American Journal of Agricultural and Biological Sciences 7 (1): 88-96, 2012
ISSN 1557-4989

A Cyanobacterium *Synechocystis* sp. PCC 6803 Glutaredoxin Gene (*slr1562*) Protects *Escherichia coli* against Abiotic Stresses

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Abstract: Problem statement: Glutaredoxins (GRXs) are ubiquitous small heat stable glutathione-dependent oxidoreductase enzymes that play a crucial role in plant development and response to oxidative stress. **Approach:** Cyanobacterium *Synechocystis* strain PCC 6803 contains two genes (*slr1562* and *ssr2061*) encoding glutaredoxins. In the present investigation the *slr1562* gene (*grxC*) was isolated and characterized. **Results:** The results revealed that the amino acid sequence deduced from GrxC protein share high identity with those of GRXs from other organisms and contain the consensus GRX family domain with a CPFC active site. Northern blotting analysis revealed that the expression of *slr1562* gene could be induced by oxidative and salt stresses. Moreover, the protein GrxC was successfully overexpressed as a soluble fraction in *Escherichia coli* JM109. The over-expression of GrxC in *Escherichia coli* cells significantly increased resistance of cells to oxidative, drought and salt stresses. **Conclusion/Recommendations:** These results suggest that the *slr1562* gene could play an important role in regulating abiotic tolerance against oxidative, drought and salt stresses in different organisms.

Key words: Luria-Bertani (LB), different stress conditions, Glutaredoxins (GRXs), *Synechocystis* PCC 6803, *slr1562*, recombinant enzyme, abiotic stress

INTRODUCTION

Glutaredoxins (Grx; EC 1.20.4.1) are glutathione dependent oxidoreductase proteins with a small (10-12 kDa) molecular weight. The Grx was discovered in *Escherichia coli* as an alternative reducing substrate of ribonucleotide reductase, the key enzyme of deoxyribonucleotide biosynthesis (Holmgren, 1976). Originally, thioredoxin had been regarded as the exclusive substrate in this process (Thelander *et al.*, 1964). Both proteins possess two redox-active cysteine residues in their active sites formed by the sequences Cys-Gly-Pro-Cys in thioredoxin and Cys-Pro-Tyr (Phe)-Cys in glutaredoxin (Holmgren, 1989). The Grx pathway requires a supply of reduced glutathione (GSH) to maintain cellular protein redox stability in the presence of Reactive Oxygen Species (ROS). ROS are generated in the cellular respiration and photosynthesis processes and increase during biotic and abiotic stresses (Able *et al*., 2000; Prasad *et al.*, 1994; Tsugane *et al*., 1999; Bolwell *et al*., 2002; Ji *et al.*, 2008). Although ROS are signals essential for plant development, high concentration of ROS can damage macromolecules such as proteins, lipids and nucleic acids and thus disrupt normal signaling in plant and eventually lead to cell death (Miller *et al.*, 2009). Many environmental stresses such as drought, salinity, heavy metals and abnormal temperature can induce excessive accumulation of ROS in plants, which will damage macromolecules and thus change normal signal transduction (Miller *et al.*, 2009; Scandalios, 2002). Grxs have been identified and isolated from various organisms such as *E. coli* (Holmgren, 1976), yeast (Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999), rice (Sha *et al.*, 1997), tomato (Guo *et al.*, 2010), bovine (Hatakeyama *et al.*, 1984) and human (Holmgren and Aslund, 1995; Lundberg *et al.*, 2001). Moreover, a large number of Grxs have been identified in various photosynthetic species based on genome comparative analysis of Grx family domain (Couturier *et al.*, 2009). Grxs were previously described to have functions in controlling plant development, DNA synthesis, signaling and stress response and [Fe-S] assembly (Rouhier *et al.*, 2004; 2007). Recently, studies have extended our knowledge on the physiological and molecular functions of Grxs in plants. The *Arabidopsis* CC type Grx GRX480 (AT1G28480) interacts with TGA2.2 and participates in Salicylic Acid

