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Expression of Two Glutathione *S*-Transferase Genes in the Yeast *Issatchenkia orientalis* Is Induced by *o*-Dinitrobenzene during Cell Growth Arrest

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Glutathione *S*-transferases (GSTs) Y-1 and Y-2 from the yeast *Issatchenkia orientalis* were purified by passage through a glutathione-agarose column, and the cDNA for GST Y-1 was cloned and sequenced. The deduced amino acid sequence consisted of 188 residues with a total calculated molecular mass of 21,001 Da and showed 36.7% identity to that of GST Y-2, another GST isoenzyme expressed in this strain. *Escherichia coli* DH5α transformed with pUC119 harboring the GST Y-1 gene under the control of the *lac* promoter exhibited 29-fold-higher GST activity than the same strain with pUC119. Northern blot analysis revealed that both genes were highly expressed in cells cultured in the presence of 200 μM *o*-dinitrobenzene (DNB), one of the substrates of GST, while only the GST Y-1 gene was expressed, and only slightly, under normal (DNB-free) culture conditions. The DNB in the medium arrested cell growth until it was reduced by conjugation with reduced glutathione. Kinetic analysis of GST gene expression during detoxification of DNB revealed that the levels of expression of both genes were elevated within 3 h after the addition of DNB and that they further increased until 12 h postaddition. The levels of expression of both genes were decreased markedly when the DNB concentration in the culture medium was lowered. These results suggest that *I. orientalis* cells sense xenobiotics and arrest cell growth as a mechanism for preventing the induction of mutations by these compounds, while the levels of expression of the GST genes are up-regulated for detoxification.

Glutathione *S*-transferase (GST; EC 2.5.1.18) catalyzes the conjugation of reduced glutathione (GSH) to a wide variety of electrophilic xenobiotics. Based on sequence similarity and substrate specificity, the cytosolic GSTs comprise a gene superfamily that includes six subclasses: alpha, mu, pi, theta, kappa, and zeta (2, 6, 9, 10, 16). GST-alpha, -mu, and -pi are abundant in human, rat, and mouse tissues and are thought to play a major role in the detoxification of electrophilic xenobiotics, including carcinogens. Some GST species were shown to be highly induced in tumor cells. High levels of GST-pi gene expression were frequently found in cell lines which became resistant to anticancer drugs, although high levels of GST-alpha and -mu expression were also often observed. The mechanism of induction of GST has also been studied in mammalian cell lines, and several *cis*-acting elements have been detected in the 5′-flanking regions of various GST genes, including the antioxidant-responsive element (ARE) (19), the 12-*O*-tetradecanoyl phorbol-13-acetate-responsive element (14), the xenobiotic-responsive element (XRE) (18), and the GST-P enhancer element (20). Thus, the relationship of GSTs to drug resistance in tumor cells has been extensively studied in mammals.

GSTs have also been found in plants (11, 21) and insects (27), and these enzymes were shown to be related to resistance to herbicides and pesticides, respectively. GST genes have been isolated from the prokaryotes *Proteus mirabilis* (17), *Methylophilus* sp. strain DM11 (1), and *Escherichia coli* (13). In the yeast *Saccharomyces cerevisiae*, the gene encoding the regulatory protein Ure2p was shown to have sequence similarity to the theta-class GSTs of maize and *Drosophila melanogaster* (15); however, no GST activity was detected in Ure2p. Recently, genes encoding two novel membrane-bound GSTs, *GTT1* and *GTT2*, were identified in *S. cerevisiae* (4). However, it is still not clear whether soluble GST plays a major role in drug resistance in yeasts.

We have found GST activity in various yeast strains (8) and have purified and characterized two GST isoenzymes, GST Y-1 and Y-2, from the yeast *Issatchenkia orientalis* (26). Also, cloning of the GST Y-2 gene and its expression in *E. coli* were carried out (24, 25). Conjugation of *o*-dinitrobenzene (DNB) to glutathione and metabolism of glutathione conjugate in the yeast *I. orientalis* were studied, and the mechanism of detoxification of this xenobiotic in *I. orientalis* was determined (23). Other investigators reported that overexpression of the GST Y-2 gene in *S. cerevisiae* led to an increase in resistance to DNB, and GST Y-2 was shown to be involved in detoxification of DNB (31). Here, we report the isolation and nucleotide sequence of the GST Y-1 gene from the yeast *I. orientalis*. Also, the expression levels of the GST Y-1 and Y-2 genes in response to the addition of DNB, an electrophilic xenobiotic, were examined.

