Marine Biology
DEC 2004; 146(1) : 53-64
http://dx.doi.org/10.1007/s00227-004-1423-6
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Characterisation and expression of four mRNA sequences encoding glutathione S-transferases pi, mu, omega and sigma classes in the Pacific oyster Crassostrea gigas exposed to hydrocarbons and pesticides

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Abstract:

Hydrocarbon and pesticide pollution in coastal ecosystems can disturb marine bivalve metabolism. In this study, we characterised four full-length cDNA sequences encoding glutathione S-transferases (GSTs) in the Pacific oyster Crassostrea gigas. A BLAST X search showed that these four sequences encode GSTs from four different classes: GST pi, sigma, mu and omega. A phylogenetic analysis of GST was made to determine the position of oyster GST compared to invertebrate and vertebrate sequences. We developed a semi-quantitative, multiplex RT-PCR to follow the expression of these four GSTs in tissues of oysters exposed to hydrocarbons and two pesticide treatments (glyphosate and a mixture composed of atrazine, diuron and isoproturon) under experimental conditions. Our results showed strong differential expression of these four GSTs that was both tissue specific as well as time and treatment dependent. We observed that expression levels were higher in digestive gland than in gill tissues in pesticide-exposed oysters. Furthermore, omega and mu class GST mRNA expression in the digestive gland might be useful as a possible marker of hydrocarbon exposure, while pi and sigma class GST mRNA expression in the digestive gland may be similarly useful as a marker of pesticide exposure in monitoring programmes.

Keywords: glutathione S-transferases; cDNA sequences; hydrocarbons; pesticides; multiplex RTPCR; gene expression; *Crassostrea gigas*.

Abstract

Hydrocarbon and pesticide pollution in coastal ecosystems can disturb marine bivalve metabolism. In this study, we characterised four full-length cDNAs encoding glutathione S-transferases (GSTs) in the Pacific oyster *Crassostrea gigas.* The homology of our sequences varied from 23% for a sigma class GST to 46.1% for a mu class GST, with those from the pi and omega classes having homologies of 27 and 37.3%, respectively. We developed a semi-quantitative multiplex RT-PCR to follow the expression of these four GSTs in tissues of oysters exposed to hydrocarbons and two pesticide treatments (glyphosate and a mixture composed of atrazine, diuron and isoproturon) under experimental conditions. Our results showed a strong differential expression of these 4 GSTs that was both tissue-specific as well as time- and treatment dependent. Furthermore, omega and mu class GST mRNA expression in digestive gland might be useful as a possible marker of hydrocarbon exposure, while pi and sigma class GST mRNA expression in digestive gland may be similarly useful as a marker of pesticide exposure in monitoring programs.

Introduction

Biotransformation processes in eukaryotic cells may be categorised by phase. Phase I is characterised by the oxygenation of xenobiotics and endogenous substrates by the inducible cytochrome P450-dependent microsomal monooxygenase. Reduction of lipophilic compounds by Cyp450 results either in directly excreted polar metabolites or in more reactive molecules which are used as substrates by phase II enzymes (Lüdeking and Köhler, 2002). Phase II enzymes catalyse the conjugation of the xenobiotic to endogenous compounds. Glutathione S-transferases (GSTs) are the major phase II enzymes that conjugate glutathione and electrophilic substrates (Lüdeking and Köhler, 2002; Van der Oost et al., 2003). A third phase has been characterised and enzymes involved in this phase are members of the multi-drug resistance and multi-xenobiotic resistance protein family (Lüdeking and Köhler, 2002). These proteins, first described in cancer cell lines (Endicott and Ling, 1989) and in marine invertebrates (McFadzen et al., 2000), act as a pump involved in the export of xenobiotics out of the cell (Lüdeking and Köhler, 2002).

Glutathione S-transferases are comprised of classes of dimeric enzymatic proteins that catalyse the conjugation of glutathione to a wide variety of hydrophobic compounds through the formation of a thioether bond with their electrophilic center (Hayes and Pulford, 1995). Based on amino acid sequence identity, enzymatic properties and immunological reactivity, there are at least eight major classes of GSTs, designated alpha, kappa, mu, pi, sigma, omega, theta and zeta (Hayes and Pulford, 1995; Pemble et al., 1996; Board et al., 1997; Sheehan et al., 2001). These enzymes have evolved as a cellular protection system against a range of xenobiotics and oxidative metabolic by-products, and in particular are known to metabolise a number of environmental carcinogens. The wide range of GST isoforms present in the various classes provide cells with an efficient way of scavenging the huge number of potentially toxic compounds encountered. They are ubiquitous enzymes reported in most animal phyla, e.g. molluscs (Fitzpatrick and Sheehan, 1993; Fitzpatrick et al., 1995; Blanchette and Shingh, 1999, Vidal et al., 2002), annelids (Stenersen et al., 1979), crustaceans

(Keeran and Lee, 1987; LeBlanc and Cochrane, 1987) and mammals (Rouimi et al., 1996). Numerous studies with molluscs have shown that GST enzyme activity is either inducible or unchanged by exposure to various xenobiotics (Khessiba et al., 2001; Alves et al., 2002; Gowlan et al., 2002; Torres et al., 2002; Cheung et al., 2002; Petushok et al., 2002; Le Pennec and Le Pennec, 2003). To our knowledge, little information is available on GST cDNA, gene sequences or mRNA expression in molluscs.

