Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 326 (2005) 618–623
BBRC
www.elsevier.com/locate/ybbrc

Overexpression of a eukaryotic glutathione reductase gene from *Brassica campestris* improved resistance to oxidative stress in *Escherichia coli*

Ho-Sung Yoon[a], In-Ae Lee[a], Hyoshin Lee[b], Byung-Hyun Lee[c], Jinki Jo[d,*]

[a] *Institute of Agricultural Science and Technology, Kyungpook National University, Daegu 702-701, Republic of Korea*
[b] *Biotechnology Division, Korea Forest Research Institute, Suwon 441-350, Republic of Korea*
[c] *Division of Applied Life Science, Department of Dairy Science, Gyeongsang National University, Chinju 660-701, Republic of Korea*
[d] *Department of Animal Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea*

Received 12 November 2004
Available online 7 December 2004

Abstract

Glutathione reductase (GR) plays an essential role in a cell's defense against reactive oxygen metabolites by sustaining the reduced status of an important antioxidant glutathione. We constructed a recombinant plasmid based on the expression vector pET-18a that overexpresses a eukaryotic GR from *Brassica campestris* (BcGR) in *Escherichia coli*. For comparative analyses, *E. coli* GR (EcGR) was also subcloned in the same manner. The transformed *E. coli* with the recombinant constructs accumulated a high level of GR transcripts upon IPTG induction. Also, Western blot analysis showed overproduction of the BcGR protein in a soluble fraction of the transformed *E. coli* extract. When treated with oxidative stress generating reagents such as paraquat, salicylic acid, and cadmium, the BcGR overproducing *E. coli* exhibited a higher level of growth and survival rate than the control *E. coli* strain, but it was not as high as the *E. coli* strain transformed with the inducible EcGR. The translated amino acid sequences of BcGR and EcGR share 37.3% identity but all the functionally known important residues are conserved. It appears that eukaryotic BcGR functions in a prokaryotic system by providing protection against oxidative damages in *E. coli*.

Keywords: Glutathione reductase; *Brassica campestris*; Oxidative stress; Overexpression of antioxidant genes

Glutathione is a thiol-containing tripeptide (γ-L-glutamyl-L-cysteinyl glycine) found in a wide range of organisms from the bacterial, animal, and plant kingdoms [1,2]. Glutathione plays multiple functions in various cellular processes including sulfur transport, protein disulfide reduction, and gene regulation in response to pathogen attacks and to environmental stress. One of the major roles of glutathione is to serve as an antioxidant by donating reducing equivalents to ascorbate in the reactive oxygen scavenging system known as the ascorbate–glutathione cycle. Harmful reactive oxygen species (ROS) such as the superoxide anion radical (O_2^-) and the hydroxyl radical (OH^-) are generated from regular biochemical and physiological reactions in the cell but are detoxified by cellular defenses including the ascorbate–glutathione cycle. The overproduction of ROS by various types of environmental stress, however, may exceed the scavenging capacity of the antioxidative systems resulting in detrimental oxidative damages to the cellular components.

To fulfill its role in the ROS detoxification, glutathione must be in a reduced form (GSH). Glutathione reductase (GR; EC 1.6.4.2) is a flavoprotein oxidoreductase which catalyzes the conversion of oxidized glutathione (GSSG) to GSH using NAD(P)H as an electron

* Corresponding author. Fax: +82 53 950 6750.
E-mail address: jkjo@kyungpook.ac.kr (J. Jo).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.11.095

donor [3]. GR thus plays a key role in sustaining the antioxidant capacity of the cells by maintaining high GSH/GSSG ratios in the cell [4,5]. Many organisms subjected to a variety of oxidative stresses respond by increasing the expression and activity of GR and other enzymes of antioxidant metabolism [6,7]. The GR protein has been purified and characterized from a large number of organisms including bacteria, fungi, plants, and human. The crystal structures of the GR proteins from *Escherichia coli* and human have been determined [8,9]. The genes and cDNAs encoding GR have been isolated from many organisms including *E. coli*, *Pseudomonas aeruginosa*, pea, *Arabidopsis thaliana*, mouse, and human [4]. The results of these studies show remarkable similarity in molecular and kinetic properties of the GR from various organisms, suggesting high evolutionary conservation and the significance of its role in cell functions.

Transgenic plants that overexpress the GR genes of *E. coli* and plants were shown to be more resistant to oxidative stress than the control plants ([7,10] and references therein). Recently, we have cloned and characterized the GR gene from *Brassica campestris* (BcGR) [6]. The BcGR mRNA level increased upon the introduction of oxidative stress such as ozone or paraquat treatment. In this paper, we report the improved tolerance of *E. coli* to oxidative stress when transformed with an IPTG inducible T7 promoter controlling the BcGR overexpression. The deduced amino acid sequence of the BcGR shares 37.3% identity with EcGR but all known essential residues for its function are conserved.