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(SA)/Jasmonic Acid (JA) cross-talk (Ndamukong *et al.*, 2007). In rice, two homologues of ROXY1, OsROXY1 and OsROXY2, were identified to complement the *Arabidopsis* roxy1 mutant phenotype. Also, over-expression of these CC type Grxs led to hyper-susceptibility to the infection of necrotrophic pathogen *Botrytis cinerea* (Wang *et al.*, 2009). Furthermore, AtGRXcp (AT3G54900), the first characterized CGFS type Grx in plants, was found to suppress the sensitivity of yeast *grx5* cells to H_2O_2 and protein oxidation and function in early seedling growth under H_2O_2 stress (Cheng *et al.*, 2006). Another *Arabidopsis* CGFS type Grx, AtGRX4 (AT3G15660), was also characterized to play important functions in plant growth and development under extreme environments (Cheng, 2008). The fern PvGRX5 was reported to increase plant tolerance to arsenic, high temperature and oxidative stresses (Sundaram *et al.*, 2007; 2009; Sundaram and Rathinasabapathi, 2010). Taken together, these studies suggest that plant Grxs have diverse functions in plant development, signal transduction and stress responses. According to Cyanobase database, the unicellular mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 possesses two Grxs encoded by the genes *slr1562* and *ssr2061*. In a previous study, we demonstrated that the over expression of the protein Grx2 encoded by *ssr2061* in *E. coli* cells showed high tolerance to NaCl compared to cells transformed with the vector alone (Gaber *et al.*, 2006). In the present study, the *slr1562* gene (GrxC) was isolated from *Synechocystis* sp. PCC 6803 and its functions were validated using gain-of-function approach by over-expression of GrxC in *E. coli*. The recombinant *E. coli* cells expressed significant increased resistance to oxidative, salt and drought stresses. Moreover, the expression pattern of the *slr1562* gene *in-vivo* was investigated by transcript analysis under different stress conditions.

MATERIALS AND METHODS

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

Bacterial strains and growth conditions: The wild-type strain of *Synechocystis* PCC 6803 was grown photoautotrophically at 27ºC in Allen's medium under a light intensity of 30 μE m^{-2} s^{-1} from fluorescent lamps. Log-phase cells of *Synechocystis* PCC 6803 (A_{730} = 0.6-1.0) were subjected to oxidative stress conditions, i.e., 2 mM H_2O_2 or 0.2 mM *t*-BuOOH and growth was continued under the same conditions. Cells were harvested at different time points (0, 30, 60 120 and 180 min). Salt stress was achieved by adding 200 mM NaCl to the medium at log-phase stage for different time periods (0, 30, 60, 120 or 180 min). In the experiments, in which the effects of low temperature and high light intensities were investigated, the cells at log-phase stage were transferred to 4 ºC under dim light intensity (30 μE m^{-2} s^{-1}) and harvested at the times given previously. The high light intensity (1200 μE m^{-2} sec^{-1}) was obtained by using white light and the cells were harvested at the same time periods as mentioned above.

Bacterial cultures of *E. coli* JM109 were stored as 25% (v/v) glycerol stocks at -80°C and maintained on Luria-Bertani (LB) plates containing 1.5% (w/v) agar. Cells harboring recombinant plasmids were grown and maintained on LB media supplemented with 50-100 μg mL^{-1} ampicillin as described by Sambrook and Russell (2001).