In this study, we characterised, for the first time, the complete cDNA sequences of four GSTs in the marine bivalve *Crassostrea gigas*, each belonging to a different class. The mRNA expression of the pi, mu, omega and sigma class GSTs, and their potential use as biomarkers of contaminant exposure were investigated. We developed a semi-quantitative multiplex RT-PCR to analyse GST mRNA expression in oysters exposed to hydrocarbons and two pesticide treatments. One pesticide exposure designated ADI was to a mixture of 2-chlor-4-ethylamino-6-isopropylamino-1,3,5,-triazin (atrazine), 3-(3,4-dichlorphenyl)-1-1-dimethyl-harnstoff (diuron) and 3-(4-isopropylphenyl)-1,1-dimethyl-harnstoff (isoproturon) and the second was to *N*-(phosphonomethyl)glycine (glyphosate).

Materials and methods

Experimental design

Adult oysters (10-11 cm), *Crassostrea gigas*, were collected from La Pointe du Château (Brittany, France) in Winter 2001 for the hydrocarbon experiment and in Fall 2002 for the pesticide experiments. After an acclimatisation period of 7 days in aerated 0.22µm-filtered seawater at constant temperature and salinity (15°C and 34‰, respectively), oysters were challenged as follows. Groups of 20 oysters were exposed for 3 weeks to a 0.1% mixture of hydrocarbon consisting of the water-soluble fraction of domestic fuel homogenised for three days in filtered seawater (Snyder et al., 2001). Other groups of 20 oysters were exposed for four weeks to either a

mixture of three herbicides (atrazine 2µg/l; diuron 0.5µg/l and isoproturon 1µg/l, mixture called ADI) or to 2µg/l of glyphosate. Another group of 20 oysters was maintained in seawater, without contaminant, as a control. No mortality was observed in the control or treated oysters.

Extraction of total RNA and cDNA synthesis (reverse transcription)

Total RNA was extracted from the digestive glands of treated oysters after 0, 7, 15 and 21 days of exposure to the hydrocarbon mixture, and from the digestive glands and gills of treated oysters after 0, 7, 15, 21 and 30 days of exposure to the pesticide treatments according to the method based on extraction in guanidium isothiocyanate (Strohman et al., 1977). Matching extractions from control oysters were done for each treatment. For each sample, 10 µg of RNA was submitted to reverse transcription using oligo dT anchor primer (GAC CAC GCG TAT CGA TGT CGA $CT_{(16)}V$) and M-MLV reverse transcriptase (Promega).

Cloning and sequencing of 5' and 3' flanking regions of omega, pi, mu and sigma GST cDNA

The procedures for the generation of 5' and 3' untranslated regions (UTR) GST cDNA were carried out according to the commercial 5’/3’ rapid amplification of cDNA ends protocol (5’/3’ RACE Kit, Roche) using specific primers designed from the sequences obtained in suppressive subtraction hybridisation (SSH) libraries (Boutet et al., in press) and reported in Table I. The 3' UTR was amplified as follows: 200 ng of reverse transcription product plus 2 mM $MgCl_2$, 10 pmol each of PCR anchor primer (GAC CAC GCG TAT CGA TGT CGA C) and specific primer was submitted to amplification using one cycle at 94°C for 2 min, 58°C for 2 min, 72°C for 1 min 30 s, then 40 cycles at 94°C for 15 s, 58°C for 30 s, and 72°C for 1 min with a final step at 72°C for 10 min. Amplification of the 5’ UTR was carried out according to the following procedure: denaturation at 94°C for 2 min, then 10 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 1 min, then 30 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 1 min with an increase of 20 s per cycle for the elongation time, and a final step at 72°C for 15 min with 2 mM $MgCl_2$ and 10 pmol each of oligo

dT anchor primer and specific primer. The resulting cDNA fragments corresponding to the 5' and 3' UTRs were cloned into pGEM-T vector (Promega) and sequenced using a Li-COR IR² (Sciencetech) and Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Bioscience).

Semi-quantitative multiplex PT-PCR

To perform semi-quantitative RT-PCR, the total amount of isolated total RNA was measured by UV-spectroscopy at 260 nm. PCR experiments in which each primer pair was omitted in the primer mix showed that the amplification of genes investigated in the PCR was not altered by the presence of the other primer pairs. Amplification was performed as follows: one cycle at 94°C for 2 min, 55°C for 1 min and 72°C for 1 min 30 s; 35 cycles at 94°C for 30 s, 55°C for 1 min and 72°C for 1 min followed by a final 10 min extension at 72°C. PCR product was separated on 1.5% agarose gels using TBE-buffer and photographed after Ethidium Bromide staining. The primer pairs used for amplification (10 pmoles each) and the length of the generated fragments are reported in Table II. A PCR amplification control (28S ribosomal DNA) was used for all experiments and was amplified as described above with primers sense AAG GGC AGG AAA AGA AAC TAA C and antisense TTT CCC TCT AAG TGG TTT CAC. Quantification of band intensities was measured using Gene Profiler 4.03 Software (Scanalytics Inc.).

Results

Identification of 4 mRNA sequences encoding GSTs in Crassostrea gigas

Sequence data was submitted to GenBank: AJ557140 1124 bp *Crassostrea gigas* GST pi mRNA, complete coding sequence (cds); AJ557141 908 bp *C. gigas* GST omega mRNA, complete cds; AJ558252 894 bp *C. gigas* GST mu mRNA, complete cds; and AJ577235 1002 bp *C. gigas* GST sigma mRNA, complete cds.