Purification of GSTs Y-1 and Y-2 by affinity chromatography. Previously, we reported the purification and properties of GSTs Y-1 and Y-2 (26) and the cloning and sequencing of GST Y-2. However, the GST Y-1 gene has not yet been cloned because of the low yield of this protein. Since GST Y-1 showed 10-fold-higher specific activity than Y-2 and has been suggested to play a major role in detoxification in cells, it was deemed important to clone this gene. We have developed methods for the purification of GSTs Y-1 and Y-2. Purification of GSTs Y-1 and Y-2 from *I. orientalis* was performed by two-step column chromatography, using DEAE-cellulose and glutathione-agarose (Table 1). Cell growth conditions, cell extract preparation, protamine treatment, DEAE-cellulose column chromatography, and GST and protein assays were done

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TABLE 1. Purification of GST Y-1 and Y-2

Fraction	Amt of protein obtained (mg)	Total activity (mU)	Sp act (mU/mg of protein)	Purification (fold)	Yield (%)
Protamine	1,000	9,700	9.7		100
DEAE-cellulose	300	8,310	27.7	2.8	85.7
GSH-agarose	0.57	1,230	2,160	223	12.7

as previously described (26). The active fractions from the DEAE-cellulose column were applied to a glutathione-agarose (Sigma Chemical Co., catalog no. G4510) column (2 by 10 cm) which was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 10 mM sodium sulfite, and 20% glycerol (stabilizing buffer). The column was washed with 4 volumes of the same buffer after sample application. GST activity was eluted with four volumes of the same buffer supplemented with 5 mM GSH and 0.5 M NaCl. There are two different types of glutathione-agarose available from commercial sources; in one type (Sigma Chemical Co.; catalog no. G4510), GSH is attached to the agarose by a sulfur moiety, while in the other (Sigma Chemical Co.; catalog no. G9761) it is attached via the α-amino group of the glutamyl residue. GST activity bound only to the former type of glutathione-agarose (Sigma catalog no. G4510) (data not shown), indicating that the α-amino group of the glutamyl residue is necessary for recognition of glutathione as a substrate by GSTs Y-1 and Y-2.

The active fraction from the GSH-agarose column showed two bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, with molecular masses of 23 and 21 kDa (data not shown), which corresponded with the sizes of GST Y-2 and GST Y-1, respectively. Both proteins were subjected to protein sequencing without further purification.

Protein sequence analysis and synthesis of an oligonucleotide probe. Proteins in the eluted fractions from the GSH-agarose column were separated by SDS-PAGE and then electroblotted onto Immobilon-PSQ polyvinylidene difluoride membranes (Millipore Co.). Two protein bands that stained with Ponceau S were cut out and applied to an automated protein sequencer. The protein with a mass of 23 kDa gave the sequence N-Thr-Phe-Ala-Thr-Val-Tyr-Ile-Lys-C, which was identical to the N-terminal sequence of GST Y-2 (25). The other protein, with a mass of 21 kDa, gave the sequence N-Thr-Phe-Gly-Thr-Leu-Tyr-Ile-Leu-Pro-Pro-C and was thought to be GST Y-1. To determine more of the sequence of the 21-kDa protein, the active fraction was subjected to SDS-PAGE and the 21-kDa protein band, which stained with Coomassie blue, was cut out and electroeluted. The isolated 21-kDa protein was subjected to treatment with lysyl endopeptidase and then purified by high-performance liquid chromatography. Two purified lysyl endopeptidase-produced fragments gave the sequences N-Trp-Leu-Ser-Phe-Ala-Asn-Ser-Asp-Leu-Cys-Gly-Ala-Met-Val-Gly-Val-Trp-Phe-Cys-Lys-C and N-Tyr-Leu-Gly-Leu-Glu-Ile-Asn-Val-Lys-C. From the partial amino acid sequence underlined in the former peptide sequence, a 17-mer degenerate oligonucleotide probe was synthesized [5′-TG(T/C)GG(G/A/T/C)GC(G/A/T/C)ATGGT(G/A/T/C)GG-3′].