Materials and methods

Construction of the overexpressing recombinant plasmids. The BcGR cDNA was cloned by screening the *B. campestris* cDNA library and the phase clones were converted to the pBluescript II SK(−) plasmid [6]. The BcGR cDNA was amplified by PCR using the forward primer BcGR-F-Nco (5′-GAT TCT GCA CAA CCA TGG CGA GGA AGA TGC-3′) and the reverse primer BcGR-R-Bam (5′-GAG TGT GAA TTG GAT CCA AAG ATA ATG C-3′). The restriction enzyme *Nco*I recognition sites were engineered in by substituting one nucleotide in the forward primer, and the *Bam*HI sites were engineered in by substituting two nucleotides in the reverse primer. *Pfu* DNA polymerase (Stratagene, USA) was used instead of the regular *Taq* DNA polymerase to achieve higher fidelity during the PCR amplification of the BcGR cDNA. The amplification reaction mixture (25 μl) contained 0.5 U *Pfu* DNA polymerase, 0.1 mM dNTP mixture, and 0.2 mM of each primer in a 10× manufacturer's PCR buffer. The reaction mixture was incubated at 94 °C for 2 min to denature the DNA and was subjected to 35 cycles of PCR with the following conditions: predenaturation at 94 °C for 30 s, annealing at 54 °C for 1 min, polymerization at 72 °C for 1 min, and final incubation for extension at 72 °C for 10 min followed by 4 °C hold until analysis. The PCR products were electrophoresed, excised, purified, and then digested with the restriction enzymes *Nco*I and *Bam*HI. The resulting fragment was ligated into the same sites of the expression vector pET-28a (Fig. 1A). The nucleotide sequence of the insert region was determined to confirm the correct reading frame of the BcGR cDNA from the T7 promoter of pET-28a. The DNA sequencing also verified the absence of any nucleotide substitutions in the inserts that could alter the amino acid sequence during the translation of the cloned gene.

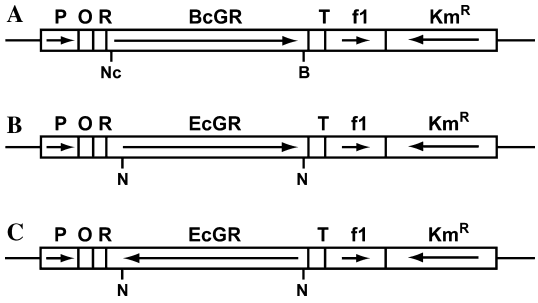

Fig. 1. Schematic diagram of the gene constructs used to overexpress in *E. coli* cells. Parts of the recombinant plasmids containing (A) the BcGR cDNA, (B) the EcGR gene in sense orientation, or (C) the EcGR gene in antisense orientation are shown. Restriction sites used to fuse GR genes to the T7 promoter were depicted under the genes as Nc (*Nco*I), B (*Bam*HI), and N (*Not*I). Arrows indicate the direction of transcription or the orientation of the genes. P, T7 promoter; O, *lac* operator; R, ribosomal binding site; T, T7 terminator; f1; f1 origin of viral replication; and Km^R, kanamycin resistant gene.

The EcGR gene was cloned by PCR amplification of the *E. coli* genomic DNA using a forward primer EGR-S (5′-GGA GTA ATT GCA GCC ATT GC-3′) and a reverse primer EGR-AS2 (5′-GCT TCT GAA CTG ATA GCG GA-3′). The amplified fragments were purified and cloned into the pGEM-T Easy Vector (Promega). The restriction enzyme, *Not*I, that flanks the insert was used to excise the cloned DNA fragments and the subsequent cloning of the fragments into the *Not*I digested and dephosphorylated pET-28a vector was carried out (Figs. 1B and C). The DNA sequencing from the vector primers identified the insertion of the EcGR gene in sense and antisense orientations with reference to the T7 promoter of pET-28a. Also the DNA sequencing confirmed the absence of substituted nucleotides and the correct reading frame of the EcGR gene of the sense orientated clones. All recombinant constructs were introduced into the *E. coli* strain BL21 by the method of Sambrook et al. [11].

Preparation of RNA and Northern blot analysis. The transformed *E. coli* cells were cultured in a 50 ml Luria–Bertani (LB) broth with vigorous shaking at 37 °C. Overexpression was induced with 0.4 mM IPTG at a cell density of 0.6 at A_{660}. The cells were left to grow for 5 h and then harvested by centrifugation. The total RNA was extracted according to the method developed by Chomczynski and Sacchi [12]. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and the concentration and purity of the samples were assessed spectrophotometrically at 260 and 280 nm. Of the extracted RNA, 50 μg was subjected to DNase digestion followed by an extraction of RNA with the following ratio of phenol:chloroform:isoamyl alcohol being 25:24:1. Sodium acetate and isopropanol were used to precipitate the RNA. The resulting RNA pellets were dissolved in 25 μl DEPC-treated water and the concentration was determined as above. Agarose gel electrophoresis was used to check the integrity and purity of the isolated RNA. Fifteen micrograms of RNA samples was denatured, separated by electrophoresis, and transferred to a Hybond-N nylon membrane (Amersham). The blot was then hybridized as previously described [6].

Overexpression, SDS–PAGE, and Western blot analysis. The *E. coli* strains containing the recombinant constructs were induced as described above and the cells were collected by centrifugation. The pellets were washed in an ice-cold PBS buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 10 mM imidazole), resuspended in the same buffer, and disrupted by sonication. The crude extract obtained after centrifugation was purified according to the product manual. Cell