Initially, partial sequences of the four GSTs were obtained from hydrocarbon-exposed *C. gigas* digestive gland SSH libraries (Boutet et al., 2003a): 384, 444, 291 and 573 base pairs encoding pi, omega, mu and sigma class GSTs, respectively. Specific primers were designed to amplify the 5' and 3' UTRs of each GST cDNA. The resulting sequences contained open reading frames of 738 bp (245 amino acids) for the pi class GST (Figure 1), 732 bp (243 amino acids) for the omega class GST (Figure 2), 648 bp (215 amino acids) for the mu class GST (Figure 3) and 609 bp (202 amino acids) for the sigma class GST (Figure 4). We also observed multiple ATTTA(G) motifs in the untranslated regions of the four GSTs correlated with transcript stability. The cDNA encoding the mu class GST had one ATTTG motif in the 3' UTR. The omega class GST had two ATTTG motifs and one ATTTA motif in the 3' UTR. The pi class GST had one ATTTA and one ATTTG motifs in the 5' UTR and two ATTTG motifs and one ATTTA motif in the 3' UTR. And, the sigma class GST had two ATTTA motifs in the 3' UTR. Moreover, the pi class GST cDNA contained 2 polyadenylation signals in its 3' UTR. The four GST cDNAs encode proteins with molecular masses of 28.4 kDa (pi), 28 kDa (omega), 25.1 kDa (mu) and 23.6 kDa (sigma).

A search for amino acid sequence homology using the ALIGNP program (Myers and Miller, 1988) revealed that the four cDNA possesses 27% (GST pi), 37.3% (GST omega), 46.1% (GST mu) and 23% (GST sigma) homology with cDNA encoding GSTs in other species.

Glutathione S-transferase expression analysis using semi-quantitative multiplex RT-PCR

Based on the sequencing information obtained, a semi-quantitative multiplex RT-PCR was designed to simultaneously assay the differential expression of the four GST genes in *C. gigas*. The results of semi-quantitative multiplex RT-PCR showed a strong differential expression between the different classes of GSTs, tissues and treatments. Analysis of GST expression in the digestive gland of oysters exposed to hydrocarbons displayed an induction of the mu (14.4-fold compared with control) and omega (4.3-fold compared to control) class GST mRNA syntheses and an inhibition of the sigma class GST mRNA synthesis (15-fold compared with control) (Figure 5). The pi class GST

mRNA synthesis seems to be induced, but this result is less clear than for other GSTs. Regarding Figure 5, we observed that omega class GST mRNA was more abundant in oyster digestive gland than were the other GST classes. No analysis was made on gill tissue since the role of digestive gland microsomes in hydrocarbon detoxification processes is well known. However, expression of GSTs was analysed in gill tissue from pesticide treated oysters because the pesticide detoxification processes are not so well known.

Results from the pesticide experiments show that the mu class GST was not expressed in either gill or digestive gland in both control and exposed oysters (Figures 6 and 7). In the digestive gland from control oysters, the omega class GST was expressed at a low rate (Figures 6 and 7). Both the omega and pi class GSTs were expressed in gill from control oysters from the two treatments (Figures 6 and 7). During the exposure to both ADI and glyphosate, expression of all GSTs was inhibited in gill tissue; a mean of 100-fold decrease was observed (Figures 6 and 7). After 30 days of exposure to ADI, we observed an induction of the pi (3.7-fold compared with control), sigma (48.4-fold compared with control) and omega (2.5-fold compared with control) class GSTs' mRNA synthesis in the digestive gland of oysters (Figure 6). The omega class GST was expressed in digestive gland in every sampling from treated oysters from the glyphosate exposure experiment, reaching a maximum value after 21 days of exposure (1.4-fold compared with control) (Figure 7). Pi and sigma class GSTs were expressed after 15 days of exposure (17.3- and 6.2-fold compared to the control, respectively), followed by an inhibition at 21 days and induction again after 30 days of exposure (2- and 3.7-fold compared to the control, respectively) (Figure 7).

Discussion

In this study, we identified complete mRNA sequences of 4 genes encoding GSTs in the Pacific oyster, *Crassostrea gigas*. These enzymes are known to be involved in Phase II biotransformation of xenobiotics. Using specific primers designed from partial sequences obtained in a previous study

related to the general response of *C. gigas* to hydrocarbon exposure (Boutet et al., in press), we amplified complete cDNAs encoding four different GSTs. The molecular masses of the corresponding proteins are in accordance with the mean value of 25 kDa observed in other mollusc species and in other phyla (Fitzpatrick and Sheehan, 1993; Blanchette and Singh, 1999; Rouimi et al., 2001; Vidal et al., 2002; Guo et al., 2002). Sequence homologies to other species were lower (23 to 46%) than for other genes investigated in previous studies in the same species. For example, we obtained a 70% homology for HSP70 gene sequence between *C. gigas* and other species (Boutet et al., 2003). A low degree of homology in GST sequences was also observed in another mollusc species, *Mytilus edulis,* ranging from 32 to 53% (Lüdeking and Köhler, 2002). An explanation for this finding might be GST specialisation within an isoform class in addition to selection for substrate specificity in various phyla (Whalen and Boyer, 1998). Nevertheless, the homologous nature of stress-gene families and their widespread occurrence among various phyla implies they have fundamental functions in cellular stress responses.

