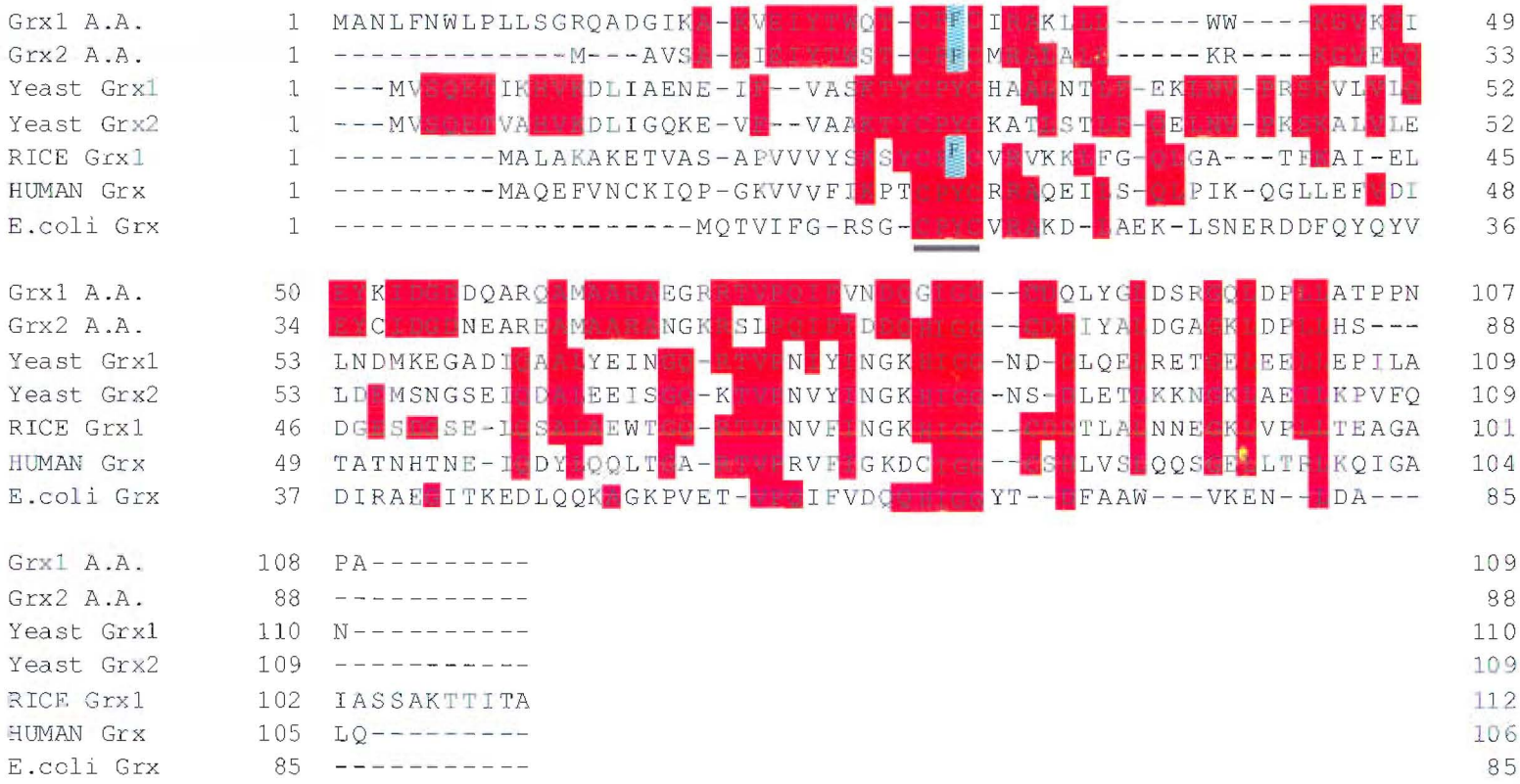

Fig. (1): Comparison of the predicted amino acid sequence of Grx1 and Grx2 with Grxs from yeast, rice, human, and E. coli. Amino acid sequences were aligned for maximal homology. Conserved residues are shown by a dark gray background. The active site, hydrophobic surface area, and a GSHbinding site are underlined. Alignments were performed using the program ClustalW, version 1.4.

