Korean J. Medicinal Crop Sci. 10(4) : 294-297(2002)

Cloning and expression of glutathione S-transferase (GST) cDNA from *Gossypium hirsutum* L.

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ABSTRACT : **A gene coding for the GST of cotton (Gh-5) was cloned into *Escherichia coli* and experssed. The enzyme remained within the cytoplasm of *E. coli*. An 696 bp open reading frame was in the 988 base pair fragment of the recombinant plasmid pET-30b(+). The deduced protein sequence consists of 232 amino acids and has a molecular mass of 30235.58 Da. The cloned enzyme conjugated reduced glutathione and 1-chloro-2,4-dinitrobenzene (CDNB). Plant GST cDNA was expressed in microbe and produced polypeptide had function as an enzyme.**

Key words : *Gossypium hirsutum* L., Glutathione S-transferases (GST), CDNB, Detoxification.

INTRODUCTION

Glutathione S-transferases (GST : EC 2.5.1.18) are a super family of enzymes that conjugate reduced glutathione to a wide variety of compounds that are lipophilic and have an electrophilic center (Coles and Ketterer, 1990; Mannervik and Danielson, 1988; Pickett and Lu, 1989). This reaction yields a GSH conjugate that is often inactive, water-soluble, and is usually less toxic than the parent compound (Droog et al., 1993; Itzhaki and Woodson, 1993). In vacuoles, glutathione conjugates can be metabolized to other intermediates by several peptidases (Lamoureux and Rusness, 1989). The inactive, water-soluble conjugates of both synthetic and natural chemicals in the cytosol can be secreted into the apoplast via an exocytosis. They may become associated with cell wall components such as pectin, hemicellulose, or lignins as an insoluble conjugate termed a "bound residue" (Lamoureux and Rusness, 1989; Sandermann, 1994).

Recently, a group of GSTs have been reported to be associated with various stress responses in plants including pathogen attack (Dudler et al., 1991) and heavy metal toxicity (Hagen et al., 1988). They are also involved in the synthesis of secondary products such as anthocyanins and cinnamic acid (Marrs et al., 1995). GSTs have been implicated in protection from oxidative damage (Bartling et al., 1993). Plants are under almost constant attack by pathogenic bacteria, virus, fungi, and feeding by insects, nematodes and other animals. Reactive oxygen species (ROS) can be induced by environmental stress, such as, high light intensity, heavy metal, ozone, pathogen attack, wounding (Allen, 1995; Tenhaken et al., 1995). Plant GSTs protect plants from oxidative damage triggered by ROS to lipid bilayers, DNA and proteins (Sandermann, 1994). In this paper, we describe the molecular cloning, nucleotide sequence of GST gene from *Gossypium hirsutum* L. (cv. Coker 312), its expression in microbe.

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Received 10 October, 2002 / Accepted 30 October, 2002

MATERIALS AND METHODS

cDNA synthesis and amplification by PCR

Fiber cDNA library from *Gossypium hirsutum* L. (cv. Coker 312) was used as a source of gst gene cloning. Both the cDNA library vector primer (T7) and 3′ oligonucleotide primer from the partial cDNA fragment were synthesized and used to amplify the putative GST cDNA by PCR-based cDNA library screening. For Gh-5 cloning, the 5′ primer, 5′-ATTATGCTGAGTGATATCCCGCT-3′, was designed and the 3′ primer had the sequence 5′-TGGTCAA GAGCCAAGAAATA-3′. Using a fiber specific cDNA library (Fig. 1), PCR-based cDNA library screening was performed. PCR product was ligated into PCR 2.1 vector. Transformations were carried out using DH5(competent cells (Invitrogen, San Diego, CA). Amplified cDNA fragments were purified and used in further analyses, such as sequencing, Northern blotting, and developing gene constructs for the expression in E coli and in transgenic plants.