The four GST cDNA sequences presented multiple ATTTA(G) motifs in their untranslated regions. These motifs are known to be correlated with transcript stability (Shaw and Kamen, 1986) and have been observed in other cDNAs, such as the sequence encoding glutamine synthetase in the sea urchin *Paracentrotus lividus* (Fucci et al., 1995) and in rat aspartate aminotransferase (Pavé-Preux et al., 1988). The GST omega cDNA sequences characterised from pig and human (Board et al., 2000; Rouimi et al., 2001) and GST mu cDNA sequence from *Xenopus laevis* (De Luca et al., 2002) did not contain these motifs in their UTRs, while the mu GST cDNA sequence from mouse contained two ATTTG motifs in the 3'UTR (Guo et al., 2002).

Another interesting 3'UTR feature seen was two polyadenylation signals in the pi class GST cDNA sequence. Multiple polyadenylation sites have already been observed for other genes (Leff et al., 1986). Caizzi et al. (1990) and Smartt et al. (1998 and 2001) suggest that the multiple polyadenylation sites indicate the presence of multiple transcripts encoding one protein. Pavé-Preux et al. (1988) found that a single aspartate aminotransferase sequence containing two

polyadenylation signals encoded two different mRNAs in rat. They postulated that the two mRNAs resulted from the differential use of these signals during the maturation of pre-mRNA. More analysis will be necessary to determine if the pi class GST sequence encode only one or two different mRNAs.

Analysis of mRNA expression by semi-quantitative multiplex RT-PCR showed that the four GSTs studied were not expressed in all tissues under normal conditions. In the present report, we observed that only the omega class GST was expressed at a low rate in both gill and digestive gland from the control oysters, whereas the mu class GST was absent in those tissues from the control oysters. In addition to tissue-specific differences, the relative abundance of the various RNAs could vary under different physiological conditions. The control oysters from the hydrocarbon and pesticide experiments did not express the same GST classes. Control oysters from the hydrocarbon experiment expressed pi, sigma and omega class GSTs, while control oysters from the pesticide experiments expressed only omega and pi class GSTs. It has been demonstrated that GSTs are associated with cell proliferation (Terrier et al., 1990; Lüdeking and Köhler, 2002) and that intracellular level of these enzymes may be co-ordinated by other genes in response to oxidative stress (Salinas and Wong, 1999). Perhaps because cell proliferation in molluscs is dependent on their physiological condition, expression of GST classes varied in the control oysters (sampled a year apart).

GST omega displayed an expression either in control or treated oysters in both tissues studied. The expression of omega class GST was observed in several tissues in human and pig (Board et al., 2000; Rouimi et al., 2001). Members of the omega class have been reported to be involved in radiation resistance in lymphoma cells (Kodym et al., 1999) and in protection against oxidative stress (Board et al., 2000; Dulhunty et al., 2001). Following an oxidative stress, a number of cellular proteins form S-thiol adducts with glutathione and cysteine (Hanson et al., 1999). The formation of these adducts can inactivate the enzymatic functions of affected polypeptides (Jahngen-Hodge et al.,

1997; Ravichandran et al., 1994). Omega class GSTs may reduce this type of S-thiol adduct and restore enzymatic function (Board et al., 2000).

Based on the pesticide exposure results, three of the four GSTs were over-expressed in the digestive glands of oysters exposed to both glyphosate and ADI. Previously, it has been demonstrated that isoproturon caused a marked induction of GST in rat liver (Schoket and Vincze, 1985; Hazarika and Sarkar, 2001) and that both atrazine and isoproturon generated strong selection in *C. gigas* populations (Moraga and Tanguy, 2000). The toxicity of these two pesticides caused a mortality rate of 60 to 70% in *C. gigas* populations at concentrations of 0.1 and 0.2 mg/l after two months of exposure. Conversely, comparable exposure to diuron does not cause mortality (Moraga and Tanguy, 2000). Moreover, pi and sigma class GSTs were not expressed in the digestive glands of control oysters. This differential expression between challenged versus control oysters, especially for pi and sigma class GSTs, may be useful as a marker of pesticide exposure. Mu and omega class GST mRNA expression may be similarly useful as a biomarker of hydrocarbon exposure.

To summarise, we characterised, for the first time, four cDNAs encoding GSTs in the oyster *Crassostrea gigas*. Their sequence data formed the basis of an expression study that used semi-quantitative multiplex RT-PCR methods to follow the simultaneous expression of the four GSTs in the same sample. The results showed tissue-specific, time- and treatment-dependent differential expression of the GSTs in oysters. Furthermore, omega and mu class GST mRNA expression may be useful as a marker of hydrocarbon exposure and pi and sigma class GST mRNA expression as a marker of pesticide exposure in monitoring programs.

Acknowledgements

This research program was supported by the Région Bretagne, the Conseil Général du Finistère and the interregional program MOREST (Summer Mortality of juvenile oyster *Crassostrea gigas*).

The authors are grateful to Brenda J. Landau for English corrections and to Monique Briand for editing the figures.

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Figure legends

Figure 1: The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class pi in oyster (AJ557140). Stop codon is marked by an asterisk and untranslated regions are in lower cases. Polyadenylation signals are underlined and messenger stability determining motifs ATTTA and ATTTG are boxed.

Figure 2: The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class omega in oyster (AJ557141). Stop codon is marked by an asterisk and untranslated regions are in lower cases. Polyadenylation signal is underlined and messenger stability determining motifs ATTTA and ATTTG are boxed.

Figure 3: The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class mu in oyster (AJ558252). Stop codon is marked by an asterisk and untranslated regions are in lower cases. Polyadenylation signal is underlined and messenger stability determining motifs ATTTA and ATTTG are boxed.

