PURIFICATION OF GLUTATHIONE S-TRANSFERASES AND GENETIC CHARACTERIZATION OF ZETA ISOZYME FROM *Pinus brutia*, Ten.

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

PURIFICATION OF GLUTATHIONE S-TRANSFERASES AND GENETIC CHARACTERIZATION OF ZETA ISOZYME FROM *Pinus brutia*, Ten.

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Glutathione *S*-transferases (GST, EC2.5.1.18) are a family of multifunctional, dimeric enzymes that catalyse the nucleophilic attack of the tripeptide glutathione (*γ*-L-glutamyl-L-cysteinyl-L-glycine) on lipophilic compounds with electrophilic centres. The primary function of GSTs is generally considered to be the detoxification of both endogenous and xenobiotic compounds. Cytosolic GSTs have been grouped into eleven distinct classes as: (A); Alpha, (M); Mu, (P); Pi, (S); Sigma, (T); Theta, (Z); Zeta, (F); Phi, (U); Tau, (B); Beta, (O); Omega and (L); Lambda.

In this study, the total RNAs from *Pinus brutia* needles were isolated, GST Zeta cDNA was prepared by RT-PCR, the length of the insert was elongated by applying 5' RACE (Rapid Amplification for cDNA ends) method and the identity of the insert was checked by sequencing. The amino acid sequence of GST-Zeta was deduced as composed of 226 amino acids. The genomic DNA was also isolated from *Pinus brutia* needles, amplified by PCR and sequenced, and compared to the sequence of cDNA. The expression level of GST-Zeta in individual trees of *Pinus brutia* were examined by Northern blot analysis, and compared to their thiol contents. The mRNA levels varied up to three-fold, whereas GSH amounts varied approximately 1.8 fold, and there were no correlation between the GST-Zeta expression and GSH concentration.

GST enzyme with activity towards CDNB was isolated and purified from *Pinus bruti*a needles in 1.95 % yield with a purification factor of 15.45-fold. The purification protocol included a sequential chromatography on Sephadex G-25 column, DEAE cellulose anion exchanger liquid chromatography column, and *S*-hexylglutathione agarose affinity columns. The purified GST showed specific activity towards CDNB as 2022 nmole/min/mg. The GST purified from needles had a molecular weight (Mr) value of about 24.000 which was confirmed by SDS-PAGE.

Key Words: Glutathione *S*-transferases, Zeta class GSTs gene sequence, Purification, SDS-PAGE Electophoresis, Characterization.

ÖZ

Pinus brutia, Ten.'den GLUTATYON S-TRANSFERAZLARIN SAFLAŞTIRILMASI VE ZETA İZOZİMİNİN GENETİK KARAKTERİZASYONU

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Glutatyon S-transferazlar (GST) dimerik konformasyona sahip, bir tripeptid olan glutatyonun lipofilik yapılardaki elektrofilik merkezlere nükleofilik olarak konjugasyonunu katalizleyen çok fonksiyonlu enzim grubudur. Birincil fonksiyonları ksenobiotiklerin ve endojenlerin detoksifikasyonlarında rol almalarıdır. Sitozolik GST ler onbir sınıf altında toplanırlar; (A); Alfa, (M); Mu, (P); Pi, (S); Sigma, (T); Teta, (Z); Zeta, (F); Fay, (U); Tau, (B); Beta, (O); Omega and (L); Lambda.

Bu çalışmada, total RNA'lar *Pinus brutia* iğne yapraklarından izole edilerek, GST Zeta cDNA'sı RT-PCR metoduyla hazırlanmış, elde edilen gen 5' RACE (cDNA uçlarının hızlı çoğaltılması) metodu ile uzatılmış ve gen dizisi tayin edilmiştir. Bu sekanstan yola çıkılarak GST-Zeta'nın amino asit dizisi ve 226 amino asitten oluştuğu belirlenmiştir. Ayrıca *Pinus brutia* yapraklarından genomik DNA izole edilerek, PCR metodu ile çoğaltılmış, sekans tayini ve cDNA sekansı ile karşılaştırması yapılmıştır. Farklı ağaçlardaki GST-Zeta gen ekspresyonları Northern blot metodu ile analiz edilmiş, içerdikleri tiol miktarları tayin edilmiş ve karşılaştırılmaları yapılmıştır. Buna göre, mRNA ekspresyonları üç kat farklılık gösterirken, GSH miktarlarının yaklaşık 1.8 kat farklılık gösterdiği ve GST-Zeta ekspresyonları ile GSH konsantrasyonlarının birbiriyle ilişkili olmadığı bulunmuştur.

CDNB'e karşı aktivite gösteren GST'nin *Pinus brutia* iğne yapraklarından %1.95'lik ürün ve 15.45 kat saf olarak izolasyonu ve saflaştırılması gerçekleştirilmiştir. Saflaştırma protokolü sırasıyla Sephadex G-25, DEAE anyon değişim, *S*-hexylglutatyon afinite kolon kromatografisinden oluşmaktadır. Saflaştırılan numune CDNB'e karşı 2022 nmole/min/mg değerinde aktivite göstermiştir. Saflaştırılan iğne yaprak GST enziminin SDS-PAGE ile moleküler ağırlığı (Mr) 24.000 Da olarak bulunmuştur.

Anahtar kelimeler: Glutatyon *S*-transferazler, Zeta sınıfı GST'lerin gen sekansı, Saflaştırma, SDS-PAGE Elektoforez, Karakterizasyon

TO THE WOMAN OF MY LIFE…

TO MY MOTHER…

ONCE AGAIN…

WITH LOVE…♥

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NOMENCLATURE

APS	Ammonium persulfate
Bis	N,N'-methylene bisacrylamide
BSA	Bovine serum albumin
CDNB	1-Chloro-2,4-dinitrobenzene
CYP	Cytochrome P450 enzyme family
CTAB	Cetyltrimethylammonium bromide
DEAE	Diethylaminoethyl
DIECA	Diethyldithiocarbamic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EA	Ethacrynic acid
EDTA	Ethylenediaminetetraacetic acid
EPNP	1,2-Epoxy-3-(p-nitrophenoxy)propane
EtBr	Ethidium Bromide
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione-S-transferase
HPLC	High-pressure liquid chromatography
LB	Luria Broth

MS	1-Menaphthyl sulfate
4-NBC	4-Nitrobenzyl chloride
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene difluoride
PVP-K 30	Polyvinylpoly pyrrolidone
5'end RACE	Rapid Amplification for cDNA Ends
ROI	Reactive Oxygen Intermediates
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylenediamine
Tris	Tris(hydroxymethl) aminomethane

CHAPTER I

INTRODUCTION

Pine refers to coniferous trees of the Genus *Pinus* in the Family Pinaceae. There are about 115 species of *Pinus*, although different authors accept anything between about 105 to 125 species (Richardson, 1998).

1.1 Taxonomy and Description of *Pinus brutia*

Pinus brutia Tenore (Turkish red pine, East Mediterranean pine and Calabrian pine have all been used as a name) is a member of Division Gymnospermae, Class Coniferae, and Family Pinaceae, Genus *Pinus* L. According to Farjon (1984), it is closely related to *Pinus halephensis*, but also showing relationships to other Mediterranean-East Asian pines, like *Pinus nigra* and *Pinus sylvestris* (Theilges, 1972).

The studies on the chemical and physical analysis of the gum terpentine as genetic markers (Gallis and Panetsos, 1997), HPLC chromatography of needles flavanoids (Shankur-Kaundun *et al*., 1997) and recently by using chloroplast genome simple sequence repeats (Morgante *et al*., 1998; Bucci *et al*., 1998) show that *P. halepensis* and *P. brutia* have different halotype profiles, therefore they are distinct species (Kandemir, 2002).