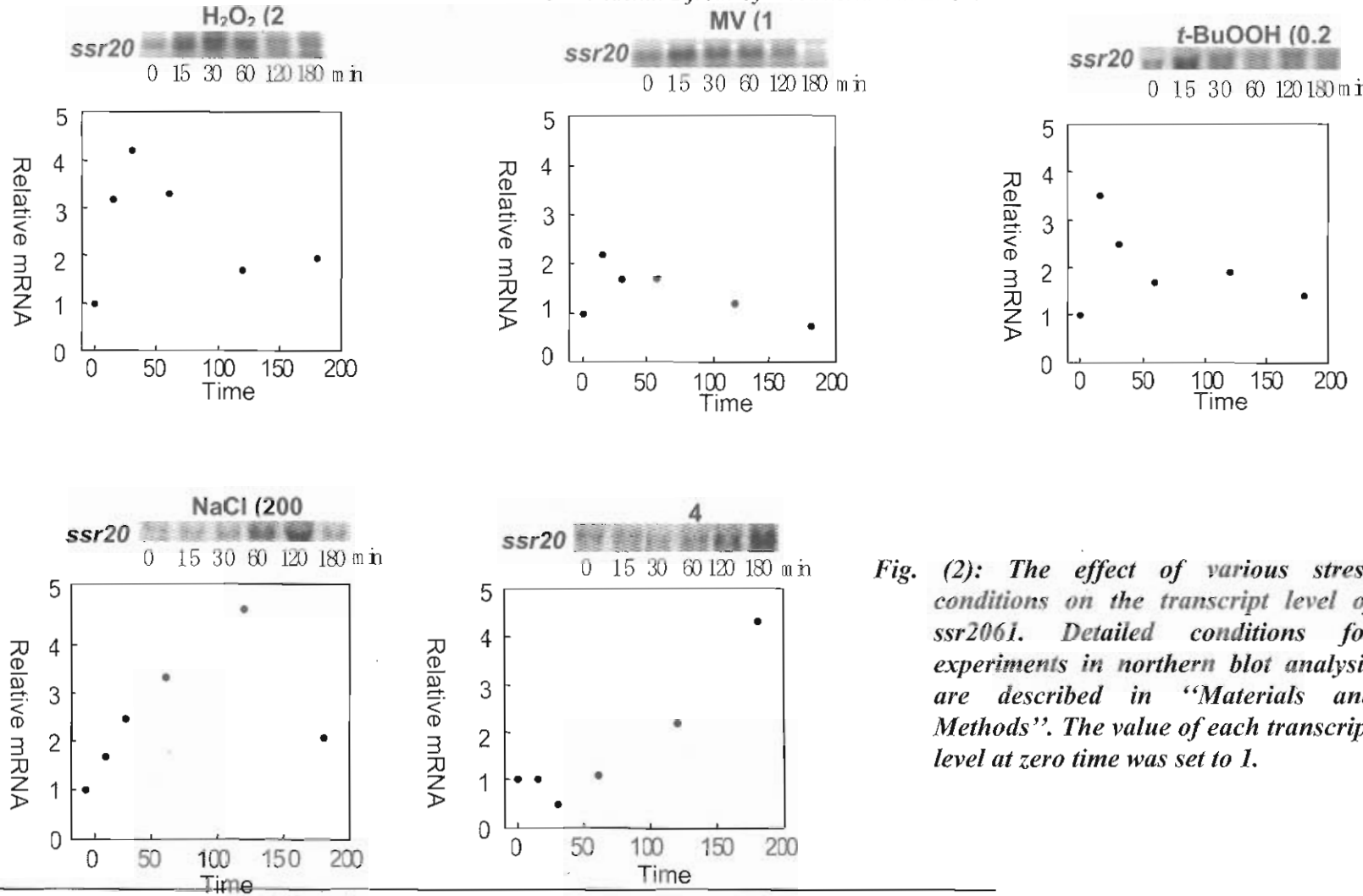

Fig. (2): The effect of various stress conditions on the transcript level of ssr2061. Detailed conditions for experiments in northern blot analysis are described in "Materials and Methods". The value of each transcript level at zero time was set to 1.