Figure 4: The nucleotide sequence and predicted amino acid sequence of glutathione S-transferase class sigma in oyster (AJ577235). Stop codon is marked by an asterisk and untranslated regions are in lower cases. Polyadenylation signal is underlined and messenger stability determining motifs ATTTA and ATTTG are boxed.

Figure 5: Expression of the four GSTs in digestive gland of oysters (n=3 for each day) exposed to hydrocarbons using semi-quantitative multiplex RT-PCR. Lane 1: 100 bp marker; Lane 2: control oysters; Lane 3: oysters exposed for 7 days; Lane 4: oysters exposed for 15 days; Lane 5: oysters exposed for 21 days.

Figure 6: Expression of the four GSTs in digestive gland (lanes 2 to 6) and gills (lanes 7 to 11) of oysters (*n*=3 for each day) exposed to ADI using semi-quantitative multiplex RT-PCR. Lane 1: 100 bp marker; Lanes 2 and 6: control oysters; Lanes 3 and 8: oysters exposed for 7 days; Lanes 4 and 9: oysters exposed for 15 days; Lanes 5 and 10: oysters exposed for 21 days; Lanes 6 and 11: oysters exposed for 30 days.

Figure 7: Expression of the four GSTs in digestive gland (lanes 2 to 6) and gills (lanes 7 to 11) of oysters (*n*=3 for each day) exposed to glyphosate using semi-quantitative multiplex RT-PCR. Lane 1: 100 bp marker; Lanes 2 and 7: control oysters; Lanes 3 and 8: oysters exposed for 7 days; Lanes 4 and 9: oysters exposed for 15 days; Lanes 5 and 10: oysters exposed for 21 days; Lanes 6 and 11: oysters exposed for 30 days.

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gacctgaacagtcatacatcatatatataggacttcaaacatttgaaagagtgttgacttttgtttaaacag     72

                                                      M   A   D   W   E        5
aagaacggaatttatcatcgtgcaaacagcgaggttttagttacacacagaa ATG GCG GAC TGG GAA     139

 I   L   Y   H   N   I   P   C   A   G   R   A   E   F   V   R   L   I        23
ATT CTT TAC CAC AAC ATA CCG TGT GCT GGA AGA GCT GAA TTT GTT CGT TTG ATC      193

 F   E   E   A   G   V   P   Y   T   E   P   M   K   T   Q   E   E   I        41
TTC GAA GAA GCT GGG GTT CCT TAT ACA GAA CCA ATG AAA ACC CAA GAG GAA ATC      247

 R   D   T   I   M   N   N   K   L   G   G   F   P   V   M   F   P   P        59
CGA GAT ACG ATC ATG AAC AAT AAA CTC GGA GGT TTT CCG GTC ATG TTC CCT CCT      301

 V   L   K   R   G   D   F   H   L   C   Q   T   S   V   I   C   K   Y        77
GTC TTG AAA CGA GGC GAT TTT CAC CTC TGT CAG ACG TCA GTG ATA TGT AAG TAC      355

 L   G   E   Q   F   R   L   M   P   K   S   E   E   E   K   W   Q   A        95
CTG GGG GAA CAA TTT AGA CTG ATG CCA AAA TCA GAA GAG GAA AAA TGG CAG GCG      409

 D   Q   V   N   A   T   I   H   D   F   V   A   E   G   R   L   E   S       113
GAT CAA GTT AAC GCC ACC ATT CAC GAC TTT GTG GCA GAA GGA AGA TTG GAA TCC      463

 R   G   A   K   S   I   N   Y   Y   F   V   G   R   L   A   F   H   G       131
CGC GGC GCT AAA AGT ATC AAC TAT TAT TTC GTA GGA AGA TTG GCC TTT CAT GGA      517

 K   H   W   V   G   S   Y   H   D   Q   K   E   E   T   Q   P   Y   I       149
AAG CAT TGG GTG GGG TCT TAC CAC GAC CAA AAG GAA GAA ACA CAG CCG TAT ATT      571

 D   W   F   V   K   E   R   L   P   K   W   L   K   H   F   E   L   V       167
GAT TGG TTT GTC AAA GAG AGG CTA CCG AAA TGG CTG AAG CAT TTT GAG TTA GTT      625

 L   K   N   N   N   G   G   N   G   F   C   F   G   E   E   V   T   Y       185
CTG AAA AAC AAC AAT GGC GGA AAC GGT TTC TGC TTT GGA GAG GAA GTG ACG TAT      679

 V   D   L   A   L   L   Q   C   L   R   G   C   E   A   S   Y   K   K       203
GTC GAC TTG GCA CTA CTC CAG TGC CTC CGC GGT TGT GAA GCG TCT TAT AAA AAG      733

 G   F   E   S   A   D   Y   C   P   S   L   K   A   F   K   A   Q   M       221
GGT TTC GAG TCG GCA GAT TAT TGC CCT TCC CTA AAG GCG TTC AAA GCC CAG ATG      787

 E   A   R   P   K   L   A   A   Y   Y   K   S   E   R   Y   P   N   T       239
GAG GCG CGT CCG AAG CTA GCG GCC TAT TAC AAG TCA GAG CGG TAC CCA AAC ACC      841