According to Frankis (1993), three varieties and one subspecies were recognized. Those are namely;

P. brutia var. *pityusa* (Steven)

P. brutia var. *stankewiczii* (Sukaczev)

P. brutia var. *pendulifolia* (Frankis)

P. brutia subsp. *eldarica* (Medwed).

The Turkish red pine is a tree which grows up to 27-35 m in height, with a usually open crown of irregular branches. The bark on the lower trunk is thick, scaly, fissured, patterned red-brown and buff, and thin, flaky and orange-red higher in the crown. The shoots are slender, 3-7 mm thick, grey-buff, and rough with persistent small decurrent scale-leaf bases. The winter buds are ovoid-acute, with red-brown scales with long free tips revolute and fringed with white hairs. The adult leaves are retained for 1.5-2.5 years, with a persistent 1-1.5 cm sheath; on most trees they are in fascicles of two, and 10-18 cm long. They are bright green to yellow-green, slender, about 1 mm thick, with serrulate margins, fine lines of stomata on both faces, and several marginal resin canals. The juvenile leaves are glaucous, 1.5-4 cn long, and continue to be grown for 2-4 years, mixed with the first adult foliage produced from 9 months from seed. The cones are erect to forward pointing on short stout stalks, symmetrical, broad conic, (4-)6-10(-12) cm long, 4-5 cm broad when closed, green, ripening shiny red-brown in April two years after pollination. They open the same summer or 1-2 years later, to 5-8 cm broad, though the seeds are often not shed till winter rain softens the scales. The scales are short, broad, thick, woody, and vey stiff; the apophysis is 10-15 x 15-20 mm, smoothly rounded, with a slight to moderate transverse ridge; the umbo is dorsal, flat to slightly raised, 5-7 mm wide, and grey-buff. The seeds are grey-brown, 7-8 x 5 mm with a broad, auricled 15-20 x 10 mm wing, yellow-buff streaked darker brown (Frankis, 1993).

The vars. *pitsuya* and *stankewiczii* differ very little from the type in morphology, but showed differences in electrophoretic tests (Conkle *et al*., 1988). Var. *pendulifolia* differs in having markedly longer leaves, 20-29 cm long, which because of their length are pendulous (Frankis, 1993).

Subsp. *eldarica* has shorter, stouter leaves 8-13 cm long, slightly smaller cones and slightly larger seeds, and is adapted to a different climatic regime (Frankis, 1993).

1.2 Biology and Economic Importance of *Pinus brutia* in Turkey

Pinus brutia Ten. (Turkish red pine) exhibits considerable variation in its form and growth characteristics. It is the most important forest tree in the north-eastern Mediterranean area. *P. brutia* forests are climax vegetation of the Mediterranean Region in Turkey. The species is also very important in terms of Turkish forestry and forest products since it has the ability to grow rapidly (with several flushes in a year). The annual shoot consists of one spring shoot arising from a winter bud and one or more summer shoots (Lanner, 1976; Kandemir, 2002). This species is also important in the cropping areas, as their resinous needles inhibit the growth of other plants (*e.g.* weeds). A sap-sucking insect *Marchalina sp*. produces large amounts of honey-dew, harvested by honeybees and sold as 'pine honey' which brings another income (Frankis, 1993).

1.3 Natural Distribution of *Pinus brutia* in Turkey

The 15% of the total forestland in Turkey is covered by *P. brutia* (3,096,064 ha.). Natural distribution of Turkish red pine in Turkey covers the Mediterranean region, almost 50% of the *P. brutia* forests found mainly in coastal areas of Mediterranean region (Neyişci, 1987; Kandemir, 2002), the Aegean region, with 40% coverage has the second largest *P. brutia* distribution and the Marmara Region, with the 10% coverage, mainly in Gelibolu and Biga Peninsula (Kandemir, 2002).

P. brutia Ten. can grow at different altitudes changing from sea level to 1300 m in stands and to 1500 m as individuals. It is possible to see between 800

to 900 m in the Mediterranean region to the northern parts of Turkey, and from 600 to 700 m in the Marmara region (Neyişci, 1987).

1.4 Genetic Variation in *Pinus brutia*

Conifers are one of the most genetically variable groups of plants as they are coming from wide range of geographical distrubition, population structure, pollination mechanism (mating system), seed dispersal (gene flow), stages of succession and fecundity features (El-Kassaby, 1991; Hamrick *et al*., 1992; Kandemir, 2002).

In recent years, attention has been given to genetic variation in various traits and improvement of the species (Işık, 1986; Işık *et al*., 1987; Kara *et al*., 1997; Kaya and Işık, 1997; Işık and Kara, 1997; Kandemir, 2002). The first study on genetic variation in seedling traits of *P. brutia* has carried out by Işık (1986). In this study a total of 60 wind-pollinated families of *P. brutia* were grouped into six populations at different elevations in southern Turkey, raised in a nursery near Antalya, and assessed for 16 seed and seedling characteristics. The study concluded that subspecies brutia has locally adapted populations with a predominatly altitudinal variation pattern.

In 1997, Işık and Kara were studied on altitudinal variation in *P. brutia* and its implication in genetic conservation and seed transfer in southern Turkey. Their report, based on growth and isoenzyme analyses, suggested that middle elevation populations (approx. between 400 and 900 m above sea level) growing in less stressful environments of Taurus Mountains perform better, represent higher total genetic diversity and have greater adaptability to high and low elevation sites than the populations from much lower and/or higher elevations.

The significant correlation between elevation and allele frequencies in certain enzyme systems (Kara *et al*., 1997), as well as between elevation and

various morphological characteristics found in previous studies (Işık, 1986; Işık, 1983; Işık *et al*., 1987; Işık and Kaya, 1993; Işık and Kara, 1997) showed that genetic variation between populations was clinical for height, and under strong genetic control, suggesting the existence of a combined selection pressure exerted by human activity and climatic factors associated with the sharp increase in altitude in the Taurus Mountains in the vicinity of Antalya.

According to Panetsos *et al*., (1998), factors such as geographic isolation, long-term negative selection due to needs in wood and resin, soil mosaic, climatic variability due to differences in altitude, as well as forest fires, are expected to have contributed to the species' present genetic structure. This suggests a certain plasticity and adaptability of *Pinus brutia* but also the existence of ecotypes adapted to different environments (Calamassi *et al*., 1988).

1.5 Stress Physiology in Plants

It is evident that if environmental conditions vary within the tolerance range of living system functioning, then they do not disturb the specialized mechanisms controlling catalytic capacities of the cell provided by regulatory enzyme action. The deviations appearing in a plant relax through operating homeostatic mechanisms. After removing the disturbance, the system rapidly returns to its initial state without any evident residual consequences. If the extent of influence exceeds the tolerance range 'stress conditions', fast adaptive homeostatic responses become insufficient for maintaining an intact living system. In this case, a higher level mechanism of adaptation 'defense' or 'stress' responses starts operating to protect themselves against biotic, abiotic and anthropogenic originly stresses (Veselevo *et al*., 1993).

As plants are confined to the place where they grow, they have to develop a broad range of defense responses to cope with those stresses. Biotic stresses like pathogen attack, abiotic stresses such as drought and desiccatiochilling, heat

shock, heavy metals, UV radiation, air pollutants like ozone and SO_2, nutrient deprivation, high light and mechanical stress, are all play an important role by shaping the life and determining the limits of adaptation. Nearly all ecosystems are subject to these periodic disturbances (Grime, 1993; Wojtaszek, 1997; Mittler, 2002).

