Structure and Antioxidant Catalytic Function of Plant Glutathione Transferases

Evangelia Chronopoulou[1], Irene Axarli[1], Irini Nianiou-Obeidat[2], Panagiotis Madesis[3], Athanasios Tsaftaris[2,3] and Nikolaos E. Labrou[1]*

[1]*Laboratory of Enzyme Technology, Department of Agricultural Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855-Athens, Greece;* [2]*Department of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, P.O. Box 261, Thessaloniki GR-54124, Greece.;* [3]*Institute of Agrobiotechnology, CERTH, 6th km Charilaou-Thermis Road, P.O. Box 361, Thermi GR-57001,Thessaloniki, Greece*

Abstract: Plant cytosolic glutathione transferases (GSTs) are an ancient enzyme superfamily with multiple and diverse functions which are important in counteracting biotic and abiotic stress. GSTs play an important role in catalyzing the conjugation of xenobiotics and endogenous electrophilic compounds with glutathione (GSH), such as pesticides, chemical carcinogens, environmental pollutants, which leads to their detoxification. GSTs not only catalyze detoxification reactions but they are also involved in GSH-dependent isomerization reactions, in GSH-dependent reduction of organic hydroperoxides formed during oxidative stress, biosynthesis of sulfur-containing secondary metabolites, and exhibit thioltransferase and dehydroascorbate reductase activity. This review focuses on plant GSTs, and attempts to give an overview of the new insights into the catalytic function and structural biology of these enzymes.

keywords: Glutathione transferase, herbicide detoxification, biotic stress, abiotic stress.

1. INTRODUCTION

GSTs are ubiquitous enzymes in aerobic organisms and are encoded by large gene families of cytosolic, mitochondrial, and microsomal proteins. GSTs catalyze the conjugation of reduced glutathione (γ-L-Glu-L-Cys-Gly; GSH) *via* the sulfhydryl group, to electrophilic centers on a wide variety of compounds, both endogenous and xenobiotic [1-4]. The conjugation of GSH to these endogenous compounds serves several important roles: (a) limit and restrict the reactivity of the chemicals; (b) increases their solubility and facilitates their membrane transport and elimination from the cell and organism; and (c) in some cases, it leads to the formation of secondary metabolites or essential biological mediators [1,5].

The GSTs comprise a complex enzyme superfamily that has been subdivided into a number of classes based on a variety of criteria (e.g. amino acid/nucleotide sequence, and immunological, kinetic and structural properties) [6]. GST genes and proteins from mammalian sources have been well characterized, but studies of GSTs from non-mammalian sources such as plants and microorganisms have revealed the existence of several different classes (for more details see Sheehan *et al*., 2001 [6]). For example, the plant soluble GSTs according to their sequence relatedness, immunological cross reactivities, kinetic properties and genome organizations can be subdivided into the following distinct classes: phi (F), tau (U), zeta (Z), theta (T), lambda (λ), dehydroascorbate reductase (DHAR), and tetrachlorohydroquinone dehalogenase (TCHQD) [4-10]. The majority of the plant GSTs belongs to the tau (GSTU) and phi (GSTF) classes, which are plant specific.

GSTs are known as promiscuous enzymes capable of catalyzing the conjugation of GSH with a broad range of electrophilic substrates [11-13]. GSTs exhibit wide substrate specificity toward electrophile molecules including organic halides, organic hydroperoxides, epoxides, arene oxides, α- and β-unsaturated carbonyls, organic nitrate esters, and organic thiocyanates [14]. GSTs not only catalyze the conjugation of GSH to electrophilic compounds but they also have more functions. For example, some members are involved in GSH-dependent isomerization reactions (e.g. in GSH-dependent isomerization of maleylacetoacetate to fumarylacetoacetate), in the synthesis of sulfur-containing secondary metabolites such as volatiles and glucosinolates, and the conjugation, transport and storage of reactive oxylipins, phenolics and flavonoids [5]. It is widely assumed that the functional promiscuity of GSTs correlates with structural flexibility, which allows for recognition of diverse structures at minimal energetic cost [15]. Typical GST-catalyzed reactions are schematized in Fig. (**1**).

GSTs play a crucial role in the protection of cells from a wide range of biotic and abiotic stresses, including pathogen attack, xenobiotic and heavy metal toxins, oxidative stress and UV radiation [16-20]. Their role in stress tolerance in plants is less characterized than their detoxification function [21], however, GSTs are thought to be evolved as part of the cell protection system against oxygen toxicity [22,23]. The

*Address correspondence to this author at the Laboratory of Enzyme Technology, Department of Agricultural Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855-Athens, Greece; Tel/Fax: +30 210 5294308; E-mail: lambrou@aua.gr