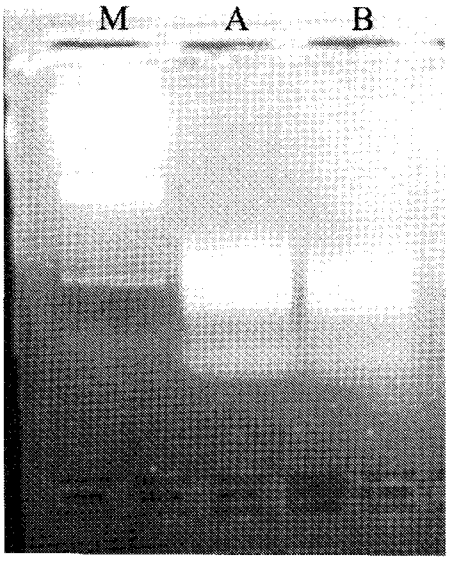

Fig. 1. Agarose gel elctrophoresis analysis of fiber cDNA library. M is lambda DNA marker digested with Hind III. Lanes A and B are amplified cotton cDNA libraries with T7 and T3 primers to analyze the size of inserts in cloning vector. One of these cDNA libraries was used to clone full length GST cDNA.

DNA sequence analysis

The nucleotide sequence of the GST gene was determined by the dideoxynucleotide-chain-termnation method using the Sequenase II kit (USB) according to the manufacturer's specifications. The DNA and deduced amino acid sequence were analyzed using the Prosite program (PC/GENE) and PSORT program (http://psort.nibb.ac.jp).

Nucleotide sequence accession number

The sequence of GST reported in this paper has been assigned accession number AF159229 in the GenBank database. The base thymine at 165 should be omitted, and as a result the start codon begins at 160.

Construction, expression, and assay of cotton GST cDNA

The Gh-5 cDNA fragment was isolated from the pCR 2.1 vector (Invitrogen, San Diego, CA) by digesting with Nco I/Bam HI and introduced into the Nco I/Bam HI sites of the expression vector pET30-b (+) (Novagen, Madison, WI) (Fig. 2) to produce a recombinant plasmid named pET-Gh-5. Subsequently, *E. coli* strain BL21DE3 was transformed with pET-Gh-5. Cells were induced in 10 ml liquid LB media to express the Gh-5 protein after 4 hours of incubation (OD_{600}=0.6) by the addition of IPTG to a final

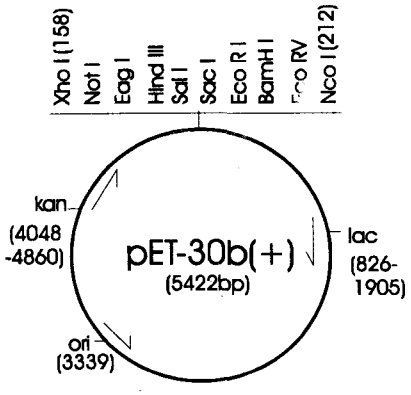

Fig. 2. A schematic diagram of the *E. coli* expression vector, pET-30b(+). Cotton GST cDNA was inserted in the sites of Nco I and Bam I. Symbols : kan, kanamycine resistance; Ori, replication origin; lac, lac promoter;

concentration of 1 mM and incubated for another four hours. The culture was centrifuged and analyzed by 10% SDS polyacrylamide gel electrophoresis (SDS/PAGE) (Fig. 4). Determination of protein concentration was determined by Bradford method (1976). The assay for GST in *E. coli* cells was carried out according to the procedure of Takahashi and Nagata (1992) with some modification of reaction temperature.

RESULTS AND DISCUSSION

Nucleotide sequence analysis of the GST cDNA

The complete nucleotide sequence of GST cDNA was determined. The 988 bp nucleotide sequence is shown in the GenBank database (AF159229). The open reading frame started with ATG codon at the position 160 and terminated with TAG stop codon at the position 856, encoding a protein of 232 amino acids. Plant GSTs are generally cytosolic and are present as homo- or hetero-dimers with a subunit molecular mass of 25-30 kDa (Marrs, 1996; Droog, 1997). Gh-5 has a molecular mass of about 31 kDa according to the SDS/PAGE analysis and calculation of 232 amino acids (Fig. 3). The theoretical molecular mass of GST is 30235.58 Da calculated by adding all amino acids in GST, and this size is well matched to the general size of plant GSTs.