The production of Reactive Oxygen Intermediates (ROIs), *e.g.* singlet oxygen (O_2^1), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-), is an unavoidable consequence of aerobic metabolism. In plants ROIs are produced in mitochondria, chloroplast and nitrogen-fixing nodules as unwanted by-products (Møller, 2001). The enhanced production of ROI during stress can pose a threat to cells, and many stress conditions enhance the expression of ROI-scavenging enzymes. However, it is also thought that during stress ROI are actively produced by cells (*e.g.* by NADPH oxidase), and act as signals for the induction of stress-response and defense pathway. Thus, ROIs can be viewed as cellular indicators of stress metabolism and as secondary messengers involved in the stress-response signal tranduction pathway (Mittler, 2002). This view, of the 'extended ROI cycle', is presented in **Figure 1**.

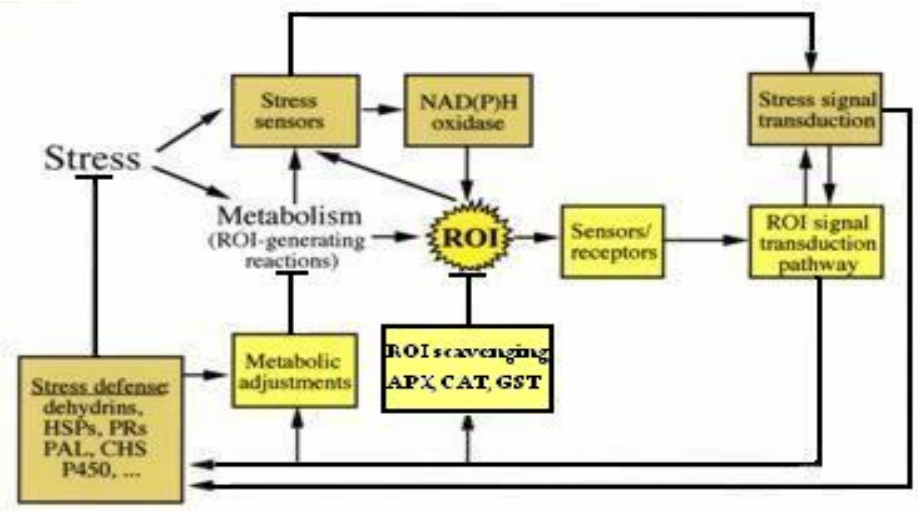


Figure 1 The extended ROI cycle (Mittler, 2002)

This cycle operates in plants during biotic or abiotic stresses. HSPs, heat shock proteins; PR, pathogenesis related proteins; PAL, phenylalanine ammonia-lyaze; CHS, chalcone synthase; P450, cytochrome 450.

Because ROIs are toxic but also participate in signaling events, plant cells require different mechanisms to regulate their intracellular ROI concentrations by scavencing of ROIs. Major ROI-scavenging mechanisms of plants include the enzymes such as superoxide dismutases (SOD), catalases (CAT), ascorbate peroxsidases (APX), glutathione S-transferases (GST) and glutathione peroxidases (GPOX) that catalyze the scavenging of ROIs (Roxas *et al*., 2000; Mittler, 2002).

Above all, responses of organism to stress differ depending on genetic compositions. Furthermore, the nature and intensity of response to a particular stress factor may vary considerably, depending upon the age, degree of adaptation, and seasonal activity of species (Larcher, 1995).

1.6 The Glutathione S-Transferase Enzyme Family

Plants actively detoxify both endogenous toxins, such as secondary metabolites and degradation products arising from oxidative stress, and exogenous man-made chemicals, such as herbicides, using a three-phase detoxification system (Sandermann, 1992; Neuefeind *et al*., 1997a). In the first phase (Phase I), oxidation, reduction, or hydrolysis reactions catalyzed by enzymes such as cytochrome P450 monooxygenases (CYP) result in the exposure, or introduction, of a functional group. Phase II enzymes then catalyze the conjugation of these metabolites with sugars or the tripeptide glutathione (GSH). In the case of GSH, glutathione S-transferases (GSTs) catalyze this conjugation reaction. In the third phase (Phase III) of metabolism, molecules "tagged" with GSH are recognized by ATP-binding cassette transporters in the tonoplast or plasma membrane, which then transfer these conjugates into the vacuole or apoplast (Martinoia *et al*., 1993; Ishikawa, 1992; Rea, 1999).

Glutathione S-Transferases (GSTs, EC.2.5.1.18) are enzymes that detoxify endobiotics and xenobiotic compounds by conjugating glutathione (GSH) to a hydrophobic substrate, forming generally, water soluble and less toxic glutathione S-conjugate. In animals, the conjugate subsequently gets catabolized and excreted (Boyland and Chasseaud, 1969), while in plants the conjugation reaction is coupled to internal compertimentation due to the lack of effective excretion pathways (Sandermann, 1992).

GSH, the tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) is first described in 1888 by de Rey-Pailhade. As he found that yeast and other cells contain a compound that spantenously reacts with elemental sulphur to yield hydrogen sulphide from the Greek words for love and sulphur he chose the name 'philothion' for his substance (Meister, 1988). Today, GSH is thought to be the most abundant low-molecular-weight soluble thiol found in most prokaryote and virtually all eukaryote cells. They are generally considered products of primary

metabolism not essential for life, but important for the detoxification of compounds of unfavorable for growth (Rennenberg and Lamoureux., 1990).

In addition to its role in intracellular detoxification of xenobiotics, GSH may also function in sulphur metabolism as transport and storage form of reduced sulphur (Smith, 1975; Bonas *et al*., 1982; Rauser *et al*., 1991), contributes to scavenging of H_2O_2 (Foyer and Halliwell, 1976; Hossain and Asada, 1987), and participates in phytochelatin synthesis (Grill *et al*., 1990; Rauser, 1990) **(Figure 2)**.

In plants, GSH is present in higher concentrations in the leaves than in the roots, but the seeds contain the highest concentrations of it (Klapheck, 1988). The distribution of GSH in plant cells has been reported by several research groups. According to these results, 7% of the GSH found in the cytoplasm, 17% in the vacuole; however, unequivocal proof that GSH is a constituent of the vacuole is lacking, and 50-76 % of it was found in the chloroplasts and the concentration of GSH in this organelle is in the 1-4mM range (Foyer and Halliwell, 1976; Wolosiuk and Buchanan, 1977; Rennenberg, 1982; Smith *et al*., 1985; Klapheck *et al*., 1987).