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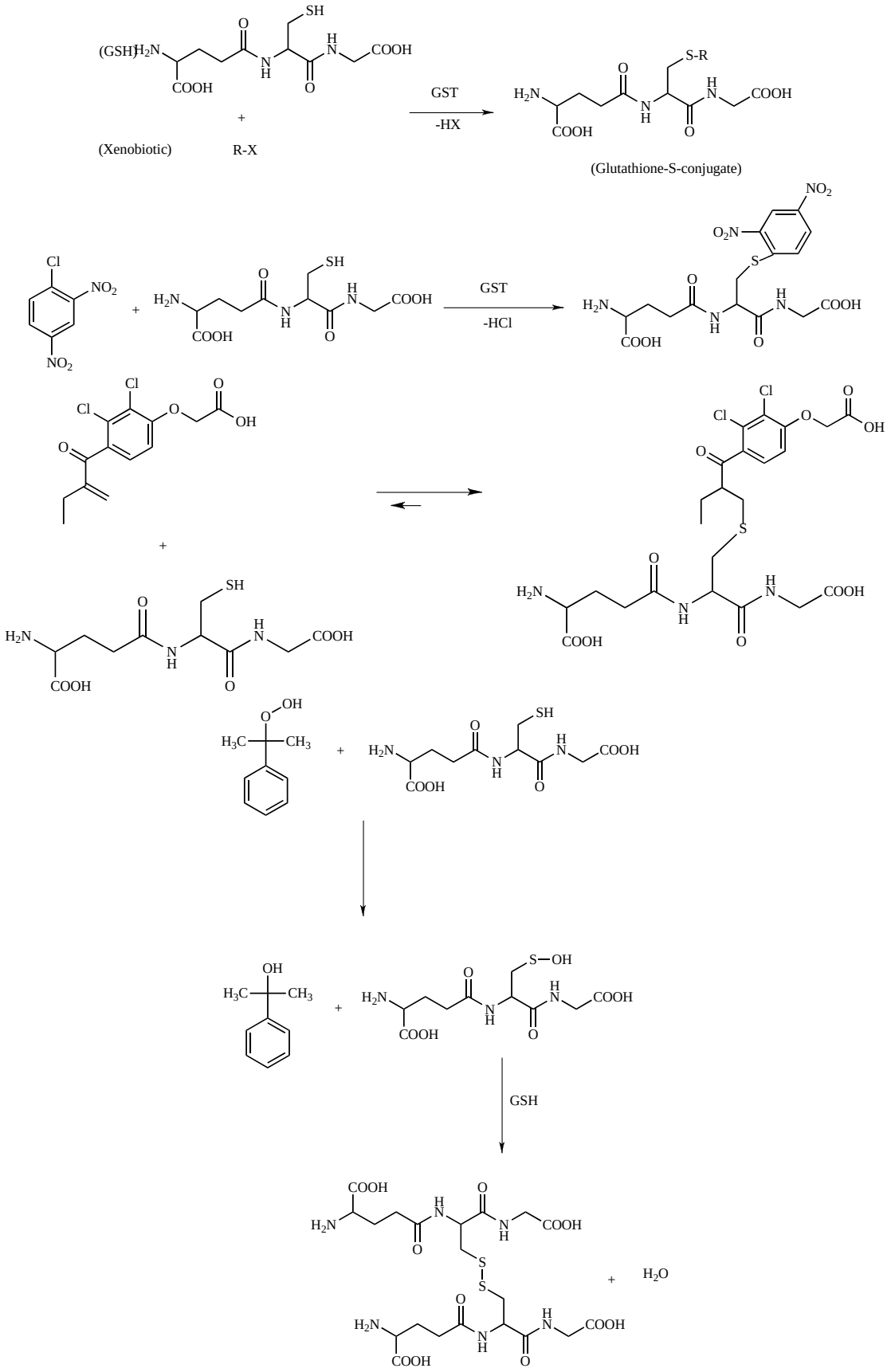


Fig. (1). A: Glutathione conjugation to a generic xenobiotic (X) catalyzed by a GST, results in the formation of a glutathione-S conjugate. B: Typical GST-catalyzed reactions. (1): nucleophilic aromatic substitution with 1-chloro-2,4-dinitrobenzene, (2): Michael-type addition reaction with ethacrynic acid, (3): hydroperoxide reduction with cumene hydroperoxide.

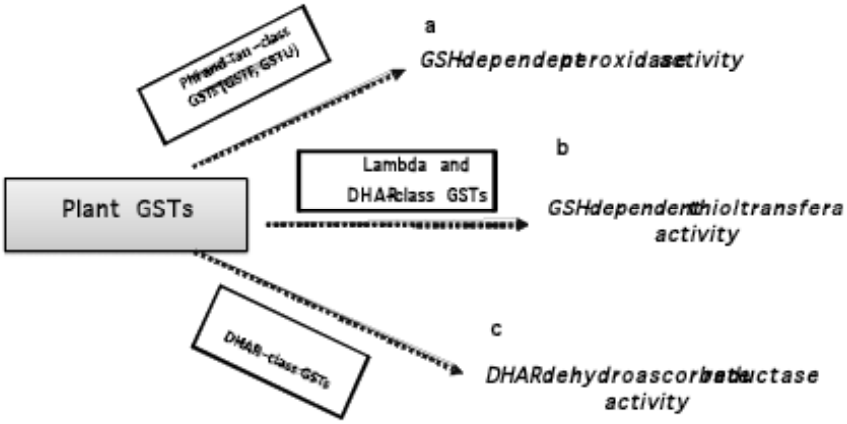


Fig. (2). Catalytic activity in relation to the antioxidant function of GSTs: a) peroxidase activity, b) GSH-dependent thioltransferase activity, and c) dehydroascorbate reductase activity.

antioxidant catalytic function of GSTs [9] is displayed through peroxidase (GPxs) [24], thioltransferase and dehydroascorbate reductase activity [21,25,26] (Fig. **2**).