 H   R   T   P   T   A   *                                                   245
CAC AGG ACA CCA ACA GCA TGA tgtgacgtggacgacaaaaaatatgacgtcatcataattgagaa     906
tttcagcaattgcttgtttttgttgttgttgtgaagtaaaacttttctgtgtagttgcttatttattt     978
gaagaattctcttgcgcttattttgctttaaaagattgatcttattgacttcattaataaatacatctatta    1050
tttggatataagatataatatatgtatatgtttatgtagaaataaacaacaaaatttgaaaaaaaaaaaaa    1122
aa                                                                          1124
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Figure 1

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                          M   P   T   Q   Q   S   F   A   T   G   S   A       12
agagagaaattattgtaaaaacca ATG CCG ACC CAA CAA TCA TTT GCT ACA GGT TCC GCC     60

 C   P   E   L   E   A   G   T   L   R   V   Y   S   M   R   F   C   P       30
TGT CCG GAA TTG GAG GCA GGG ACT CTC CGA GTA TAC AGC ATG AGG TTC TGT CCG     114

 Y   A   Q   R   A   L   L   V   L   T   Y   K   N   I   P   H   E   V       48
TAT GCT CAA CGA GCC CTG CTG GTC CTG ACA TAC AAA AAT ATA CCA CAT GAA GTG     168

 V   N   I   N   L   K   N   K   P   E   W   F   L   Q   K   N   P   L       66
GTC AAC ATC AAT CTG AAA AAT AAA CCG GAA TGG TTT CTG CAG AAG AAC CCA CTG     222

 G   R   V   P   T   L   E   K   D   D   R   I   V   Y   E   S   A   I       84
GGG CGG GTT CCC ACC TTA GAG AAA GAT GAC AGA ATC GTG TAC GAG TCC GCT ATC     276

 C   C   D   Y   L   D   Q   V   Y   P   D   N   K   L   T   P   D   D      102
TGC TGT GAC TAT TTG GAC CAG GTG TAT CCC GAT AAC AAG CTG ACC CCA GAT GAC     330

 P   Y   R   Q   A   R   D   K   M   T   V   E   V   F   S   Q   F   V      120
CCT TAC CGT CAG GCC CGG GAC AAG ATG ACC GTG GAG GTC TTC TCT CAG TTT GTT     384

 S   D   F   Q   K   M   M   S   S   P   P   Q   E   K   P   E   S   L      138
TCG GAT TTT CAA AAA ATG ATG AGT TCA CCA CCG CAA GAG AAA CCC GAG AGT TTA     438

 Q   K   I   K   N   N   L   C   E   F   E   S   S   L   T   A   R   Q      156
CAA AAG ATC AAA AAC AAC TTA TGT GAG TTT GAG AGC AGC CTA ACA GCA AGG CAA     492

 G   A   Y   F   G   G   N   A   V   Q   M   L   D   F   L   L   W   P      174
GGC GCC TAC TTT GGA GGG AAC GCA GTG CAG ATG CTA GAC TTC CTG CTG TGG CCA     546

 W   F   E   R   I   L   I   F   A   K   V   V   P   L   T   F   S   L      192
TGG TTT GAA CGT ATT CTC ATC TTC GCA AAA GTT GTT CCG CTG ACG TTC TCT TTA     600

 E   D   Y   P   A   L   C   E   W   T   K   K   M   P   E   C   P   A      210
GAG GAC TAT CCA GCT TTG TGT GAA TGG ACA AAG AAA ATG CCG GAA TGT CCG GCC     654

 V   Q   K   C   R   L   D   P   Q   Q   F   L   E   F   Y   K   S   T      228
GTC CAA AAA TGT CGA TTG GAC CCT CAG CAG TTT TTG GAA TTT TAC AAA AGT ACA     708

 K   A   G   A   P   D   Y   D   V   P   K   T   S   Q   S   *              244
AAA GCT GGC GCA CCT GAT TAT GAC GTA CCT AAA ACT TCA CAG TCA TAA acgaagag    764
aaagtgaacaacttggtactaacatgtatatacatttttatgttctgtaacacaatatttggttttacaatt    836
tctgtttcaaaattattttcttccgctgtatttgatttctcaaataaattaatttaaaataaaaaaaaaaaa    908
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Figure 2

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                                                             M   S   T     3
gggagtcgctggtgcgtgcacatgctagttcgattcgctgcacttttttcatcaagcaaa ATG TCG ACG    69

 L   G   Y   W   N   I   R   G   L   G   Q   P   I   R   L   L   L   N    21
CTT GGC TAC TGG AAC ATT AGA GGG CTT GGC CAG CCA ATC AGA TTG CTG CTG AAC   123

 Y   V   G   E   E   F   D   D   V   Q   Y   E   L   G   D   A   P   D    39
TAT GTC GGA GAG GAA TTC GAT GAT GTA CAG TAT GAA CTT GGA GAT GCA CCC GAC   177

 Y   S   R   E   E   W   L   S   V   K   N   T   L   G   L   A   F   P    57
TAT AGC AGA GAA GAA TGG CTC TCT GTC AAA AAC ACT CTA GGA CTA GCC TTC CCA   231

 N   I   P   Y   Y   I   D   D   D   I   K   I   T   Q   S   N   S   I    75
AAT ATT CCC TAT TAC ATT GAT GAT GAT ATA AAA ATT ACA CAA AGT AAC TCC ATA   285

 L   R   Y   I   G   D   K   H   G   L   L   G   K   T   P   R   D   K    93
TTG AGG TAT ATT GGA GAT AAA CAT GGC CTG TTA GGA AAA ACT CCC CGA GAC AAA   339