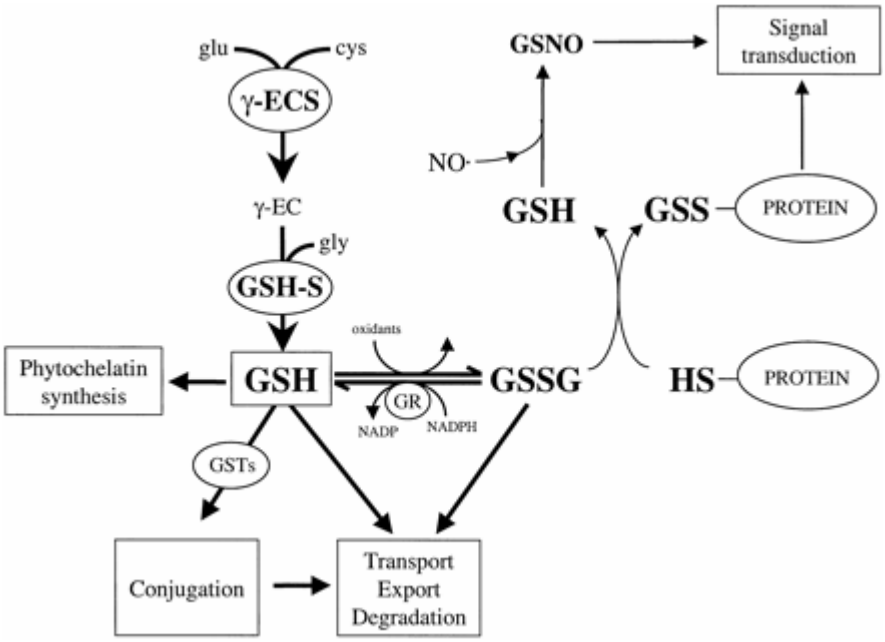


Figure 2 Glutathione biosynthesis and interacting processes in plant cells (Noctor *et al*., 2002)

γ-EC, γ- glutamylcsytein; γ-ECS, γ-gluyamylcystein synthetase; GR, glutathione reductase; GSH, reduced glutatkione; GSSG, glutathione disulphide (oxidized glutathione); GSH-S, glutathione synhetase.

The glutathione pool in the plant tissue is very dynamic and dependent to the rate of synthesis, degradation, translocation and utilization for biosynthetic processes (Rennenberg and Lamoureux., 1990) **(Figure 3)**.

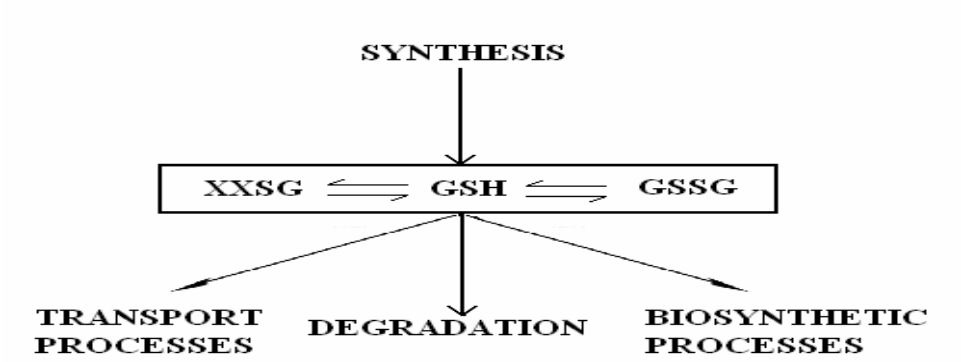


Figure 3 Processes that modulate the concentration of GSH

(Rennenberg and Lamoureux., 1990).

XSSG, glutathione mixed disulphides; GSSG, oxidized glutathione

GSTs have been found in all living species, including plants, animals and bacteria (Hayes and Pulford, 1995). Most GSTs exist as soluble enzymes, although a small family of trimeric microsomal GSTs has been characterized (Andersson *et al*., 1994; Jakobsson *et al*., 1996) and a mitochondrial GST, referred to as GST Kappa, has also been identified (Pemble *et al*., 1996).

The soluble forms exist as dimeric proteins, with subunit molecular weights of approx. 25 kDa. GST subunits appear to be only capable of hybridizing with subunits from the same class (Hayes and Pulford., 1995). Formation of homo- and hetero-dimers presumably allows the formation of a larger number of enzymes from a limited number of genes (Sheehan *et al*., 2001).

The GSTs comprise a complex and widespread enzyme superfamily that has been subdivided further into an ever-increasing number of classess based on their biochemical and immunological characteristics over the last 30 years (Wilce and Parker, 1994). Sequencing studies were used to extend this system and at

least 11 distinct classess of soluble GSTs have been identified so far; (A); Alpha, (M); Mu, (P); Pi, (S); Sigma, (T); Theta, (Z); Zeta, (F); Phi, (U); Tau, (B); Beta, (O); Omega and (L); Lambda from mammals, plants and insects (Dixon *et al*., 2002a).

In mammals, the amino acid sequence identity within class is greater than 70% and interclass identity is usually less than 30% (Rossjhon *et al*., 1998), whereas in plants, identity between classes is remain the same as in mammals, but within classes identity can be as low as 30% as gathered from the full genome sequence of *A.thaliana*.

The plant GSTs, in addition to their enzymatic activities, have less well characterized roles in endogenous metabolism including functioning as glutathione peroxidases counteracting oxidative stress (Cummins *et al*., 1999; Roxas *et al*., 1997), and also acting as flavonoid-binding proteins (Mueller *et al*., 2000), stress signalling proteins (Loyall *et al*., 2000), regulators of apoptosis (Kampranis *et al*., 2000), and catalyze the GSH-dependent isomerization reactions (Dixon *et al*., 2000).

GSTs are distributed in a wide range of organisms ranging from *E.coli* to mammals (Mannervik and Danielson, 1988). They were first discovered in rat tissues (Booth *et al*., 1961), and their presence in plants was first recognized shortly afterwards in 1970, when a GST activity from maize was shown to be responsible for protecting the crop from injury against herbicide atrazine (Frear and Swanson, 1970). Since then GSTs have been identified and characterized with a differential and overlapping substrate specifities in many plants such as maize (Edwards and Owen 1986; Rossini *et al.,* 1996; Jablonkai and Hatzios 1991; Scarponi *et al.,* 1992; Jepson *et al.,* 1994; Holt *et al.,* 1995; Marrs *et al.,* 1995; Hatton *et al.,* 1996; Dixon *et al.,* 1997; Marrs and Walbot, 1997), wheat (Jablonkai and Hatzios, 1991; Mauch and Dudler, 1993, Romano *et al.,* 1993; Edwards and Cole, 1996; Riechers *et al.,* 1996; Riechers *et al.,* 1997), tobacco

(Droog *et al.,* 1995), dwarf pine (Schröder and Rennenberg, 1992), soybean (Ulmasov *et al.,* 1995; Andrews *et al.,* 1997), *Arabidopsis thaliana* (Reinemer *et al.,* 1996), barley (Romano *et al.,* 1993; Wolf *et al.,* 1996), Setaria spp. (Wang and Dekker, 1995), carnation (Meyer *et al.,* 1991a), potato (Hahn and Strittmatter, 1994), chickpea (Hunatti and Ali, 1990; 1991), sorghum (Gronwald *et al.,* 1987; Dean *et al,.* 1990), velvetleaf (Anderson and Gronwald, 1991) and sugarcane (Singhal *et al.,* 1991).

1.6.1 Nomenclature and Classification of GSTs in Plants

Using a classification system based on immunological cross-reactivity and sequence relatedness, soluble mammalian GSTs have been divided into the alpha, mu, pi, sigma, theta and zeta classes (Dixon, *et al*., 1998; Hayes, and McLellan, 1999). Most non-mammalian GSTs were lumped into the highly heterogeneous theta class, which was viewed as closest to the original progenitor of all eukaryotic GSTs. However, the view that plant GSTs are a primitive subgroup is far from the truth.