Proteins able to participate in unrelated biological processes have been grouped under the generic name of moonlighting proteins [27,28]. Work with different organisms has uncovered a great number of GST isoenzymes that are able to participate in unrelated biological processes. In addition to their role in catalyzing the conjugation of electrophilic substrates to GSH, these enzymes also carry out a range of other functions. Different activities of GST isoenzymes include their role as modulators of signal transduction pathways that control cell proliferation and cell death, regulation of the metabolic pathways, bind non-catalytically and transfer a wide range of endogenous and exogenous ligands [5,6,29,30,31]. For example, the isoenzyme GSTP1-1 from human is an ubiquitously expressed protein that plays an important role in the detoxification and xenobiotics metabolism. This isoenzyme, has been associated with the development of tumor resistance to anticancer drugs, acts as a repressor of JNK and other protein kinases involved in stress response, cell proliferation, and apoptosis, and plays an important regulatory role in TNF-α-induced signaling by forming ligand-binding interactions with TRAF2 [32,33]. Another example of moonlight activity comes from the protein Ure2 [34]. Ure2 is an important regulator of nitrogen catabolite repression, the process that controls the utilization of available nitrogen sources by *S. cerevisiae*. Ure2 does not have a typical GST substrate specificity but belongs to a subset of GST proteins that exhibits glutathione peroxidase activity and are active against different oxidants [35].

2. ANTIOXIDANT CATALYTIC FUNCTION OF GSTs

GSH can function as an antioxidant and as a substrate or cofactor of GSTs [7,36-41]. GSH is mainly known for its antioxidant function against Reactive Oxygen Species (ROS) and hydrogen peroxide (H_2O_2) [42,43]. The high concentration of ROS can lead to a non-controlled oxidation of DNA, proteins and membrane lipids which can cause disruption of metabolism and cellular structure destruction [41,44].

Plant GSTs exhibit GSH-dependent peroxidase activity (GPx , EC 1.11.1.9) [24,45] and act protectively against cytotoxicity by reducing organic hydroperoxides of fatty acids and nucleic acids to monohydroxyalcohols which are less toxic [1,10,25]. This reaction is important as prevents the formation of cytotoxic aldehyde derivatives from organic hydroperoxides degradation [10].

Plant GSTs with GPx activity contribute to defence against oxidative injury during various stresses, including oxidative stress, pathogen attack, herbicide treatment, and to abiotic stresses [46]. It was suggested that in addition to the direct protective effect of the GPx activity, the enhanced tolerance may be due to the GPx-mediated increase in GSSG concentration in the cells, which then function as a signal to activate further protective stress responses [47-49].

The GPxs in plants can be divided into three types. These are the selenium-dependent GPxs identified in *Aloe vera* [50], the non-selenium dependent phospholipids hydroperoxide glutathione peroxidases (PHGPxs) and glutathione transferases showing glutathione peroxidase activity [51]. The selenium-dependent GPxs composed of four 16 kDa subunits contain selenocysteine at the catalytic site and appears to be similar to mammalian cytosolic GPx. PHGPx contain cysteine at the catalytic site and appears to be different to the mammalian type PHGPxs. These enzymes can be widely found in plant cells including chloroplasts, mitochondria, cytoplasm, peroxisome and apoplast [51-53].

Plant *theta* and *tau* class GSTs exhibit high GPx activities toward organic hydroperoxides [54]. For example, the isoenzymes from wheat [16], peas [8], soybean [55], monocot weeds such as *Alopecurus myosuroides* (blackgrass), and dicot weeds such as *Arabidopsis thaliana* [51,56] display wide substrate specificity towards organic hydroperoxides. In particular, the *phi* and *tau* class GSTs from *Arabidopsis thaliana* have shown high peroxidase activity with linoleic acid hydroperoxides (13-hydroperoxy-9,11,15-octadecatrienoic acid and 13-hydroperoxy-9,11-octadecadienoic acid) [56].

The isoenzymes of the GST-like class with dehydroascorbate reductase (DHAR) activity catalyze the reduction of dehydroascorbate (DHA) to ascorbic acid using GSH. Members of this class have already been found in *Arabidopsis* [9], rice and soybean [21]. The DHARs do not exhibit

GSH conjugating activity. Unlike most other GSTs, DHARs are monomeric and form mixed disulfides with GSH [9].

Members of the lambda and DHARs classes of GSTs, exhibit thioltransferase activity using the 2-hydroxyethyl disulfide (HED) as a substrate [9]. In cases of oxidative stress, when there is a lack of GSH, some protein thiols are S-thiolated making protein-thiol disulfides (Fig. **3**). This modification affects the activity of the proteins or enzymes. Whereas many proteins are active when the key sulfhydryls are in the thiol form, others require them to be in the oxidized, disulfide form [57,58]. For example, glutathione disulfide (GSSG) can activate enzymes such as glucose-6-phosphatase, acid phosphatase, γ-aminolaevulinate synthetase, creatine kinase, etc. On the other hand, glutathione disulfide inhibits glycogen synthetase, pyruvate kinase, adenylate cyclase, phosphorylase/phosphatase, ribonucleotide reductase, phosphofructokinase, etc [10,57,59,60,61].