Northern-blot analysis: The cyanobacterial cultures were collected at different times during stress conditions as previously indicated. After centrifugation of 50 ml cell culture in 50 mL tubes with crushed ice at 3000 X*g* for 10 min, the cell sediments were immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Total RNAs (20 mg) were isolated from the cyanobacterial cells as described by Los *et al*. (1997). Total RNA (30 μL each) was subjected to electrophoresis on 1.2% (w/v) agarose gels containing 2.2M formaldehyde and transferred to a Hybond N membrane (Amersham Biosciences, NY, USA). The membrane was pre-hybridized at 55°C for 3 h in a buffer containing 6×SSC, 5×Denhardt's solution, 1% (w/v) SDS and 100 μg mL^{-1} denatured salmon sperm DNA. The membrane was probed with ^{32}P-primed DNA encoding GrxC protein at 55°C for 12 h. Blots were washed two times at room temperature in 2×SSC, 0.1% SDS for 10 min each and in 0.1×SSC, 0.1% SDS at 60°C for 1 h. The membrane was then exposed to an imaging plate and the relative expression of GrxC transcript was calculated using a Mac BAS 1000 image scanner (Fuji Photo Film, Tokyo, Japan).

Expression of *slr1562* gene in *E. coli*: The chromosomal DNA was isolated from *Synechocystis* PCC 6803 according to the method described by Williams (1988). One DNA fragment containing the open reading frame of *slr1562* was amplified by PCR. The forward primer (5'-AGGTGATCATATGGCTAATT-3') and reverse primer (5'-ACAGGGACAAATAATACCTA-3') were deduced from the nucleotide sequence of *slr1562* of *Synechocystis* PCC 6803. The forward primer was designed to introduce an *NdeI* site with an ATG codon

for the initiation of translation (bold nucleotides sequence). Amplified DNA fragment was 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 *slr1562* gene, the plasmid was digested with *NdeI* and *Bam*H1 and the resultant 330 bp DNA fragment was cloned into a pET3a vector (Novagen, Madison, NI, USA) digested with the same restriction enzymes. The resulting construct, designated pET/GpxC, was introduced into the *E. coli* strain JM109. The recombinant enzyme in *E. coli* was produced by the method described by Tamoi *et al*. (1996).

Response of *E. coli* to environmental stress conditions: For the oxidative stress experiment, overnight cultures of wild type *E. coli* JM109 cells or pET/GpxC cells were grown in fresh LB liquid medium containing 100 μg mL^{-1} ampicillin under continuous shaking condition at 37°C. When the A_{600} reached a value of 0.1, the desired concentrations of H_2O_2 (0, 20 or 30 mM) and 0.5 mM IPTG were added simultaneously. Growth was measured at different time intervals (0, 1, 2 or 3 h) at 37°C in the absence and presence of H_2O_2. For salinity and drought stress experiments, when the A_{600} reached a value of 0.6, the wild type and pET/GpxC cells were striped on LB agar plates medium containing NaCl (0 ,0.34, 0.51, 0.68 or 0.85 M) or agar (1.5, 2.5, 3 or 3.5%) and incubated overnight at 37°C.

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 4ºC. The supernatants (40 mg) were analyzed in a 15% (w/v) SDS-PAGE according to Laemmli (1970) and Li *et al.* (2010). While, the Tricine-SDS-PAGE was performed as described by Schagger and Jagow (1987).

Database search and sequence evaluation: The genome of *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996) was searched for proteins representing Grxs. The two candidate proteins of putative Grxs were verified by alignment with the protein sequence of Grxs from several organisms in ClustalW. Protein size of GrxC as number of amino acid, molecular mass and pI value was calculated utilizing the ProtParamTools of the ExPASy-Proteomics Server under www.expasy.org/tools/protparam.html.

RESULTS

Sequence analysis of *slr1562* gene: Analysis of the complete genome sequence of *Synechocystis* PCC 6803 (Kaneko *et al.*, 1996) revealed two ORFs (*slr1562* and *ssr2061*) encoding two Grx proteins (GrxC and Grx2, respectively). Overall, the ORF *slr1562* coding sequence is 330 bp long, resulting in a protein of 109 amino acids, including the initiator methionine. The theoretical molecular weight, isoelectric point (p*I*) and molar extinction coefficient of GrxC have been determined by the program Protparam tool from Expasy (http:// www.expasy.org/tools/protparam.html) and are respectively 12,222 Da, 8.52 and 26470 M cm^{-1} at 280 nm. The deduced amino acid sequence of GrxC possesses a consensus Grx family domain with a CPFC catalytic residue at the C-terminus (Fig. 1). In order to determine the relationship between GrxC and other CPFC type Grxs, multiple sequence alignment was performed based on the amino acid sequences of GrxC and related CPFC type Grxs from yeast, rice, human and *E. coli*. Amino acid sequence alignment suggested that GrxC share high consensus domains including CPFC catalytic residue with other Grxs (Fig. 1).