Three distinct types of plant GSTs were recognized initially (Marrs, 1996). Type I included GSTs with herbicide-detoxifying activity; these genes have three exons. The other large group, type III, consisted mainly of auxin induced GSTs, with the genes containing two exons. Types I and III GSTs show >50% sequence divergence and have now been placed in separate classes (Hayes and McLellan, 1999; Droog, 1997). Type II GSTs have ten exons and are much closer to the mammalian zeta GSTs (Dixon *et al*., 1998). Recently, a Type-IV grouping was proposed for several *Arabidopsis* genes that are similar to classical mammalian theta enzymes (Dixon, *et al.,* 1998). This diversity makes the catch-all theta classification inappropriate. Some plant GSTs clearly group with specific mammalian types but there are two distinct plant-specific types (Dixon, *et al*., 1998). Because the principle of Greek letter designations is widely used for non-plant GSTs (Hayes and McLellan, 1999), Edwards *et al*., (2000) suggested that a

new nomenclature system be adopted for plant GST genes (**Table 1**). Therefore, the current classification system recognizes four main classess, two of which are plant-specific and two of which are more phylogenetically widespread. The plant-specific classes are Phi (previously Type I) and Tau (previously Type III). The Theta class includes enzymes previously designated as Type IV, while the Zeta class includes those previously classified as Type II (Edwards *et al*., 2000).

• Phi (F) – a plant-specific class replacing Type I.

• Zeta (Z) – replacing Type II.

• Tau (U) – a plant-specific class replacing Type III.

• Theta (T) – replacing Type IV.

Table 1 Suggested new nomenclature applied to maize and *Arabidopsis* GSTs (Edwards *et al*., 2000)

Species	Old name	Proposed name
Zea mays	*Zm*GST I	*Zm*GSTF1
Zea mays	*Zm*GST II	*Zm*GSTF2
Zea mays	*Zm*GST III	*Zm*GSTF3
Zea mays	*Zm*GST V	*Zm*GSTU1
Zea mays	*Zm*GST VI	*Zm*GSTU2
Zea mays	*Zm*GST VII	*Zm*GSTU3
Zea mays	*Bronze2*	*Zm*GSTU4
Arabidopsis	*At*GST10	*At*GSTT1
Arabidopsis	GST1, PM239x14	*At*GSTF1
Arabidopsis	GST2, PM24.1	*At*GSTF2
Arabidopsis	GST3, ERD11	*At*GSTF3
Arabidopsis	GST4, ERD13	*At*GSTF4
Arabidopsis	GST6	*At*GSTF5
Arabidopsis	GST7	*At*GSTF6
Arabidopsis	GST8	*At*GSTF7
Arabidopsis	GST11	*At*GSTF8
Arabidopsis	GST5, 103-1a	*At*GSTU1

Very recently, two outlying classes of the GST superfamily in *A.thaliana* which differed from all other plant GSTs by containing a cysteine in place of serine at the active site have been identified by Dixon *et al*., (2002 b). Those are

namely; glutathione-dependent dehydroascorbate reductases (DHARs) and the Lambda (L) GSTs (**Figure 4**).

Using the GSTs of *Arabidopsis* as an example, the nomenclature of the system is explained in **Figure 5**. A remaining problem lies in the numbering of the subunits. In organisms such as *Arabidopsis*, for which comprehensive genome information is available, it is possible to assign the numbering of the genes encoding the GST subunits of each class on the basis of their location on the chromosomes. In plants for which genome information is incomplete or unavailable, however, the current numbering system is based on the order of discovery of the GST genes for each class in the given plant species (Edwards *et al*., 2000).

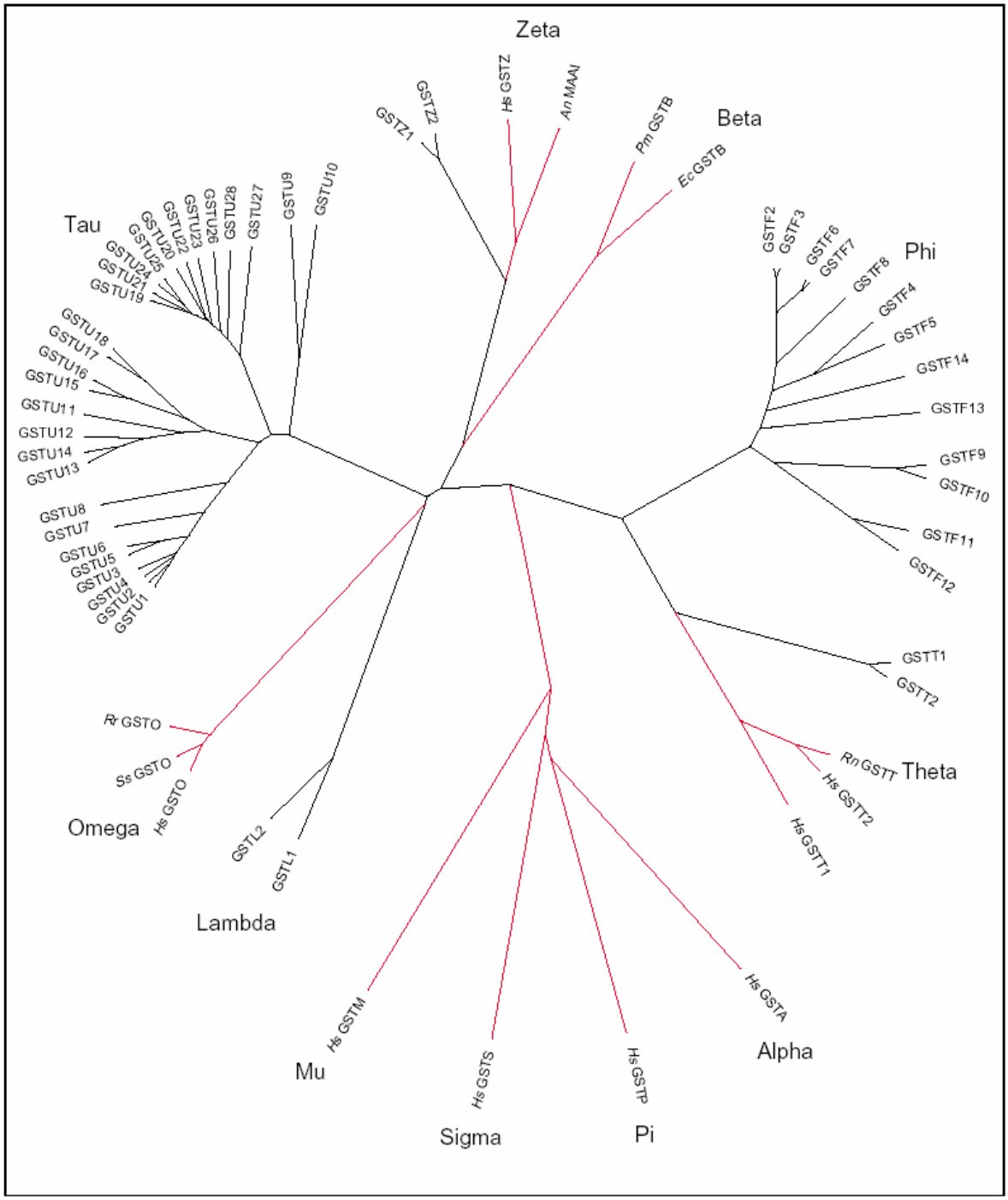


Figure 4 Phylogenetic tree illustrating the diversity of GSTs and the relationships between classes (Dixon *et al*., 2002 a).

All the GSTs identified from *Arabidopsis* are shown in black; representative GSTs from other classes and organisms are shown in red, and their names are prefixed with two letters denoting the source organism: *Hs*, *Homo sapiens*; *Rr*, *Rattus rattus*; *Rn*, *Rattus norvegicus*; *Ss*, *Sus scrofa*; *An*, *Aspergillus nidulans*; *Pm*, *Proteus mirabilis*; *Ec*, *Escherichia coli*. Branch lengths correspond to the estimated evolutionary distance between protein sequences.