cDNA cloning and sequencing of the GST Y-1 gene. A λgt10 cDNA library, constructed from poly(A)$^+$ RNA purified from *I. orientalis* cultured with DNB, was screened with the degenerate oligonucleotide probe described above. Several positive clones were obtained from the 50,000 plaques screened. One of the positive clones, containing a 0.7-kb cDNA fragment, was isolated, and the cDNA fragment was subcloned into the *Eco*RI site of pUC119 to construct pGS148. *E. coli* DH5α transformed with pGS148 showed 29-fold-higher GST activity (94 mU/mg) than the same strain transformed with pUC119 (3.2 mU/mg) when cultured with isopropyl-β-D-thiogalactopyranoside (IPTG), and this suggested that the cDNA contained the GST Y-1 gene. A 0.7-kb cDNA fragment sufficient to cover the GST Y-1 gene was sequenced in both directions. A single open reading frame, consisting of 188 amino acid residues with a calculated total molecular mass of 21,001 Da, was found, which agreed with the results obtained by SDS-PAGE. This open reading frame contained amino acid sequences showing complete identity with those of the N-terminal and lysyl endopeptidase-produced fragments from the purified 21-kDa protein (Fig. 1).

The deduced amino acid sequence of GST Y-1 was compared with those of GSTs from other species. GST Y-1 showed the highest degree of homology (36.7% over 188 amino acid residues) to GST Y-2, another GST isoenzyme in *I. orientalis*.

The major cytosolic GSTs were grouped into four classes, designated alpha, mu, pi (9), and theta (10), according to their primary structures and other properties. GST Y-1 did not show any significant homology to mammalian GSTs of the alpha, mu, or pi classes (data not shown) but showed a slight sequence similarity to theta-class enzymes, such as bacterial GSTs from *E. coli* (20.5% over 166 amino acids) (13) and *P. mirabilis* (26.4% over 159 amino acids) (17) as well as GSTs I (21) and III (11) from maize (25.9% over 143 amino acids and 24.0% over 104 amino acids, respectively) (Fig. 1).

X-ray crystallographic studies have shown that the alpha-, mu-, and pi-class GSTs exhibit similar topological patterns despite their low degree of identity at the primary-structure level (30). The N-terminal domain of each class of GST has been considered to be very important for GSH binding, and site-directed mutagenesis studies have suggested that the highly conserved Tyr residue near the N terminus is essential for catalytic activity which may activate GSH by promoting thiolate anion formation (7, 22). Recently, the crystal structure of a theta-class enzyme from *Lucilia cuprina* was reported (29). Although its structure was similar to those of the other GSTs, and the Tyr residue near the N terminus was conserved, the hydroxyl group of the Ser residue in the N-terminal domain was found to be close to the position of the conserved Tyr residue of the mammalian GSTs (alpha, mu, and pi) on superposition of the GST crystal structures. Site-directed mutagenesis experiments also revealed that the Ser residue in the N-terminal region of theta-class GSTs plays the same important role in catalysis as the Tyr residue in alpha-, mu-, and pi-class GSTs (3).

Although the Ser residue was conserved in GST Y-1, it was replaced by a Thr residue in GST Y-2. Since the replacement of a Ser residue with Thr in theta-class enzymes reduced activity (3), the difference in the specific activities of GSTs Y-1 and Y-2 (GST Y-1 showed 10-fold-higher specific activity with 1-chloro-2,4-dinitrobenzene [CDNB] than did GST Y-2) might have been due to this amino acid residue.