Fig. 1: Alignment of the predicted amino acid sequence of *S.* PCC 6803 GrxC with Grxs from *S.* PCC 6803 Grx2, *E. coli*, rice and yeast. Amino acid sequences were aligned for maximal homology. The alignment was performed with GENETYX-MAC ver. 14.0.6 software using the Clustal W method. Amino acids are given with standard single-letter designation and dashes (-) indicate no consensus. Numbers indicate protein length in amino acids. The active site, hydrophobic surface area and a GSH binding site are underlined

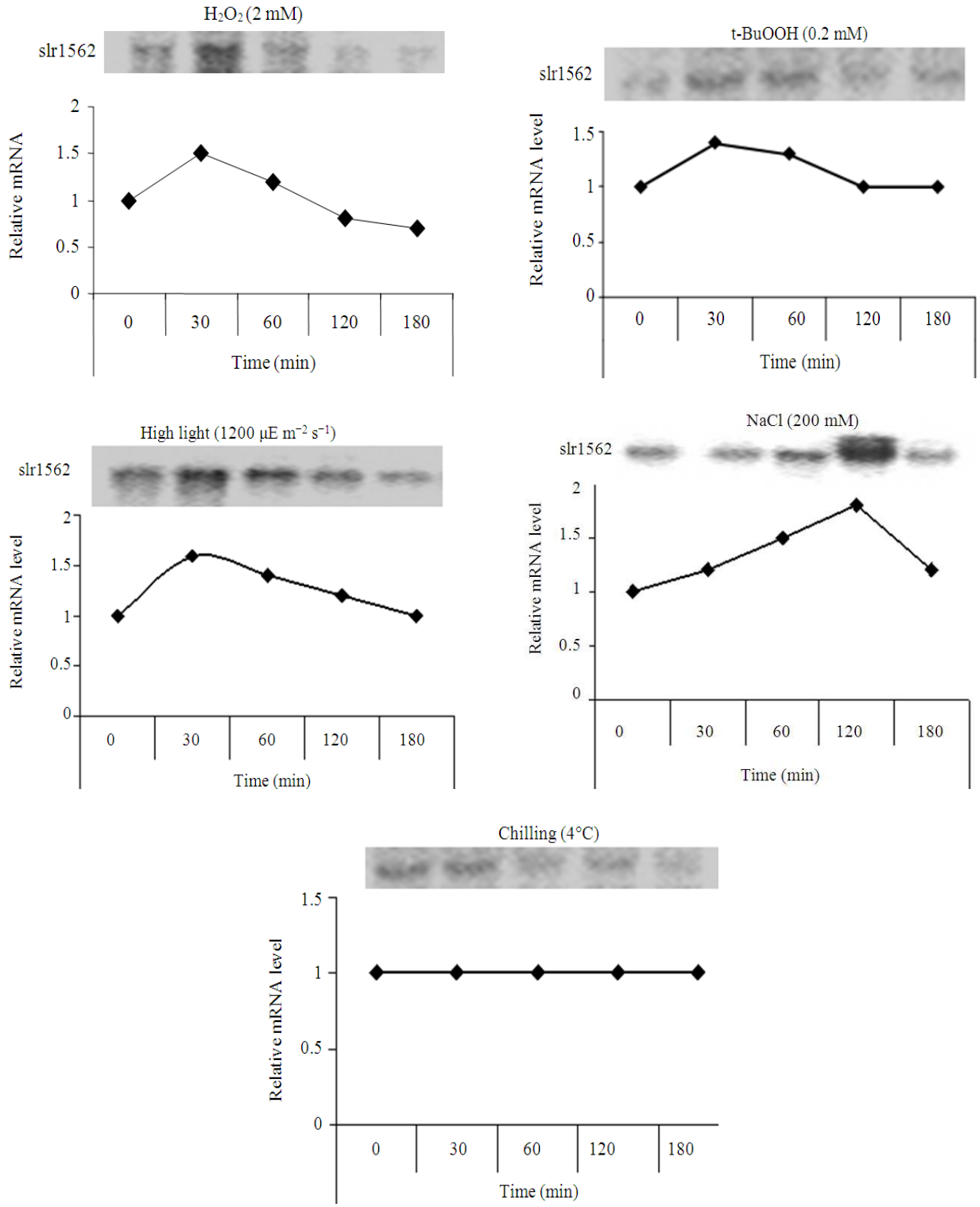


Fig. 2: The effect of various stress conditions on the transcript level of *slr1562*. Detailed conditions for experiments in Northern blot analysis are described in ‘‘Materials and Methods’’. The value of each transcript level at zero time was set to 1

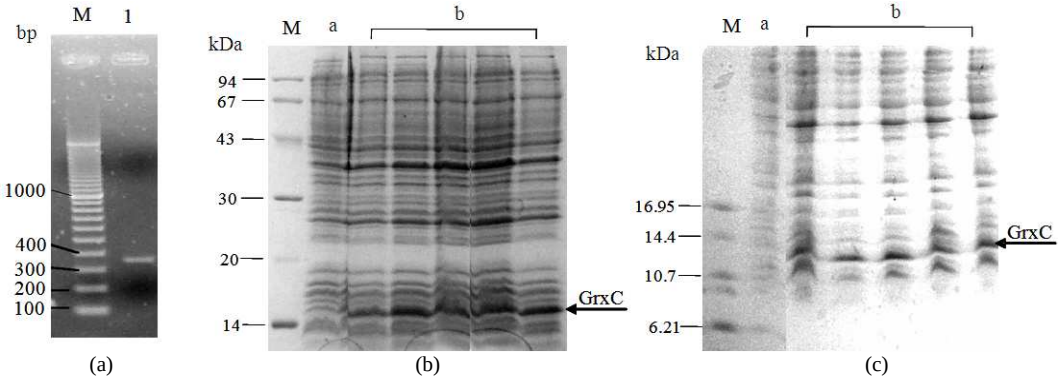


Fig. 3: Analysis of the recombinant GrxC expressed in *E. coli* cells. (A) PCR amplification of the *Synechocystis* ORF *slr1562*. Genomic DNA from *Synechocystis* sp. PCC 6803 was isolated and amplified as described in ''Materials and Methods''. Lane 1, PCR product of *slr1562* (about 330 bp); lane M, 100 bp DNA ladder. (B) SDS-PAGE analysis of proteins expressed in *E. coli* JM109 containing the pET/GrxC plasmid on IPTG induction as described in ''Materials and Methods''. Protein samples derived from 200 μL of culture were run on 15% SDS-PAGE gel and visualized by staining with Coomassie blue. (C) Trycine-SDS-PAGE of GrxC proteins expressed in *E. coli* cells. Detailed conditions for experiments are described in ''Materials and Methods''. Lane a, pellet fraction of *E.coli* wild type cells; lane b, pellet fraction of pET/GrxC transformed cells induced by IPTG; lane M, protein molecular weight markers