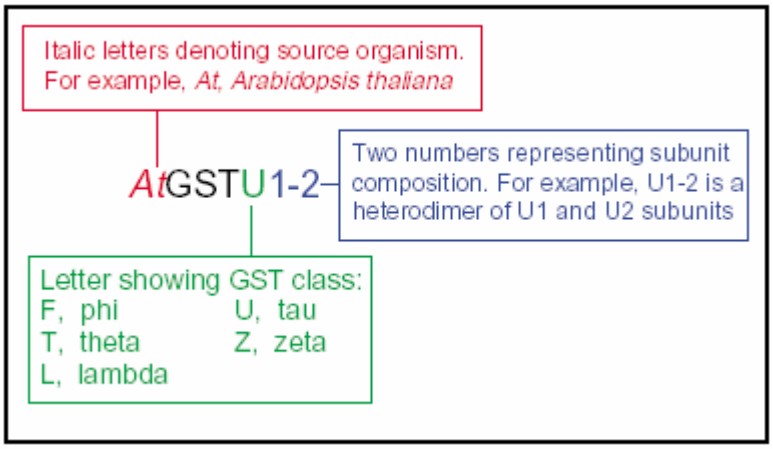


Figure 5 Nomenclature for *Arabidopsis* and other plant GSTs

(Dixon *et al*., 2002a).

Adaptod from the mammalian GST classification system

1.6.2 Structures of GSTs in Plants

The structural biology of GSTs derived from the different classes has been studied in detail, with high-resolution crystal structures available for the mammalian alpha, mu, pi, zeta, sigma and theta GSTs, as well as the bacterial beta GSTs (Dirr *et al*., 1994; Sheehan *et al*., 2001). Structural information on plant GSTs is available for phi GSTs from *Arabidopsis* (Reinemer *et al*., 1996) and maize (Neuefeind *et al*., 1997 b and c) and for a zeta-class GST from *Arabidopsis* (Thom *et al*., 2001). Despite the extreme sequence divergence between the GST classes the overall structures of the enzymes are remarkably similar (**Figure 6**), suggesting a strong evolutionary pressure to retain structural

motifs involved in binding GSH at the active site (Dirr *et al*., 1994; Armstrong, 1997; Sheehan *et al*., 2001).

Each GST subunit of the protein dimer contains an independent catalytic site composed of two components (domains) (**Figure 7a**). The first is a binding site specific for GSH or a closely related homolog (the G site) formed from a conserved group of amino-acid residues in the amino-terminal domain of the polypeptide. The second component is a site that binds the hydrophobic substrate (the H site), which is much more structurally variable and is formed from residues in the carboxy-terminal domain. Between the two domains is a short variable linker region of 5-10 residues (**Figure 7a**).

The subunits that make up the dimer are related by two-fold symmetry as shown in **Figure 7b**. The dimer interface is large, with a buried surface area of between 2,700 and 3,400Å. Most classes of GST have one of two types of subunit interface, either a hydrophobic ball-and-socket interface (alpha, mu, pi, and phi classes; as illustrated in **Figure 7**), or a hydrophilic interface (theta, sigma and beta classes) due to the absence of Phe residue and the hydrophobic socket between the helices 4 and 5 (Armstrong, 1997). Subunits from different classes of GST are not able to dimerize because of the incompatibility of the interfacial residues. As the active sites of each subunit are normally catalytically independent, the reasons that all classes of active soluble GSTs described so are dimers, rather than monomers, remain unclear.

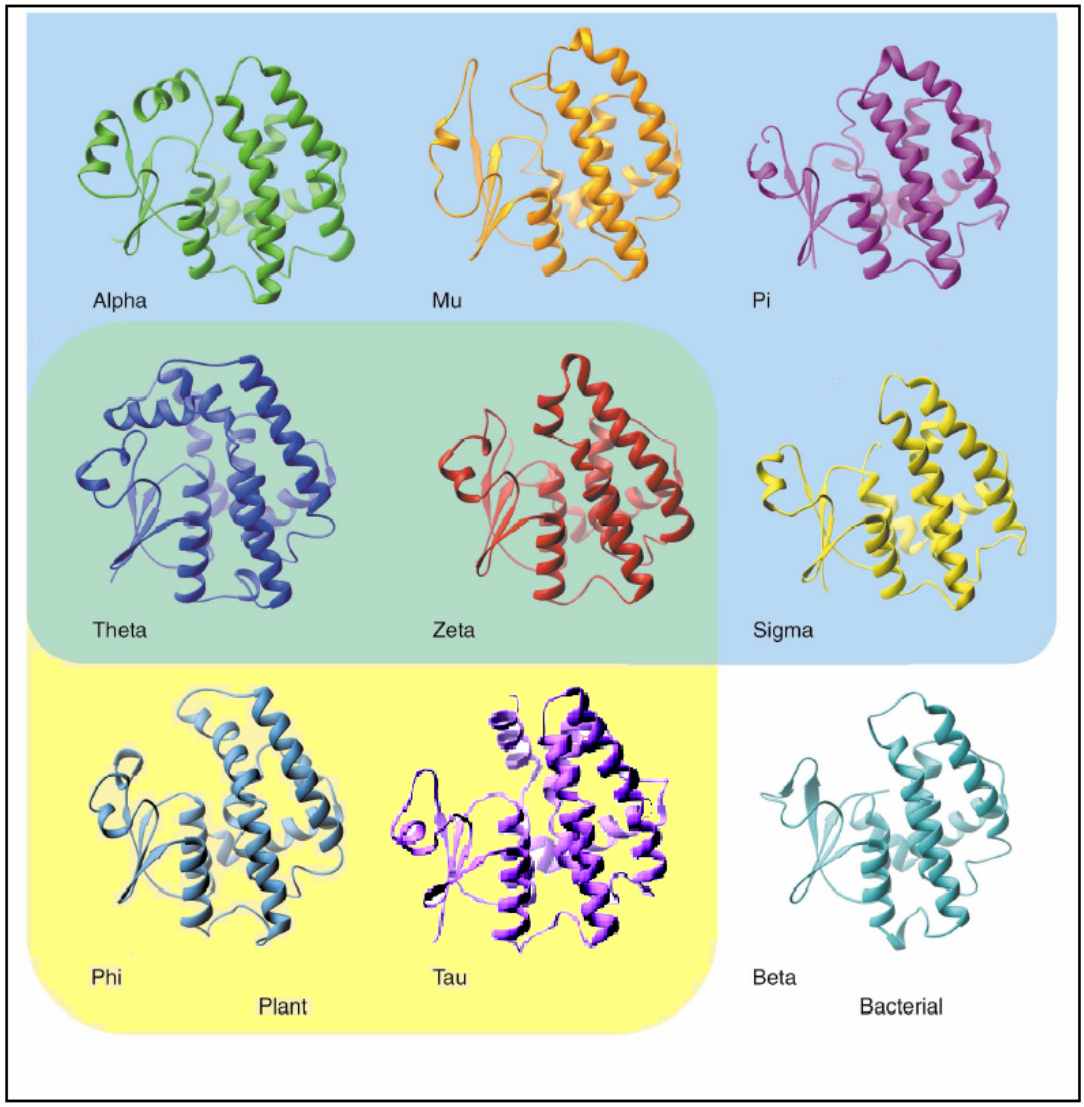


Figure 6 Ribbon representations of the structures of GST subunits

(Modified from Dixon *et al*., 2002a)

The GSTs specific to mammals (alpha, mu, pi and sigma) have a blue background; the plant specific (phi and tau) GSTs have a yellow background; and bacteria-specific (beta) GST have white background; GSTs (theta and zeta) that have counterparts in both animals and plants have green backgrounds. Although there is little sequence similarity between enzymes of different classes, there is significant conservation in overall structure.