Effects of DNB on GST gene expression. We previously reported that cells cultured with DNB showed increased GST activity (8). To determine whether DNB affects GST Y-1 or Y-2 gene expression, Northern blot analysis was performed. Total RNA was prepared from exponential-phase cells cultured with or without 200 μM DNB, and 20 μg of each RNA sample was subjected to Northern blot analysis with GST Y-1 or Y-2 cDNA as a probe (Fig. 2). The expression level of GST Y-1 in cells cultured with DNB was 7.7-fold higher than that in cells cultured without DNB. High levels of GST Y-2 gene

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*     *     ‡          *    *                          *    *
MTFGTLYILPP-SPRSAWLPK-LAKYLGLEINVKSM-LEVE------DFKSKFPLGKAPAFEGSD  56
MTFATVYIKPH-TPRGDWLAS-LGQYVGLEIKTVDY-KSAE----ASKFEELFPLKRVPALVTPN  58
MAPMKLYGAVM-SWNVTRCATAL-EEAGSDYEIVPINFATA-EHKSPEHLVRNPFGQVPALQDGD  62
MAPLKLYGMPL-SPNVVRVATVL-NEKGLDFEIVPVDLTTG-AHKQPDFLALNPFGQIPALVDGD  62
MK--LFYKPGACSLASHI---TLRES-GKDFTLVSVDLMKKRLENGDDYFAVNPKGQVPALLLDD  59
MK--LYYTPGSCSLSPHI---VLRET-GLDFSIERIDLRTKKTESGKDFLAINPKGQVPVLQLDN  59

   * *  **  *                    *
GFRLTETLAIIKYFIDSSSKPEF-AGSSLKEKALNEKWLSFANSDLCGAMVGVWF---------- 110
GFQLTELIAIVEYIVAKGSKPEL-SGKTTEERATNTRWLSFFNSDFVQAAGGYFM---------- 112
-LYLFESRAICKYAARK-NKPELLREGNLEEAAMVDVWIEVEANQYTAALNPILFXVLISPMLGG 125
-EVLFESRAINRYIASKYASEGTDLLPATASAAKLEVWLEVESHHFHPNASPLVFQLLVRPLLGG 126
GTLLTEGVAIMQYLADSVPDRQLLAPVNSISRYKTIEWLNYIATELHKGFTPLFRP---------- 115
GDILTEGVAIVQYLADLKPDRNLIAPPKALERYHQIEWLNFLASEVHKGYSPLFSS---------- 115

                          *           **
CKDESKKPELVSKLNSLLQYIDNELNNSKFLVGDSVLVADILLYVTLQHIVEIGVDISSFSHLKK 175
GPNDEIKQQSLQTMLSLLEYIDKHLSQSKYFTNNTILTADIFAFQIFAMAKQFGVDFTHYPNVER 177
TTDQKVVDENLEKLKKVLEVYEARLTKCKYLAGDFLSLADLNHVSVTLCLFATPYASVLDAYPHV 190
APDAAVVEKHAEQLAKVLDVYEAHLARNKYLAGDEFTLADANHALLPALTSARPPRPGCVAARPH 191
DTPEEYKPTVRAQLEKKLQYVNEALKDEHWICGQRFTIADAYLFTVLRWAYAVKLNLEGLEHIAA 180
DTPESYLPVVKNKLKSKFVYINDVLSKQKCVCGDHFTVADAYLFTLSQWAPHVALDLTDLSHLQD 180

YSEEVAKHELLAE                          188   GST Y-1
FTGEVSQHPIIKNM                         191   GST Y-2
KAWWSGLMERPSVQKVAALMKPSA               214   Maize GST I
VKAWWEAIAARPAFQKTVAAIPLPPPPSSSA        222   Maize GST III
FMQRMAERPEVQDALSAEGLK                  201   E.coli GST
YLARIAQRPNVHSALVTEGLIKE                203   P. mirabilis GST
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FIG. 1. Alignment of amino acid sequences of several theta-class GSTs. Each amino acid sequence was deduced from the cloned-DNA sequence (GST Y-2 [25], maize GST I [21], maize GST III [11], *P. mirabilis* GST [17], and *E. coli* GST [13]). Amino acid positions are indicated on the right. The amino acid residues conserved with those of GST Y-1 are shaded. Asterisks indicate the amino acids completely conserved in the six GSTs. The double dagger indicates conserved serine residues in theta-class GSTs.

expression were also observed in cells cultured with DNB, while no expression of GST Y-2 was detected under normal (DNB-free) culture conditions. These results indicate that expression of both GST genes is strongly induced by DNB.