3. STRUCTURE OF GSTs

GSTs belong to the thioredoxin superfamily (also including thioredoxin, glutaredoxin, and disulfide-bond formation facilitator) classified by the common GSH binding domain-adopted thioredoxin fold (Fig. **4**) [62,63]. So far, the available three-dimensional (3D) that have been solved can be summarized as follows: (i) one phi class GSTs from *Arabodopsis thaliana* [64], two from maize (*Zm*GSTF1 and *Zm*GSTF3) [65,66], (ii) a zeta class GST from *Arabodopsis thaliana* [67], (iii) and three tau class GSTs, one from wheat (*Ta*GSTU4) active in herbicide detoxification [19], one from rice (*Os*GSTU1), and more recently one from *Glycine max* (*Gm*GSTU4-4) [68,69]. Because of the important role of the tau class GSTs, the structure of the *Gm*GSTU4-4 [68,69] will be presented and discussed with regards to the other plant classes.

3.1. Overall Structure

Each soluble GST is, in general, active as dimer of approximately 23–30 kDa subunits of and an average length of 200–250 aminoacids [70] (Fig. **4**). Sequence identity within class is typically >40%. For example, sequence identity within tau class GSTs is shown in Fig. (**5a**). Interclass identities are significantly lower, usually <20% in plants (Fig. **5b**). Although there is little sequence similarity between enzymes of different classes, there is significant conservation in overall structure (Fig. **6**).

Each subunit adopts the same folding pattern, which is called 'GST fold', and consists of two distinct domains: the N-terminal domain (approximately one third of the protein sequence), consisting of β-strands and α-helices as secondary structure elements, usually βαβαββα, similar to the thioredoxin fold [63,64,71] and the all helical C-terminal domain composed of α-helices arranged in a right-handed spiral (Fig. **4**) [23,72,73]. Each subunit has an independent active site, consisting of two regions: a GSH binding site (G-site) in the N-terminal domain and a xenobiotic (hydrophobic) substrate binding site (H-site) in the C-terminal domain [19,65,66,68,69,74] (Fig. **4a,c**).

3.2. Interactions Between Subunits

The interactions that are involved in assembling the quaternary structure of GSTs include salt bridges, hydrogen bonds, hydrophilic and hydrophobic interactions, including a lock-and-key motif that physically anchors the two subunits together [75-77]. The lock-and-key motif is a common feature of GSTs of the tau, phi, alpha, mu and pi classes [67,75,76]. Only subunits with the same interfacing type appear to be compatible for dimerization. Subunits from different classes of GST are not able to dimerize because of the incompatibility of the interfacial residues [78,79].

3.3. GSH Binding Site (G-site)

In each monomer the G-site is located in a polar region, formed by the beginning of helices H1, H2, and H3 in the N-terminal domain, (Figs. **4A, 7**) [68]. The G-site contains specific residues critical for GSH binding and catalytic activity. In particular, a highly conserved, catalytically essential Ser of the tau (Ser13 in *Gm*GSTU4-4) [68,80], phi, zeta, and theta classes plant and of insect delta class GSTs and Tyr of the mammalian alpha, mu, pi classes GSTs have a crucial role in the mechanism of GSH activation [6]. The Ser/Tyr hydroxyl group acts as hydrogen bond donor to the thiol group of GSH, contributing to stabilization of reactive thiolate anion which is the nucleophile group for the electrophilic substrate [72,81]. GSTs that belong to the, omega,

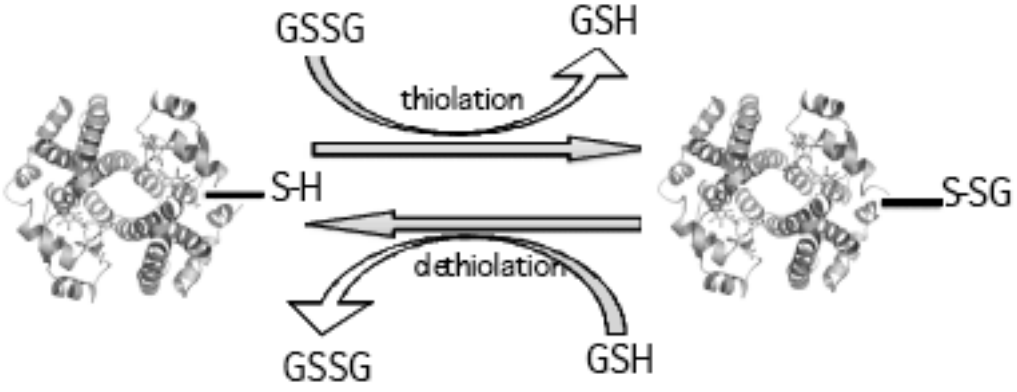


Fig. (3). Thioltransferase activity plays regulatory and protective role through reversible thiolation and dethiolation reactions.