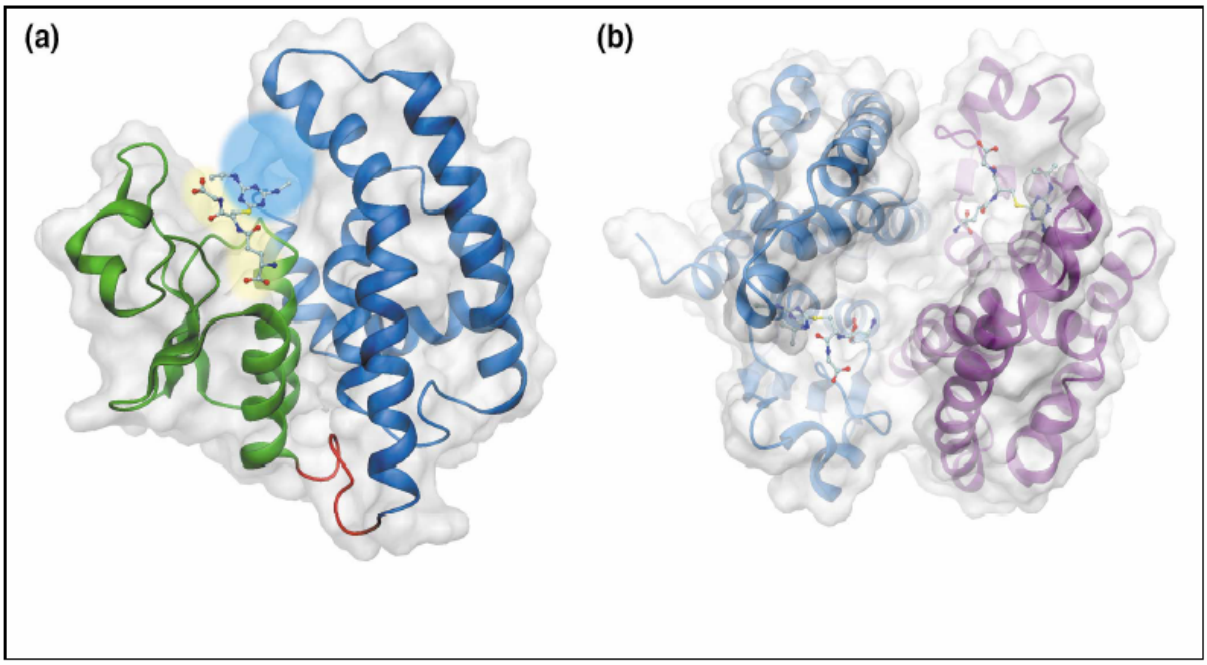


Figure 7 Overview of GST dimer structure and substrate binding
(Dixon *et al*., 2002a)

(a) A ribbon/surface representation of a typical GST subunit (*Z. mays* GSTF1, pdb 1BYE), with the amino-terminal domain in green, the linker region in red, the carboxy-terminal domain in blue and the protein surface in gray. A glutathione conjugate of the herbicide atrazine in ball-and-stick representation is shown binding at the active site; the GSH-binding site (G site) is highlighted in yellow and the hydrophobic site (H site) is highlighted in blue. **(b)** A ribbon/surface representation of the *Zm*GSTF1 homodimer oriented with the amino-terminal domains at bottom left and top right and the subunits in blue and purple. The atrazine-glutathione conjugates are shown in ball-and-stick representation, bound at the active site of each subunit. The dimer is formed by a ball-and-socket interaction between the amino- and carboxy-terminal domains of the different subunits ; the deep cleft between subunits is characteristic of phi GSTs.

The N-terminal domain 1 (approx. residues 1-80) adopts a topology similar to that of the thioredoxin fold (Katti *et al*., 1990; Wilce and Parker,1994; Martin, 1995), consisting of four β-sheets with three flanking α-helices (**Figure 8**). The fold consists of distinct N-terminal and C-terminal motifs which have a

βαβ and *ββα* arrangement respectively, and which are linked by an α -helix (*α* -2 in **Figure 8**). The former begins with an N-terminal *β*-strand (*β* -1), followed by an *α*-helix (*α* -1) and then a second *β*-strand (*β* -2) which is parallel to *β* -1. A loop region leads into a second *α* -helix (*α* -2), which connects with the C-terminal motif. This motif consists of two sequential *β* -strands (*β* -3 and *β* - 4), which are antiparallel and which are followed by a third *α*-helix (*α* -3) at the C-terminus of the fold. The four *β* -sheets are essentially in the same plane, with two helices (*α* -1 and *α* -3) below this plane and *α* -2 above it, facing the solvent. The loop that connects *α* -2 to *β* -3 features a characteristic proline residue which is in the less favoured *cis* conformation and is highly conserved in all GSTs. This is referred to as the *cis*-Pro loop which, while playing no direct role in catalysis, appears to be important in maintaining the protein in a catalytically competent structure (Allocati *et al*., 1999). In GSTs, domain 1 is highly conserved and provides most of the GSH binding site.

Domain 2 (approx. residues 87 - 210) begins at the C-terminus of the linker sequence, and consists of five *α*-helices in the case of the Pi and Mu classes (Reinemer *et al*., 1991; Ji *et al*., 1992) and six - *α* helices in the case of the Alpha class (Sinning *et al*., 1993) (the number of helices in domain 2 varies widely between classes). The C-terminal domain is less similar between the three mammalian classes than the N-terminal domain (**Figures 6 and 7**) (Dirr *et al*., 1994; Wilce and Parker, 1994). It contributes most of the residues that interact with the hydrophobic second substrate, as well as contributing a highly conserved aspartic acid residue (occurring in helix *α* -4) to the GSH binding site. Differences in the C-terminal domain may be responsible for differences in substrate specificity between the three classes (Wilce and Parker, 1994).