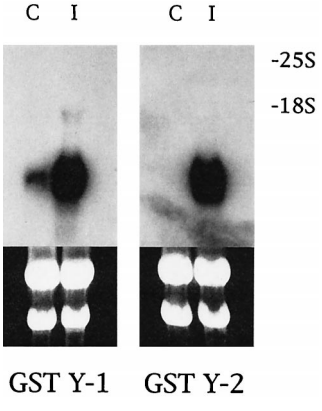

FIG. 2. Northern blot analysis. Twenty-microgram portions of total RNA extracted from *I. orientalis* cultured with (induced [I]) or without (control [C]) 200 μM DNB were loaded onto 1% agarose gels and, after electrophoresis, stained with ethidium bromide (bottom). After proteins were blotted onto Hybond N^+ nylon membranes, hybridization was performed with ^{32}P-labeled GST Y-1 (top left) or Y-2 (top right) cDNA probe (2×10^6 cpm/ml). The positions of 25S and 18S rRNAs are indicated on the right.

We also reported previously that when DNB was added to the culture medium, cell growth was repressed for about 48 h and then cells began to grow with highly induced GST activity (23). To examine the responses of the GST Y-1 and Y-2 genes to DNB, two sets of time course experiments were performed. First, the early response to addition of DNB was analyzed. Cells were grown in 20 liters of yeast extract-peptone-dextrose (YEPD) medium containing 200 μM DNB, and every 3 h after DNB addition 2 liters of culture was removed and cell growth was monitored by determining the optical density at 600 nm. Cells in each sample were precipitated by centrifugation and flash frozen in liquid nitrogen prior to RNA extraction, and each supernatant was subjected to high-performance liquid chromatography as described previously (23). Once DNB was added to the culture medium, cell growth was repressed immediately, and this growth arrest lasted for at least 48 h (Fig. 3C and D). The DNB concentration in the culture medium did not change for 48 h (Fig. 3D), while the levels of expression of both the GST Y-1 and Y-2 genes were already increased at 3 h after DNB addition (Fig. 3A). The levels of expression of both genes increased and reached a plateau at 12 h after addition of DNB. After a lag phase caused by DNB addition, *I. orientalis* started to grow again, detoxifying DNB (as a glutathione conjugate), which was enzymatically digested to *S*-2-nitrophenyl cysteine and secreted into the culture medium (23). We next monitored the genetic responses when cells again started to grow with a high detoxifying capability. Cells started to grow