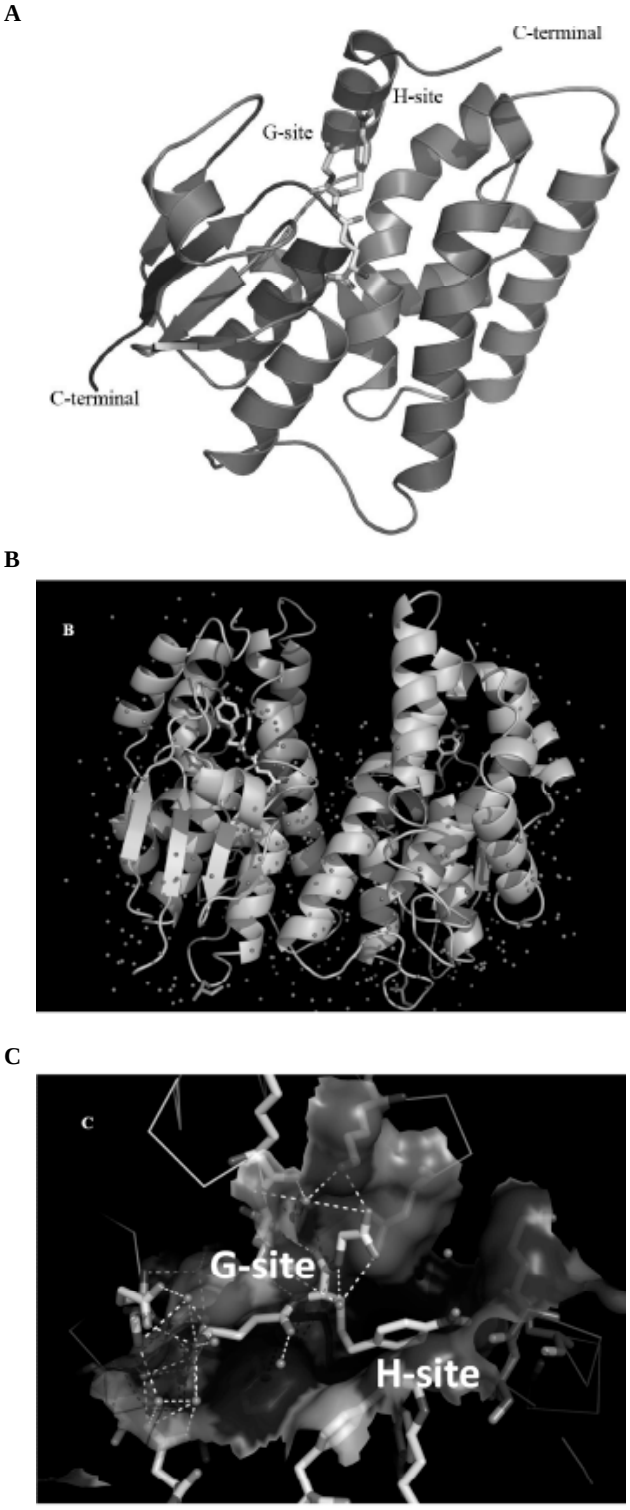


Fig. (4). A cartoon representation of the tau class *Gm*GSTU4-4 monomer (A), dimer (B) and the substrate binding site (C). Secondary structure elements and the location of G- and H-site are labelled. The water molecules are represented by spheres. The bound inhibitor S-(p-nitrobenzyl)-glutathione (Nb-GSH) is shown in a stick representation. The figures were produced using PyMol.

beta and lambda classes contain instead of Ser/Tyr, a catalytically essential Cys, which is involved in forming a mixed disulfide with GSH [9].

The analysis of crystal structures of soluble GSTs clearly demonstrates that, several active-site residues and a functionally conserved electron-sharing network contributes to the formation and stabilization of the thiolate anion. Amino acids mainly with positive charges for instance Arg18 (α-helix H1) located at the bottom of the G-site, which is conserved among all tau GST sequences, although not involved directly in the formation of the G-site, seems to have an indirect role in GSH binding, and in stabilization of G-site architecture through a network of hydrogen bonds and electrostatic interactions [68].

3.4. Electrophilic Binding Site (H-Site)

The H-site is composed of non-conserved residues from the C-terminal domain (Figs. **4** and **7B**). In general, the H-site of GSTs exhibits a low degree of sequence identity that determines substrate specificity (Fig. **5A**). For example, the H-site of *Gm*GSTU4-4 is typically hydrophobic, and is built predominantly by hydrophobic residues from the C-terminal domain: helix H4a, (Tyr107, Arg111), helix H6 (Trp163) helix H9 (Phe208, Leu212, Lys215 and Leu216), and Phe10 and Leu37 from the N-terminal domain [68,69].

3.5. Ligand Binding Site (L-Site)

In addition to their catalytic function GSTs act as ligand-binding proteins and bind hydrophobic molecules (azo-dyes, bilirubin, heme, polycyclic aromatic hydrocarbons, steroids, thyroid hormones, plant hormones and flavonoids) in a non-substrate manner into a distinct site. This site is termed L-site [7,68,82-87].