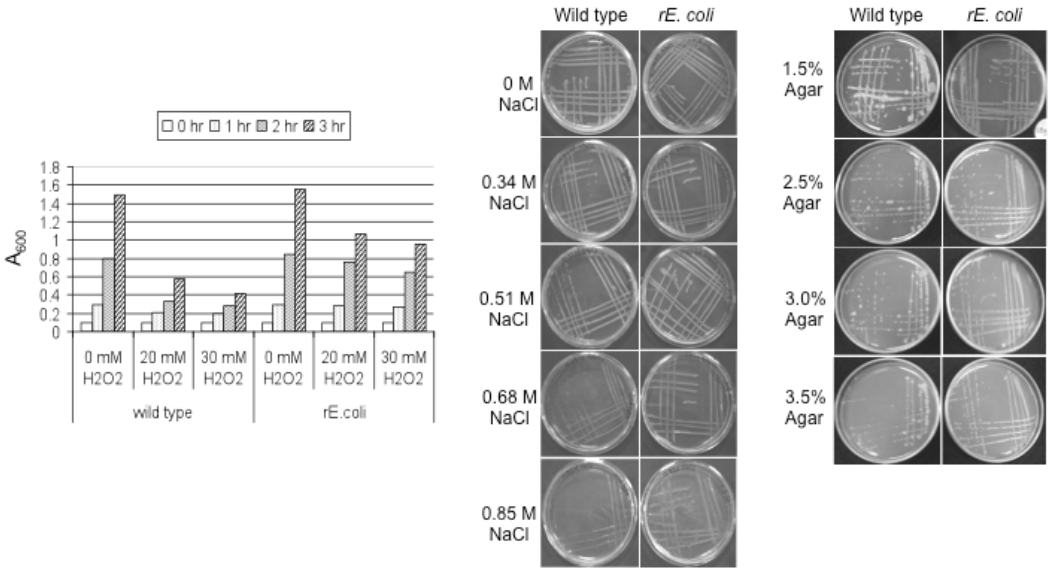
The deduced amino acid sequence of GrxC revealed 66% identity to that of Grx2. The putative protein GrxC shared 20-36% identity with the amino acid sequence of Grxs from *E. coli* (29.9%), yeast Grx1 (30%), yeast Grx2 (20%) and rice (36%) (Fig. 1).

Expression profile of *slr1562* in *Synechocystis* PCC 6803 under stress conditions: To investigate the expression pattern of *slr1562 in vivo*, the transcript level of *slr1562* was assayed by northern blot analysis under standard growth condition and under growth conditions, which induce oxidative stress. The latter include H_2O_2 and *t*-BuOOH treatments as well as growth under NaCl, chilling and high light intensity stress. The results showed that the transcript levels of *slr1562* were increased within 30 min in response to high light intensity (1200 μE m^{-2} s^{-1}) and oxidative stress caused by the addition of 2 mM H_2O_2 or 0.2 mM *t*-BuOOH followed by its decline until it reached a level comparable to that of same value as the untreated cells. In contrast, the treatment with 200 mM NaCl induced a significant up-regulation of the *slr1562* mRNA level, which was steadily increased with time until 2 h (Fig. 2). Interestingly, the level of *slr1562* transcript did not change in response to chilling stress (4°C) (Fig. 2).

Expression of the *Synechocystis slr1562* gene in *E.coli*: The 330 bp DNA fragment corresponding to the ORF *slr1562* was amplified from the genomic DNA of *Synechocystis* sp. PCC 6803 by PCR (Fig. 3A). The amplified product was cloned into pET3a expression vector at the *Nde*I-*BamH*1 site resulting in the pET/GrxC plasmid. The recombinant plasmid with the correct reading frame was confirmed by DNA sequencing. This plasmid was used to transform *E. coli* JM109 strain to characterize the expression pattern of the GrxC.

The optimum conditions for the expression of GrxC in *E . coli* were examined. After induction with IPTG, the recombinant enzyme was expressed at a high level in *E. coli* cells. SDS⁄PAGE analysis of the soluble proteins from the host cells showed that the proteins are successfully expressed using this system (Fig. 3B). The recombinant GrxC protein was found to be expressed approximately 20% of the total proteins in *E. coli* quantified by band scan. the apparent molecular mass of this fusion protein was about 14.5 kDa. This is not in good agreement with the theoretically predicted molecular mass of GrxC protein (12, 2 kDa). However, as shown in Fig. 3C, by using the Tricine-SDS-PAGE, the protein profiles corresponding to the recombinant GrxC were correlated with the molecular weight (12, 2 kDa) calculated from the deduced amino acid sequence of its clone.