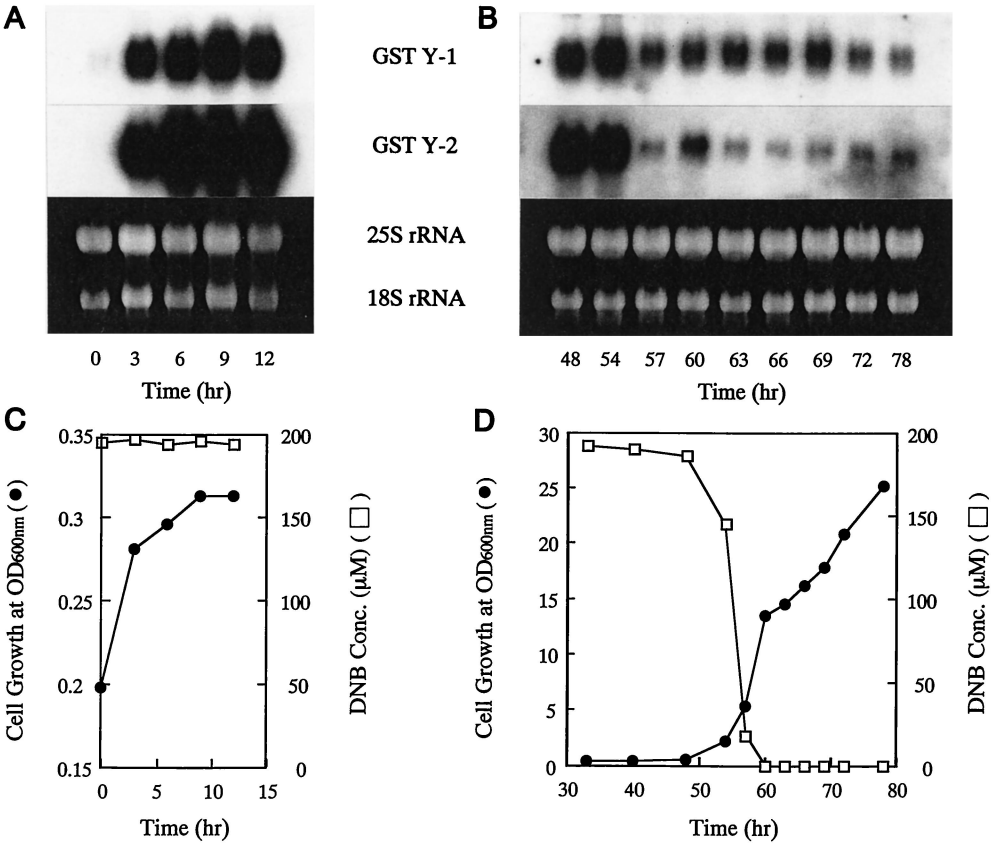

FIG. 3. Effects of DNB on cell growth and GST gene expression. *I. orientalis* cells grown in YPD medium to late exponential phase were inoculated into a jar fermentor containing 20 liters of YPD medium, and the cells were further cultivated after DNB was added to 200 μM. (A and B) Before and after DNB addition, cell cultures were sampled at the indicated times and samples were subjected to Northern blot analysis. Levels of GST Y-1 (top) and GST Y-2 (middle) mRNAs are shown. Total RNA was loaded onto agarose gels, electrophoresed, and stained with ethidium bromide (bottom). (A) Cells cultured for 0 to 12 h; (B) cells cultured for 32 to 78 h. (C and D) Cell growth and DNB concentration (conc.) in the culture medium were also analyzed. (C) Cells cultured for 0 to 12 h; (D) cells cultured for 32 to 78 h. OD600nm, optical density at 600 nm.

exponentially after a 48-h lag phase, and the DNB concentration in the culture medium decreased in inverse proportion to cell population growth (Fig. 3D). Both the GST Y-1 and Y-2 genes were highly expressed at early exponential phase (48 and 54 h), but the levels of expression of both genes were markedly reduced 3 h later, in the middle of the exponential growth phase (57 h), at which time most of the DNB in the culture medium had been detoxified by glutathione conjugation (Fig. 3B). These results indicated that the levels of expression of both GST genes were regulated by the DNB concentration in the medium and that the response of these genes to DNB was very rapid.

The mechanisms of induction of several GST isoenzymes from mammals have been studied. Analysis of the 5′-flanking region of the rat liver GST Ya subunit gene revealed two *cis*-acting regulatory elements (12): an XRE (18) and an ARE (12). The XRE is also found in the 5′-flanking region of the cytochrome P-450IA1 gene and has been shown to be responsible for activation by planar aromatic compounds. The ARE is distinct from the XRE and mediates induction by the metabolites of several planar aromatic compounds as well as reactive oxygen species. In this study, the activities of both GST Y-1 and GST Y-2 were induced markedly by the addition of DNB, an electrophilic compound. Since the induction of both genes was dependent on the presence of DNB in the medium, there might be an upstream regulatory element(s) such as an XRE or ARE in these genes. Further studies are necessary to elucidate the mechanisms of induction of GSTs in yeast cells.

In this study, DNB was shown to induce the expression of two GST genes. Some electrophiles are also known to cause DNA damage which affects the cell cycle. It has been reported that many types of cells respond to DNA damage by regulating progression through subsequent mitotic cell cycles (28). Regulation of cell cycle transitions in response to damage is a result of signal transduction pathways called checkpoints (5). In the yeast *S. cerevisiae*, checkpoints responding to DNA damage or to inhibition of DNA replication regulate entry into and progression through S phase and mitosis. Since DNB is an electrophilic compounds which is known to react with nucleophilic residues in nucleotides and proteins, it is possible that DNB causes DNA damage or inhibits DNA replication, resulting in G_1 or G_2 arrest. Our observation that the addition of DNB repressed cell growth is consistent with the cell cycle arrest mechanism of electrophiles. Thus, it is plausible that *I. orientalis* cells monitor toxic compounds in the environment and arrest the cell cycle as a means of preventing the induction of mutation by these compounds while inducing detoxifying systems such as GST to protect themselves.

Nucleotide sequence accession number. The nucleotide sequence of the 711-bp cDNA encoding the GST Y-1 gene has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AB021655.

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