Little information is available about the exact localization and the nature of the L-site in GSTs. Variation in the location of L-site between different GST isoenzymes is a well-known feature of GSTs. For example, the L-site in *Gm*GSTU4-4 was found to bind the molecule (4-nitrophenyl) methanethiol [68] and is located in a hydrophobic surface pocket formed by Trp11, Arg20, Tyr30, Tyr32, Leu199 and Pro200 (Fig. **8**). The main binding residues (Trp11, Arg20, Tyr30 and Tyr32) are, in general, conserved within the tau GST family (Fig. **5A**). On the other hand, the L-site of GST from *Schistosoma japonica* [82] is located at the dimer interface. In the case of the *Arabidopsis* enzyme [88], the L-site is located next to the G-site between the side chains of helices α3''/α3''' and α5'', whereas the L-site of the human pi class GST and the maize GST I is located into the H-site [83,86].

The precise role of L-site is unclear. However, it has been proposed that binding of non-substrate ligands to GST prevents modification (e.g. degradation, oxidation) of the molecules *in vivo*. Another possibility is that GST prevents cellular damage that may be caused by cytotoxic and genotoxic compounds. The other possibility is that binding to L-site may help to the delivery of the ligands to specific cellular protein receptors or compartments [83,84,86,68]. Lu and Atkins (2004) have demonstrated the possible antioxidant role for the ligandin activity of GSTs [29]. More recently, Dixon and Edwards have shown that GSTUs from *Arabidopsis thaliana* are able to bind tightly thioester of faty acids

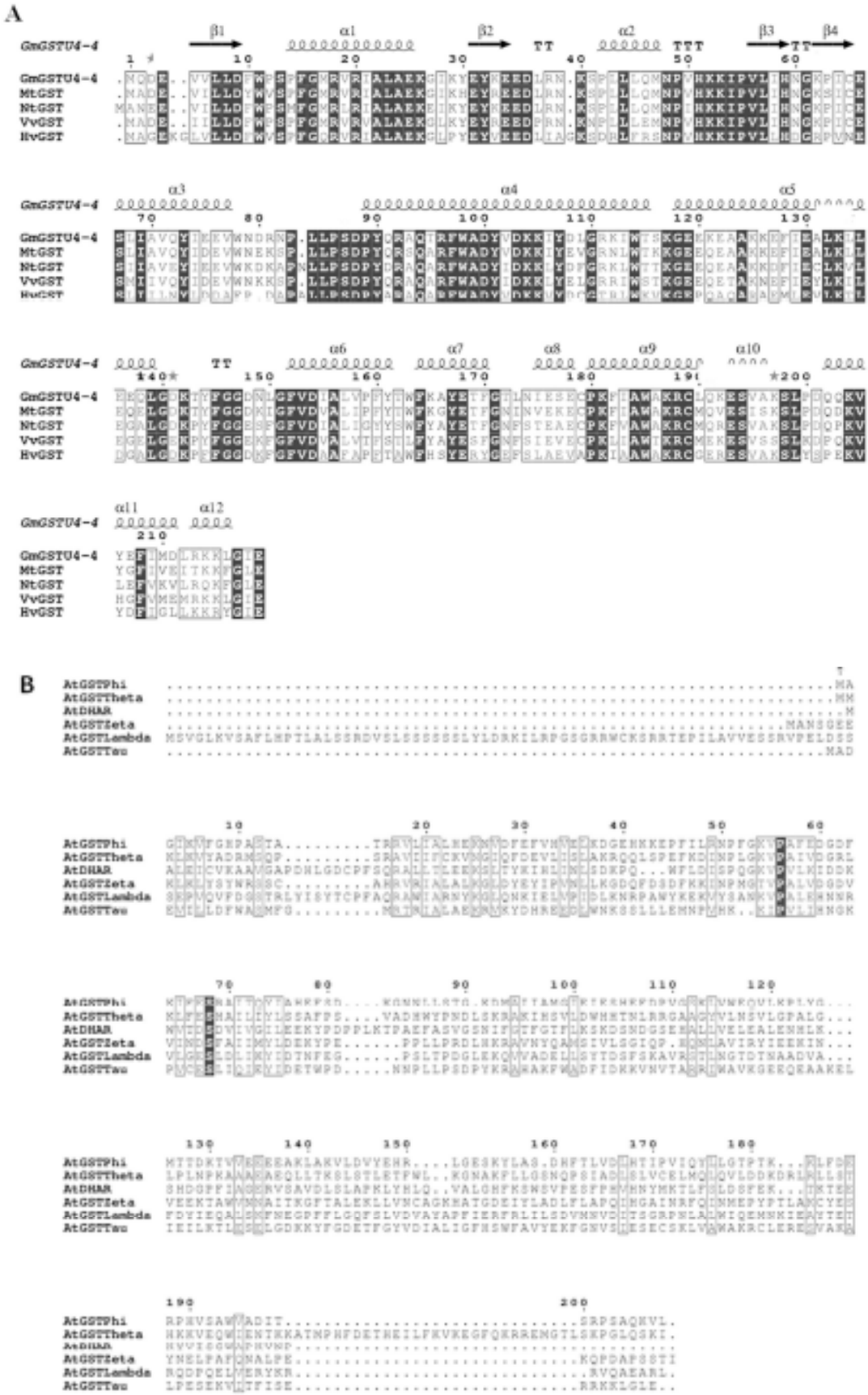

A
GmGSTU4-4
MtGST
NtGST
VvGST
HvGST
B
AtGSTPhi
AtGSTtheta
AtDHAR
AtGSTzeta
AtGSTLambda
AtGSTTau

Fig. (5). Contd….