Effect of recombinant GrxC on tolerance to oxidative, salt and drought stresses in *E. coli* cells: In the present study, *E. coli* was used as a model system to test whether the recombinant GrxC can protect *E. coli* against oxidative stress.



(a) (b) (c)

Fig. 4: Effect of different environmental stress conditions on the growth of recombinant pET/GrxC and *E. coli* wild type cells. (A) Growth of recombinant pET/GrxC and wild type *E. coli* cells after 3 h of incubation in the presence of 20 or 30 mM H_2O_2 started at 0.1 OD in liquid LB-medium. (B) and (C) Growth of recombinant pET/GrxC and wild type *E. coli* cells in different concentrations of NaCl and Agar in solid LB-medium, respectively. Detailed conditions for experiments are described in ''Materials and Methods''.

Such oxidative stress was produced *in vitro* by H_2O_2, a known oxidative stress inducer. For this purpose, the growth of recombinant *E. coli* cells was compared to the growth of the wild type in the presence of H_2O_2. The growth rate of the pET/GrxC transformed *E. coli* cells showed increased resistance, approximately 2 and 2.5 fold, higher than that of the wild type cells within 2 and 3 h of the investigation in the presence of 20 mM or 30 mM of H_2O_2, respectively (Fig. 4A). Next, in order to find out the protective efficiency of GrxC on cell growth under salt stress, *E. coli* wild type and recombinant cells were grown under various concentrations of NaCl (Fig. 4B). Interestingly, the growth of wild type cells was abolished in the presence of 0.68 M and 0. 85 M NaCl (Fig. 4B). On the other hand, the recombinant pET/GrxC could rescue the growth of the *E. coli* strain JM109 on the plate supplemented with same comcentration of NaCl. The growth was somehow better than those of the wild type *E. coli* cells (Fig. 4B).

Additionally, the requirement of *slr1562* gene for the survival of recombinant *E. coli* cells under drought stress was examined. In this experiment, the effect of drought stress was analyzed by using different concentrations of solid agar medium (1.5, 2.5, 3 and 3.5 %). As expected, pET/GrxC over expressing cells grew better than the wild type cells in the presence of 3 and 3.5% LB solid agar medium (Fig. 3C). All these results clearly indicate that the over-expression of GrxC in *E.coli* cells can improve their tolerance to oxidative, salt and drought stresses.

DISCUSSION

Glutaredoxin was originally identified as an electron donor for ribonucleotide reductase (Holmgren, 1979) and this remains its best characterized function. Nevertheless, not all glutaredoxins serve as hydrogen donors for ribonucleotide reductase, as shown for glutaredoxins from rabbit bone marrow, pig liver, *E. coli* and SlGRX1 from tomato (Hopper *et al.*, 1989; Vlamis-Gardikas *et al.*, 1997; Guo *et al.*, 2010). This study describes the cloning and sequence information of *S.* PCC 6803 *slr1562* and presents findings on the characterization of recombinant GrxC. As reported in other Grxs family (Xia *et al.*, 1992; Holmgren and Aslund, 1995) three conserved regions have been identified in GrxC that contain the active site (CxxC or CxxS), hydrophobic surface area and a GSH or ribonucleotide reductase binding site (underlined, Fig. 1). However, the amino acid residues between the active cysteine residues, which are highly conserved in known *E. coli*, yeast and mammalian Grx, were different in GrxC, where the Tyr residue was replaced by Phe

residue. The same difference at active site has also been shown in rice Grx and *Synechocystis* Grx2 (Sha *et al*., 1992). Data base searches showed that Grx2 was weakly homologous to sequences of Grx from *E. coli* (29.9%), yeast Grx1 (30%), yeast Grx2 (20%) and rice (36%) (Fig. 1). The significance of this may be due to the change of the secondary structure of GrxC with the other Grxs as described previously by Vlamis-Gardikas *et al.* (1997).