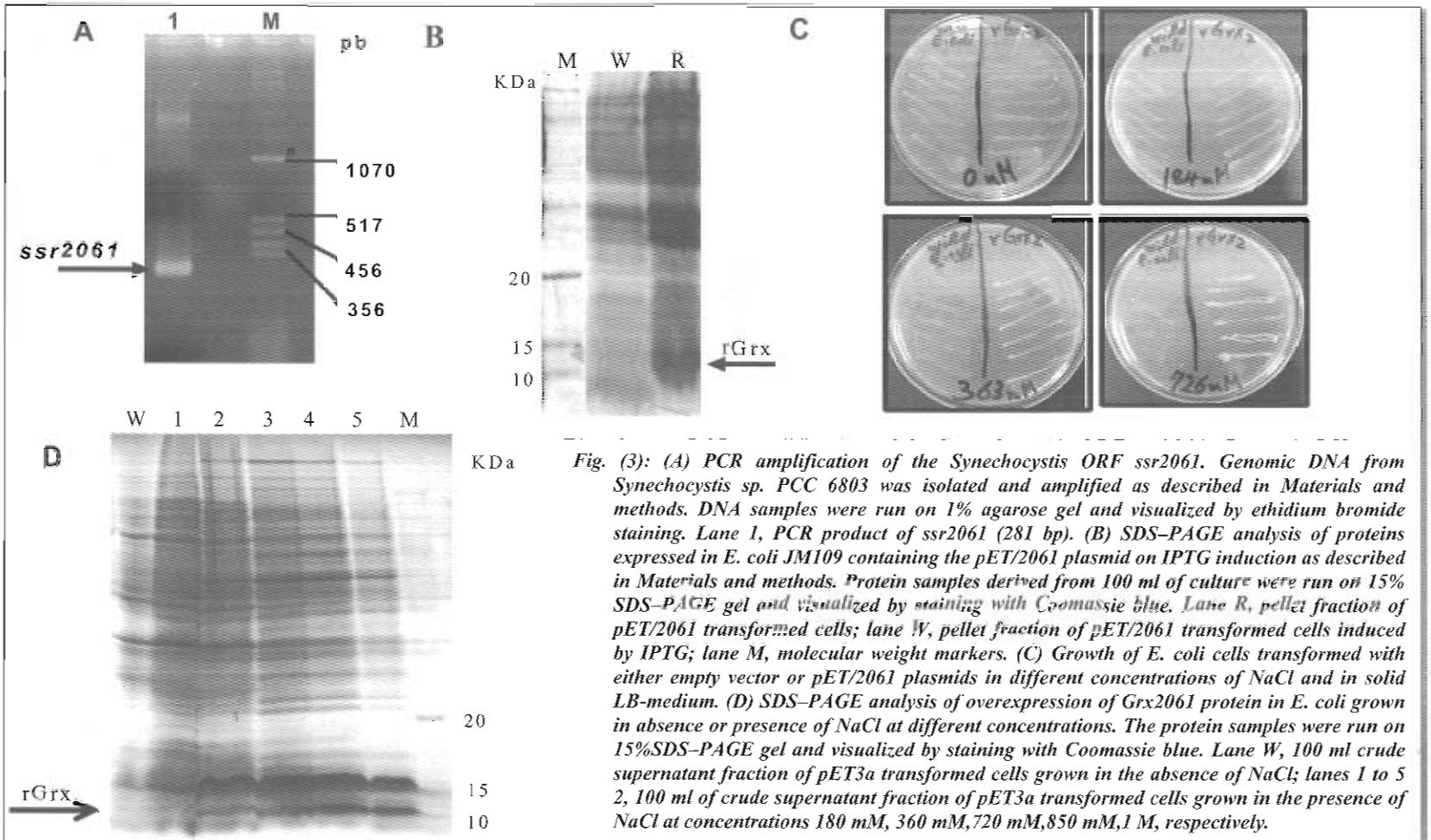

Fig. (3): (A) PCR amplification of the Synechocystis ORF ssr2061. Genomic DNA from Synechocystis sp. PCC 6803 was isolated and amplified as described in Materials and methods. DNA samples were run on 1% agarose gel and visualized by ethidium bromide staining. Lane 1, PCR product of ssr2061 (281 bp). (B) SDS–PAGE analysis of proteins expressed in E. coli JM109 containing the pET/2061 plasmid on IPTG induction as described in Materials and methods. Protein samples derived from 100 ml of culture were run on 15% SDS–PAGE gel and visualized by staining with Coomassie blue. Lane R, pellet fraction of pET/2061 transformed cells; lane W, pellet fraction of pET/2061 transformed cells induced by IPTG; lane M, molecular weight markers. (C) Growth of E. coli cells transformed with either empty vector or pET/2061 plasmids in different concentrations of NaCl and in solid LB-medium. (D) SDS–PAGE analysis of overexpression of Grx2061 protein in E. coli grown in absence or presence of NaCl at different concentrations. The protein samples were run on 15%SDS–PAGE gel and visualized by staining with Coomassie blue. Lane W, 100 ml crude supernatant fraction of pET3a transformed cells grown in the absence of NaCl; lanes 1 to 5 2, 100 ml of crude supernatant fraction of pET3a transformed cells grown in the presence of NaCl at concentrations 180 mM, 360 mM,720 mM,850 mM,1 M, respectively.