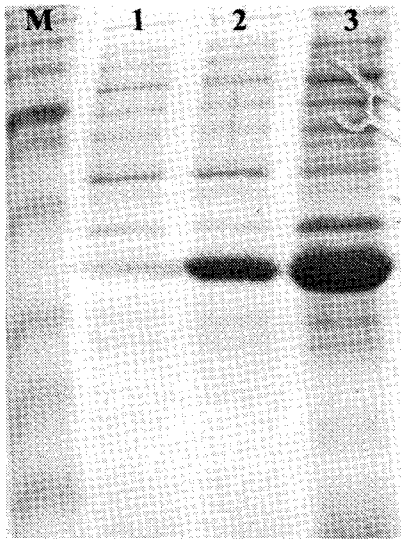

Fig. 3. SDS-PAGE analysis of the GST produced by *E. coli* DH-5-*α* The *E. coli* cells were grown for 16 h at 37℃ in LB medium. Whole cell extracts or culture supernatants were appropriately concentrated and applied to 10% (w/v) polyacrylamide gel. Lanes 1-2, Coomassie Blue stained gel. Cell extracts of *E. coli* DH-5 -*α* are shown in lanes 2 and 3. Cell extracts of *E. coli* DH-5-*α* are shown in lanes 2 and 3, and its supernatant in lane 1, as a control. Lane 2 supernatants were taken 1 hr later after adding inducer, ITPG. Lane 3 is 4 hrs later. M is molecular weight markers (bottom to top; 99, 69, 57, 43, 29, 23 and 18 kDa). The migration position of GST is indicated as about 31 kDa.

Gh-5 expresison in *E. coli* and GST Assay

Gh-5 was inserted into the expression vector, pET30-b(+), and expressed in BL21DE3 host *E. coli*. Bacterial cultures with 10 mM IPTG yielded functional GST. SDS/PAGE of recombinant cotton GST showed a strong dominant band with an apparent molecular mass of approximately 32 kDa, which was not found in the supernatant from non-induced (Fig. 3). Cell extracts of *E. coli* DH5(are shown in lanes 2 and 3. Cell extracts of *E. coli* DH5(are shown in lanes 2 and 3, and its supernatant in lane 1, as a control. Lane 2 supernatants were taken 1 hr later after adding inducer, ITPG. Lane 3 samples were taken 4 hrs later. The size of the band did not agree with the calculated value for the Gh-5 polypeptide. The expressed protein was found most abundantly in the supernatant fraction of the *E. coli* extracts (Data not shown). So the protein is likely to be in soluble form. Results of the k-NN prediction from PSORT are that cotton GST is cytoplasmic protein (65.2%) rather than nuclear (21.7%), vesicles of secretory system (8.7%), or mitochondrial protein (4.3%).

GST activity assay

When CDNB was used as substrate, this putative cotton GST (pET-Gh-5) showed significant GST activity (Figure 4). The total GST specific activity of the *E. coli* transformant was approximately 40 times higher than that of the non-induced control. The low level of GST activity of the control is considered to spontaneous conjugation of glutathione and CDNB.

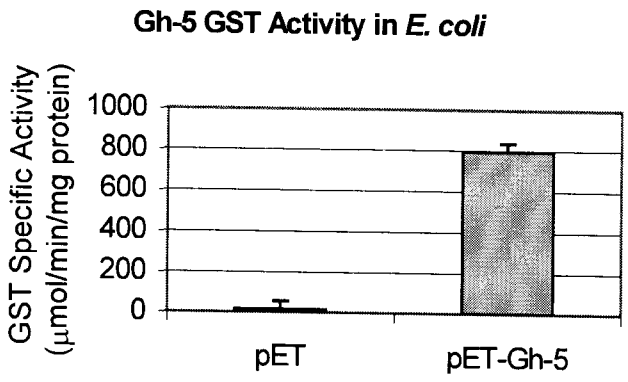

Fig. 4. GST specific activity of protein extracts from *E. coli* cells containing pET-Gh-5 gene construct using CDNB as substrate. Producton of GST by control and *E. coli* Dh-5-αtransformants after four hours growth. The two strains were grown aerobically in LB medium at 37℃. Whole cell extracts were prepared and GST specific activities of them were assayed.

ACKNOWLEDGEMENTS

This work was supported as part of a grant from Rural Development Administration, Korea(BioGreen 21 Project)

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