A large number of Grxs have been found to be functional in plant development and play an important role in plant responses to environmental stress especially oxidative, drought and salt stresses (Guo *et al.*, 2010; Cheng *et al.*, 2006; Cheng, 2008; Xing and Zachgo, 2008). In this present study, *Synechocystis slr1562* shown to be required *in vivo* for protection against ROS. GrxC was found to function in protection against H_2O_2, t-BuOOH, high light and salinity but not under chilling stress. In a contrast, in a previous work the transcript level of *ssr2061* (Grx2*) in vivo* started to increase after 1 h from the chilling stress condition and steadily increased with time till 3 h (Gaber *et al.*, 2006). These data suggested that *slr1562* and *ssr2061* may have some different functions, at least in the case of chilling stress and *ssr2061* may be more important than *slr1562* for protection against this stress. These results are in agreement with the results reported by Guo *et al*. (2010), which demonstrated that the tomato glutaredoxin gene, SlGRX1, plays an important role in plant responses to oxidative, drought and salt stresses. Thus, similarly to the suggested function of Grx in plants, it may be hypothesized that the cyanobacterial GrxC is involved in protecting cells from oxidative damage, particularly under stress (Guo *et al*., 2010).

The apparent molecular mass of GRxC fusion protein was about 14.5 kDa (Fig. 3B). This is not in good agreement with the theoretically predicted molecular mass of GrxC protein (12, 2 kDa). It has been reported that the high concentration (15 %) acrylamide Laemmli gels cannot be used to access the small protein range, because the stacking limit in the Laemmli system is too high and small proteins usually appear in different molecular size. Therefore, the molecular weight of GrxC protein was confirmed by carrying out Tricine-SDS-PAGE, which is a useful technique for the low molecular weight proteins (Schagger and Jagow, 1987).

The requirement for glutaredoxins in protection against ROS may reflect a specific role in the regulation of a cellular antioxidant(s), or a more general role in protection against oxidants as a result of their disulfide oxidoreductase activity. In a previous study, we showed that *Synechocystis* Grx2 had a specific role in salinity tolerance (Gaber *et al*., 2006). Additionally, in the present research, we found that GrxC-expressing recombinant *E. coli* had greater tolerance to oxidative, salt and drought stresses than the *E. coli* wild type cells (Fig. 4). It has been proposed that some Grx enzymes participate in protection against oxidative stress (Cotgreave and Gerdes, 1998; Luikenhuis *et al.*, 1998; Rodriguez-Manzaneque *et al.*, 1999; Guo *et al.*, 2010). It may be worth to know that both the wild type and the pET/GrxC transformed strains had endogenous Grx genes in their chromosomes. However, the normal level of Grx was not sufficient to protect the wild type cells against H_2O_2 mediated toxicity and the increased level of tolerance observed in the IPTG-induced *E. coli* cells transformed with pET/GrxC might be due to the over expressed GrxC protein. These results suggest that a GrxC protein from the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803, a heterologous source, can confer oxidative stress tolerance to the non photosynthetic *E. coli*.

CONCLUSION

The present research work describes cloning of the CPFC type Grx gene *slr1562* of *Synechocystis* sp. PCC 6803, over expression of the protein in the cytoplasm of *E. coli* and its functions in *Synechocystis* cells were confirmed by Northern blot studies. The results indicate that *Synechocystis* GrxC confers oxidative, salt and drought stress tolerance to *E. coli*, thus, confirming absence of species barrier in terms of the Grx functioning. To my knowledge, this is the first research showing that the *Synechocystis* GrxC plays a crucial role in oxidative, salt and drought stresses and thus, provide useful information for genetic engineering of plant crops tolerant to abiotic stress.

ACKNOWLEDGMENT

The researchers would like to express their thanks and appreciation to Prof. Dr. Shigeru Shigeoka, Department of Advanced Bioscience, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara, Japan, for providing the pET/GrxC construct.

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