Overexpression and purification of the recombinant protein

The 281 bp DNA fragment corresponding to the ORF *ssr2061* was amplified from the genomic DNA of *Synechocystis* sp. PCC 6803 by PCR (Fig. 3A). The amplified product was cloned into pET3a prokaryotic expression vector at the *Nde*I–*BamH*1 site resulting in the pET/2061 plasmid. This plasmid was used to transform *E. coli* JM109 strain to characterize the expression pattern of the cyanobacterium Grx2. The optimum conditions for the expression of Grx2 in *E. coli* were examined. After induction with IPTG, recombinant Grx2 was found to be expressed approximately 20% of total proteins in *E. coli.* As shown in Fig. (3B), the protein profiles corresponding to Grx2 were correlated with the molecular weight (10 kDa) calculated from the deduced amino acid sequence of its clone. Moreover, the transformed *E. coli* cells have been checked by the isolation of Miniprep and the treatment of restriction enzymes (data not shown).

Antioxidative properties of Grx2

The environment within cells predominantly gets reduced due to high levels of GSH. However, oxidative stress can alter this environment allowing exposed cellular thiols to become oxidized (Chrestensen *et al.*, 1995). It has been proposed elsewhere that some Grx systems participated in protection against oxidation or repairing sensitive sulfhydryls for maintaining the adequate redox state of proteins in the intracellular environment and thus for regulating various cellular activities (Holmgren, 1989; Cotgreave and Gerdes, 1998; Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999; Rouhier *et al.*, 2003). In this study, *E. coli* was used as a model system to test whether the *Synechocystis* Grx2, on transformation to *E. coli*, can circumvent salt stress. Such salt stress was generated *in vitro* by NaCl (0-726 mM), known as salt stress inducer. For this purpose, we compared the growth of *E. coli* transformed with either pET/Grx2 or empty vector in presence of NaCl. Within the range of NaCl concentrations tested during this study, growth rate in liquid medium of the pET/Grx2 transformed *E. coli* cells was higher than that of the cells transformed with empty vector (data not shown). The effect of NaCl was also analyzed on solid medium with the growth of empty vector and pET/Grx2 transformed cells with different concentrations of NaCl. As expected, Grx2 overexpressing cells grew better than the empty vector transformed cells in the presence of 148 mM, 363 mM, and 726 mM of NaCl concentrations (Fig. 3C). It may be noted that both the empty vector and the pET/Grx2 transformed strains had endogenous *grx* genes in their chromosomes. However, the normal level of Grx was not sufficient in the empty vector transformed *E. coli* to protect the cells against NaCl mediated toxicity and the increased level of tolerance observed in the pET/Grx2 *E. coli* cells transformed with pET/Grx2 might be due to the overexpressed Grx2 protein. The following evidence favored such an assumption. As judged by SDS-PAGE analysis, pET/Grx2 transformed in *E. coli* cells showed increased levels of Grx2 polypeptide under different concentrations of NaCl, compared to the *E. coli* wild-type cells (Fig. 3D). These results suggest that a Grx gene/protein from the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803, a heterologous source, can confer abiotic stress expecially salt tolerance to the non photosynthetic *E. coli.*

In conclusion, the present study describes the study of transcript level of *ssr2061* in *Synechocystis* sp. PCC 6803 cell under different stress conditions, and cloning

of the Grx gene of *Synechocystis* sp. PCC 6803 in the cytoplasm of *E. coli*. Further experiments demonstrate that introducing of the *Synechocystis* Grx can confer salt stress tolerance to *E. coli* confirming absence of species barrier in terms of the Grx function.

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الملخص العربي

تحسين صفة المقاومة للملوحة في بكتريا القولون المحولة وراثيا بجين الجلوتاريدوكسين 2 المفصول من سلالة السيانو بكتريا PCC 6803

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الهدف من هذه الدراسة التعرف علي دور ووظيفة إنزيم الجلوتاريدوكسين -2 المفصول من السيانوبكتريا وعلاقتها بالمقاومة للملوحة. أكدت الأبحاث علي وجود الأحماض الأمينية الهامة المتماثلة والمحافظة علي وظيفة الجين في الكائنات المختلفة مثل البكتريا والنبات وكذلك الثدييات . وبدراسة التعبير الجيني علي مستوي عملية النسخ في السيانوبكتريا وجد زيادة معدل التعبير الجيني تحت الظروف البيئية غير الملائمة وخاصة زيادة الملوحة . وتم كلونة هذا الجين في بكتريا القولون باستخدام التفاعل المتسلسل والبادئات المتخصصة تحت سيطرة المحفز المتخصص بزيادة معدل البروتين .

اثبتت التجارب قدرة البكتريا المحولة وراثيا علي النمو تحت تركيزات مختلفة للملح قد تصل الي 700 ملي مول في حين لم تستطع السلالات البرية الخالية من الجين النمو علي تركيزات ملح مرتفعة .