 V   D   C   D   M   M   V   E   N   A   M   D   F   R   N   G   V   I   111
GTG GAC TGT GAT ATG ATG GTG GAG AAC GCC ATG GAT TTT AGA AAT GGG GTC ATT   393

 R   L   C   Y   D   N   D   Y   E   K   I   K   D   D   Y   F   A   N   129
CGG TTG TGC TAC GAC AAC GAC TAC GAA AAG ATC AAG GAC GAC TAC TTT GCC AAT   447

 V   K   D   K   L   R   Q   F   D   T   F   L   G   D   K   P   W   F   147
GTC AAG GAC AAA CTA AGA CAG TTT GAC ACG TTC CTT GGA GAC AAA CCT TGG TTC   501

 A   G   D   G   I   T   I   C   D   F   P   L   Y   E   L   L   D   Q   165
GCT GGA GAT GGT ATC ACC ATC TGT GAC TTC CCA TTG TAC GAG TTA CTA GAC CAG   555

 H   R   L   M   K   P   G   I   L   D   D   Y   P   N   L   T   K   F   183
CAC AGA CTG ATG AAG CCT GGG ATA CTA GAC GAT TAC CCC AAC CTG ACC AAG TTT   609

 V   E   R   F   E   N   L   P   K   I   K   A   Y   M   A   S   D   K   201
GTG GAG AGA TTC GAG AAC CTT CCT AAA ATT AAG GCC TAC ATG GCG TCC GAT AAA   663

 F   M   A   R   P   V   N   N   K   F   A   L   F   K   *                215
TTC ATG GCC AGA CCC GTC AAC AAC AAG TTT GCA TTG TTC AAG TGA tttttaacaaaa   720
tatgttaaataattacactttaacatgtagccataaggacatagaaccctacatacctgtatgtgtacatgt   792
attggagaacaacagttttgtcactctgtatgggtctgtaaaaaaaaattagattctgctattataatatta   864
aacaacacgcatttgcaaaaaaaaaaaaaa                                             894
```

Figure 3

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                 M   A   S   Y   R   L   H   Y   F   D   V   R   G   R      14
acactggggataaaar ATG GCC AGC TAC CGA CTT CAC TAC TTC GAC GTT AGG GGC AGG    58

 G   E   I   V   R   M   L   F   K   L   A   Q   A   E   F   G   D   I      32
GGA GAA ATA GTA CGA ATG CTC TTC AAA CTG GCC CAG GCT GAG TTT GGG GAT ATT    112

 R   V   T   Q   G   E   W   T   D   V   K   H   D   T   P   T   G   E      50
CGA GTT ACT CAG GGT GAA TGG ACT GAT GTT AAG CAT GAC ACC CCT ACT GGA GAA    166

 L   P   Y   L   E   V   G   E   K   Q   L   T   Q   S   L   T   I   A      68
CTA CCG TAC TTA GAG GTT GGT GAA AAG CAG CTG ACA CAG AGT CTG ACC ATC GCC    220

 R   Y   L   A   R   E   F   G   L   A   G   D   T   N   W   E   R   A      86
CGC TAC TTG GCC AGG GAG TTC GGT TTA GCT GGG GAC ACG AAC TGG GAG CGC GCT    274

 L   V   E   Q   V   V   D   T   C   D   D   L   R   A   E   N   A   K     104
CTT GTG GAG CAA GTG GTG GAC ACA TGT GAT GAC CTG AGA GCA GAG AAC GCC AAG    328

 I   I   H   E   R   D   P   V   R   L   A   L   M   K   S   K   M   K     122
ATC ATC CAT GAA AGA GAC CCG GTC AGG CTG GCA CTA ATG AAA TCA AAG ATG AAA    382

 D   Q   I   L   P   K   Y   L   N   R   L   T   K   F   L   N   E   H     140
GAC CAA ATA CTT CCC AAA TAC TTG AAC AGA CTT ACT AAA TTT CTA AAT GAA CAT    436

 G   D   R   Y   F   I   G   S   K   I   T   S   A   D   I   A   V   H     158
GGA GAT AGA TAT TTC ATC GGA TCA AAG ATA ACG TCG GCA GAC ATT GCT GTC CAT    490

 E   V   L   T   T   F   L   Q   N   D   P   S   C   L   D   K   H   D     176
GAG GTA TTG ACC ACC TTC CTC CAG AAC GAC CCG TCA TGC CTT GAC AAG CAC GAC    544

 V   L   R   K   H   R   Q   L   V   E   H   H   P   N   L   S   E   Y     194
GTA CTA CGG AAA CAT CGA CAG TTA GTA GAG CAC CAC CCC AAC CTG AGT GAA TAC    598

 L   S   S   R   P   R   F   V   V   *                                     203
CTC TCC TCC AGA CCC CGC TTT GTC GTA TAA ctcgttacctgaatcatcagagccttgcaggc   660
cgtcaatacaggcgatataccgcgatatatcaggggagacaaactgtactgcgcgataccagaggaaaagaa   732
ttaacgtacgatttaatagtgttactatttttcattcagtataaattaatgtaaggcacgtggtatggttta   804
caaagcgtaaactgtcccctgttatactcgtataacatagaaataatatgattcattccttatactttaatt   876
ttccatttataagtaagaacttatttcctttaatggattttcagaaaacttcaaaatgatgttaaaattgcc   948
atcaaaggcagaaagcgataacagtaaactgtttctcgagaaaaaaaaaaaaaa                    1002
```