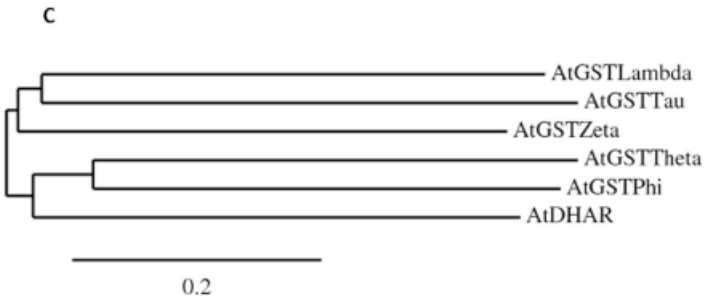


Fig. (5). A: Sequence alignment of members of the tau family of GSTs compared with the secondary structure of *Gm*GSTU4-4 (PDB code 2VO4) produced using ESPript (http://espript.ibcp.fr/ESPript/ESPript/). *Gm*GSTU4-4 numbering is shown above the alignment. Alpha helices and beta strands are represented as helices and arrows, respectively, and beta turns are marked with TT. Conserved areas are shown shaded. A column is framed, if more than 70 % of its residues are similar according to physico-chemical properties. This sequence alignment was created using the following sequences (NCBI accession numbers are in parentheses): *Gm*GSTU4-4: *Glycine max* (AAC18566), *Nt*GST: *Nicotiana tabacum* (CAA39707), VvGST: *Vitis vinifera* (XP_002263395), *Mt*GST: *Medicago truncatula* (ACJ85907), *Hv*GST: *Hordeum vulgare* (ABI18247). B: Sequence alignment of representative members of the *Arabidopsis thaliana* GST family (phi, theta, DHAR, lambda and tau). Conserved areas are shown shaded. A column is framed, if more than 70 % of its residues are similar according to physico-chemical properties. This sequence alignment was created using the following sequences (NCBI accession numbers are in parentheses): *At*GST Phi (NP_171792); *At*GST theta (NP_198937); *At*DHAR (Q9FWR4); *At*GST zeta (Q9ZVQ3); *At*GST tau (AAS76278); *At*GST lambda (NP_191064). C: Phylogenetic analysis of representative members of the *Arabidopsis thaliana* GST family (phi, theta, DHAR, lambda and tau) (TreeDyn program run at http://www.phylogeny.fr/).

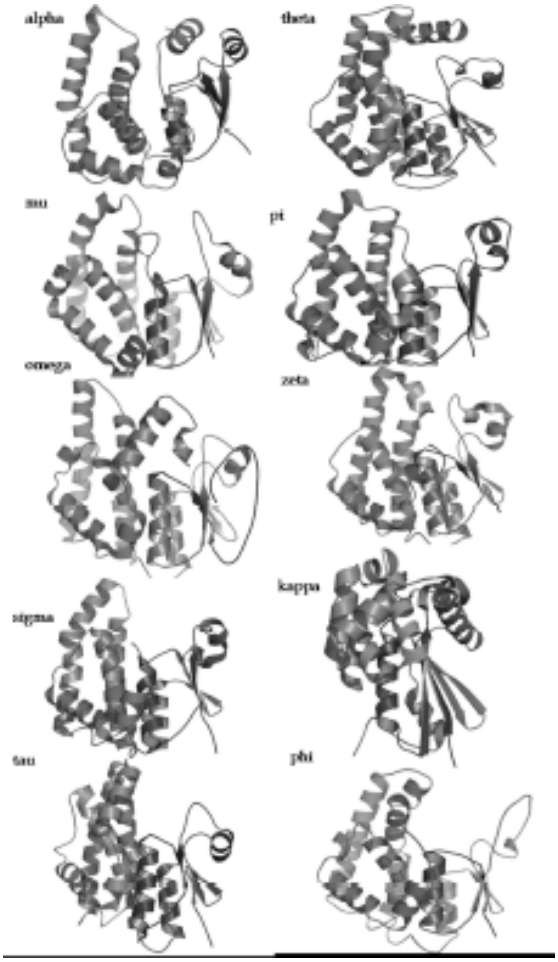
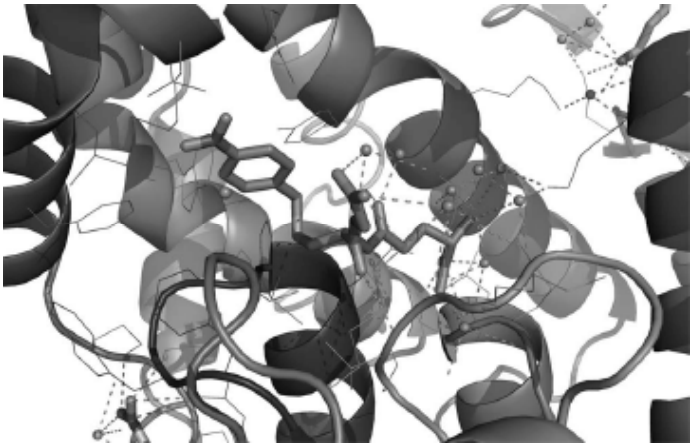


Fig. (6). Ribbon representations of the structures of the GST classes: alpha (PDB code: 1gse), mu (PDB code: 1hna), pi (PDB code: 1glp), theta (PDB code: 1ljr), zeta (PDB code: 1fw1), omega (PDB code: 1eem), sigma (PDB code: 1mou), kappa (PDB code: 1yzx), phi (PDB code: 1aw9), tau (PDB code: 1gwc). The figure was produced using PyMol.

A



B

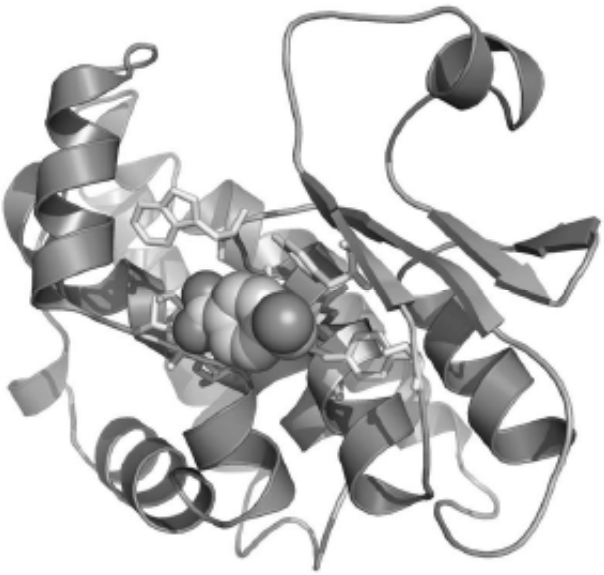


Fig. (7). Cartoon representation of the G- and H-site of *Gm*GSTU4-4 with the inhibitor S-(p-nitrobenzyl)-glutathione. Amino acid side chains that contribute directly to G and H-site formation are shown in a stick representation. The figure was produced using PyMol.

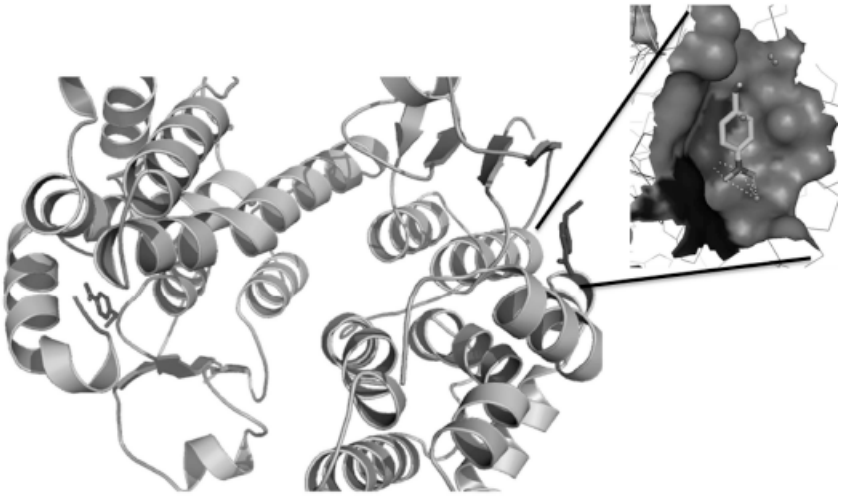


Fig. (8). A representation of the putative L-site of *Gm*GSTU4-4 with the ligand (4-nitrophenyl)-methanethiol. The ligand (4-nitrophenyl)-methanethiol is represented as a stick. The figure was produced using PyMol.

with varied chain length ($C_{(6)}$ to $C_{(18)}$), oxygen content, and desaturation, with $K_{(d)}$ approximately 1 μM [89]. The strong and binding of various fatty acids by each GSTU and the conservation in binding observed in the different hosts suggest that GSTUs have selective roles in binding and conjugating these unstable metabolites *in vivo*. In addition, the same group of researchers has shown that the ability of GSTs to act as ligand binding proteins of porphyrins *in vitro* [90] results in highly specific interactions with porphyrinogen intermediates, which can be demonstrated in both plants and bacteria *in vivo* [91].

In conclusion, the plant GST family of enzymes belongs to the thioredoxin superfamily classified by the common GSH binding domain-adopted thioredoxin fold. The GST family represents a group of catalysts with multiple roles many of which are important in counteracting biotic and abiotic stress. These roles can be relevant to maintaining cellular homeostasis as well as in the direct detoxification of toxic compounds. The detoxification roles of GSTs arise for their ability to catalyze the conjugation of GSH to a large number of electrophilic molecules. The antioxidant catalytic function of GSTs is exhibited through peroxidase, thioltransferase and dehydroascorbate reductase activity. Further analysis and study of this protein family will inevitable reveal many examples of functional and catalytic diversification and will highlight the importance of these enzymes in the protection against the oxidative stress and in other cellular processes.

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ABBREVIATIONS

CDNB	=	1-chloro-2,4-dinitrobenzene
GSH	=	Glutathione
GST	=	Glutathione transferase
G-site	=	GSH binding site
GPx	=	Glutathione peroxidase
H-site	=	Hydrophobic binding site; S-(p-nitrobenzyl)-glutathione
Nb-GSH	=	Sec, Selenocysteine
ROS	=	Reactive Oxygen Species

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