Figure 4

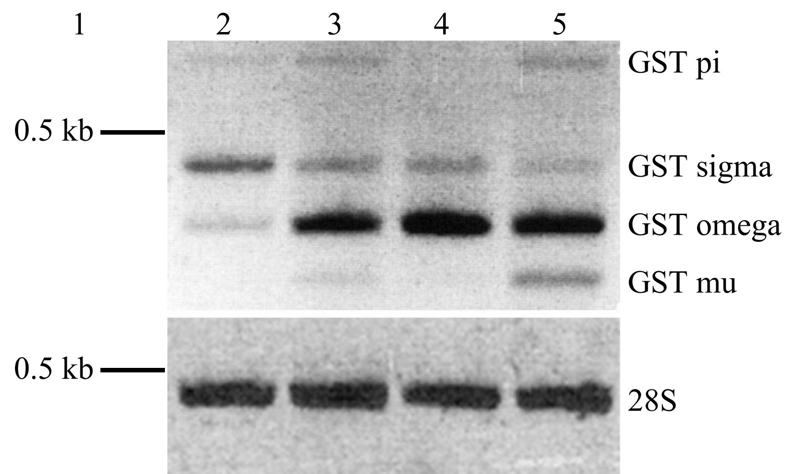

Figure 5

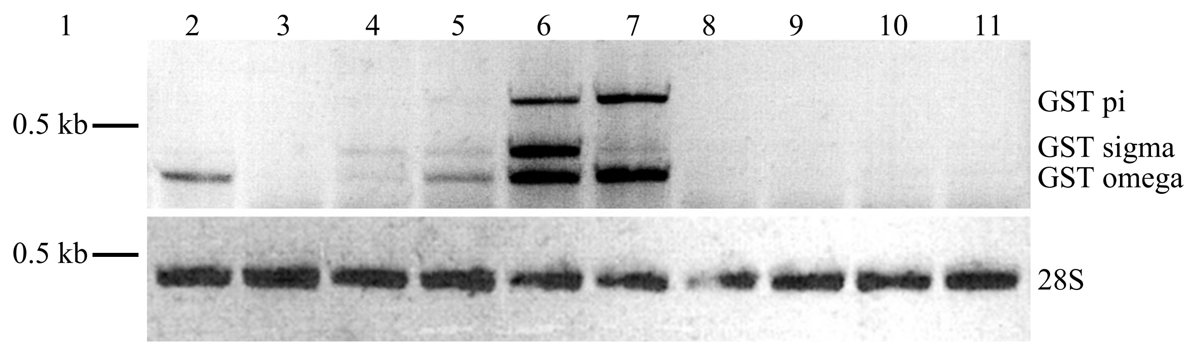

Figure 6

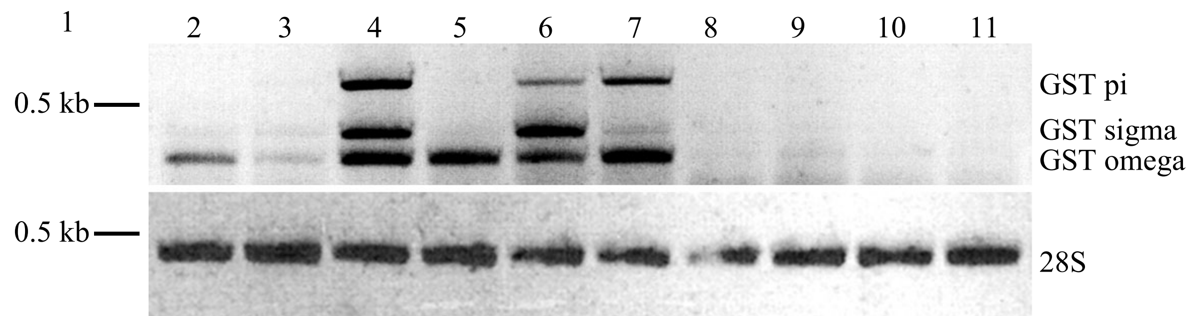

Figure 7

Table I. Combinations of primers used in the amplification of the 5' and 3' UTR of the cDNA encoding the four GSTs.

Genes	Primer sequences
GST mu	sense GGGCTTGGCCAGCCAATCAGATTGCTGCT
	antisense TCTGATTGGCTGGCCAAGCCCTC
GST pi	sense GAGGCGCGTCCGAAGCTAGCGGC
	antisense TTTCCATGAAAGGCCAATCTTCC
GST omega	sense TGGCCATGGTTTGAACGTATTCT
	antisense TCGGGATACACCTGGTCCAAATA
GST sigma	sense AACCTGAGTGAATACCTCTCCTCCAGACC
	antisense CCAGCTAAACCGAACTCCCTGGCCAAGTA

Table II. Combinations of primers used in semi-quantitative multiplex RT-PCR and length of the generated fragments.

Genes	Primer sequences	length of generated fragments (bp)
GST mu	sense ATGTCGACGCTTGGCTACTGGAACATTAG	200
	antisense TTGAACAATGCAAACTTGTTGTTGACGGG	
GST pi	sense ATGGCGGACTGGGAAATTCTTTACCACAA	690
	antisense GCTGTTGGTGTCCTGTGGGTGTTTGGGTA	
GST omega	sense TATTTGGACCAGGTGTATCCCGA	280
	antisense AGAATACGTTCAAACCATGGCCA	
GST sigma	sense TACTTGGCCAGGGAGTTCGGTTTAGCTGG	390
	antisense GGTCTGGAGGAGAGGTATTCACTCAGGTT	