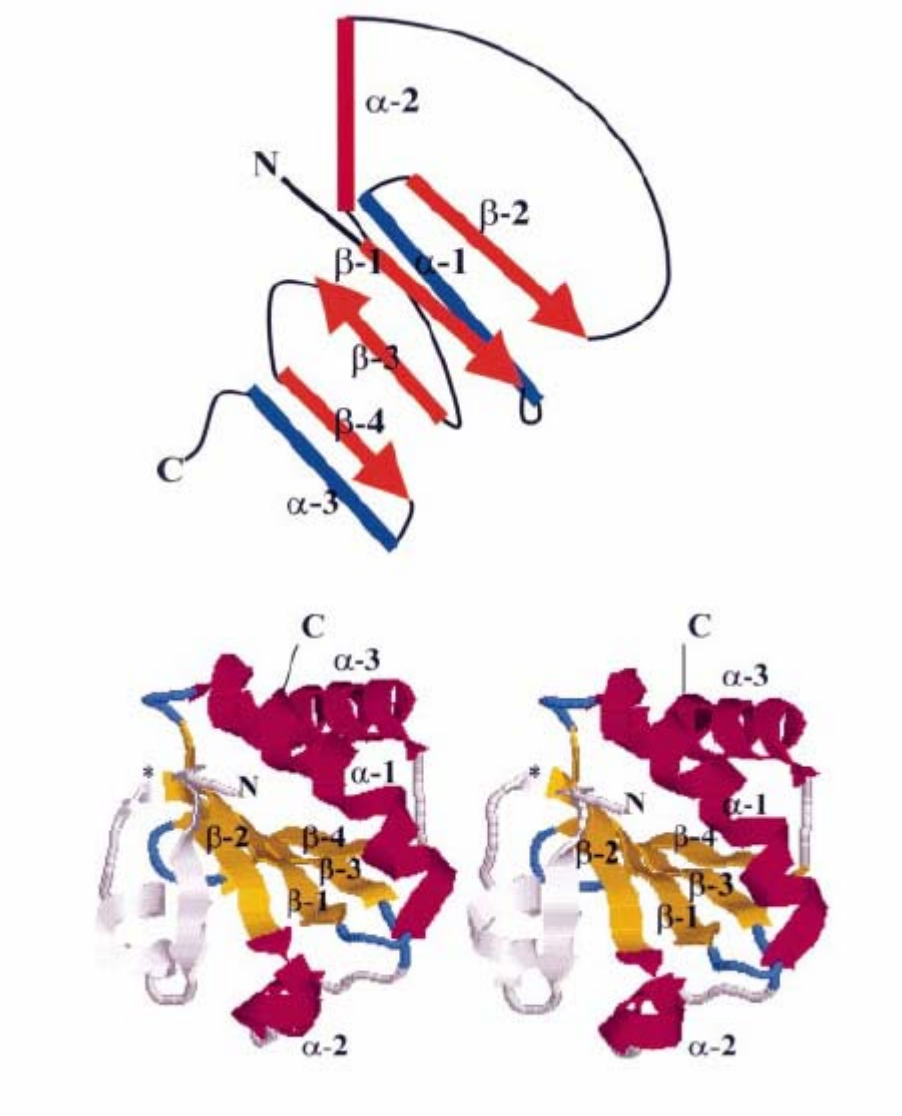


Figure 8 The thioredoxin fold (Sheehan *et al.*, 2001)

A schematic diagram representing the thioredoxin fold is shown above a RasMol depiction of the thioredoxin dimer (Katti *et al.*, 1990). In the diagram, α-helices are shown as cylinders, while *β*-sheets are shown as orange arrows. The four *β* -sheets are essentially co-planar, with one helix (α -2) shown in red above this plane and the other two α -helices (α -1 and α -3) shown in blue below the plane. The *cis*-Pro loop links α -2 to *β* -3. In GSTs, domain 2 is connected to the C-terminus by a short linker peptide. In thioredoxin itself, *β* -sheets are coloured yellow, while α -helices are magenta. The thioredoxin fold has an extra *β* -sheet and α -helix at the N-terminus (residues 1-21) ending at the point denoted by * where the fold proper begins. These additional N-terminal features are coloured grey.

The enzyme uses the binding interactions with GSH to activate the sulfur for nucleophilic attack. The peptide is bound in an extended conformation, with the γ-glutamyl residue pointing down toward the dimer interface, the cysteinyl sulfur pointing to the subunit to which it is bound, and the glycyl residue residing near the surface of the protein. The molecule is anchored by over a dozen electrostatic interactions utilizing virtually all of the hydrogen bond donor and acceptor sites on the peptide. Even though the orientation of the peptide in the active site is approximately the same for all of the isoenzymes, there are substantial differences in the details of the hydrogen-bonding interactions (Armstrong, 1997).

In spite of similar overall topologies, the structures differ considerably with respect to a number of details. The most notable topological differences include the mu-loop and the α-9 helix of the class mu and alpha enzymes, respectively. Both of these structural elements are located adjacent to the substrate binding sites and contribute to a more constricted active site of these two enzyme types when compared to examples of pi, sigma and the theta classes. The features of subunit-subunit recognition also differ between enzyme families. In addition, many of the groups involved in the binding of GSH have been altered through evolution.

The most conserved region of structure in all of the cytosolic enzymes is a core $\beta\beta\alpha$ motif that is responsible for recognition of the γ- glutamyl portion of the peptide (**Figure 9).** The *cis*-prolyl residue that precedes this region helps to conserve the overall fold of the domain. The only other highly conserved interaction is between the carbonyl and NH groups of the cysteinyl residue and the main chain of the protein just preceding the conserved *cis*-prolyl residue. The similarity in this region of structure for the two most divergent classes of cytosolic enzyme, theta and alpha, is obvious and much more highly conserved (**Figure 9)** than are the interactions between the proteins and sulphur of GSH (**Figure 10).** The most fundamental difference among the GSH binding sites of

the various enzyme classes involves the interaction of protein with the sulfur of the peptide (**Figure 10).**

The theta class enzymes thought to be the evolutionary precursor of the alpha, mu, pi, and sigma class proteins, utilizes the hydroxyl group of a serine residue located near the N-terminus of the polypeptide to activate the sulphydryl group of bound GSH, as zeta class GSTs (**Figure 11**). In contrast, the class alpha, mu, pi, and sigma enzymes have recruited the hydroxyl group of a tyrosyl residue, located in a slightly different position, to act as a hydrogen bond donor to the sulphur which lowers the pKa of the thiol in the E•GSH complex so that it is ionized at physiological pH. In the class alpha additional stabilization gathered from positive charge of Arg 15 (Armstrong 1997).

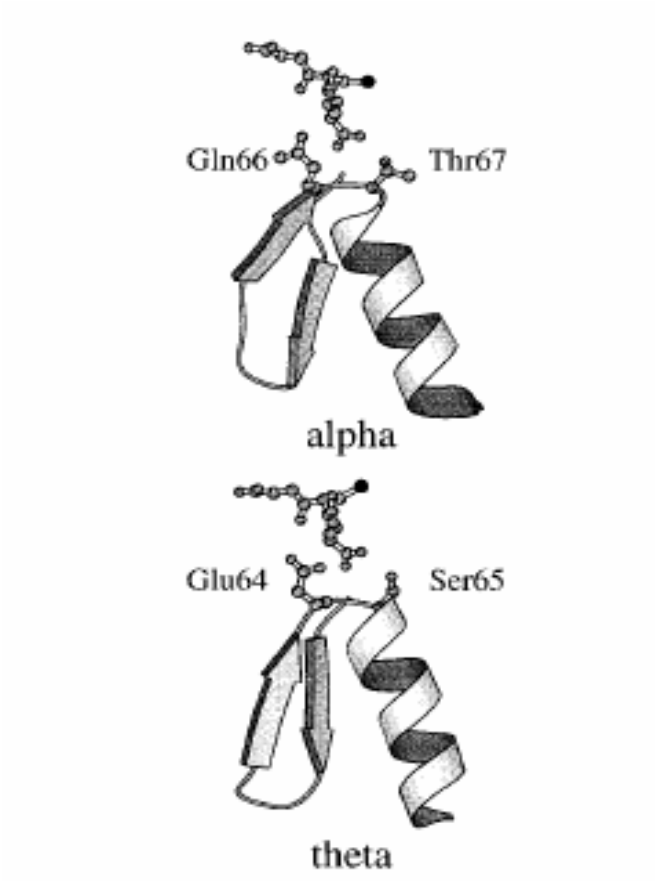


Figure 9 Representation of the highly conserved core *ββα* motif

(Armstrong, 1997)

The motif is responsible for the recognition of the *γ*-glutamyl residue of GSH. Glutathione and the side chains located at the turn between the *β*4-strand and *α*3-helix involved in hydrogen-bonding interactions with the *α*-amino and *α*-carboxyl moieties of the *γ*-glutamyl residue are illustrated in ball-and-stick. The two motifs represented are from what are considered to be the most divergent classes of cytosolic GSH transferases, the theta and alpha classes.

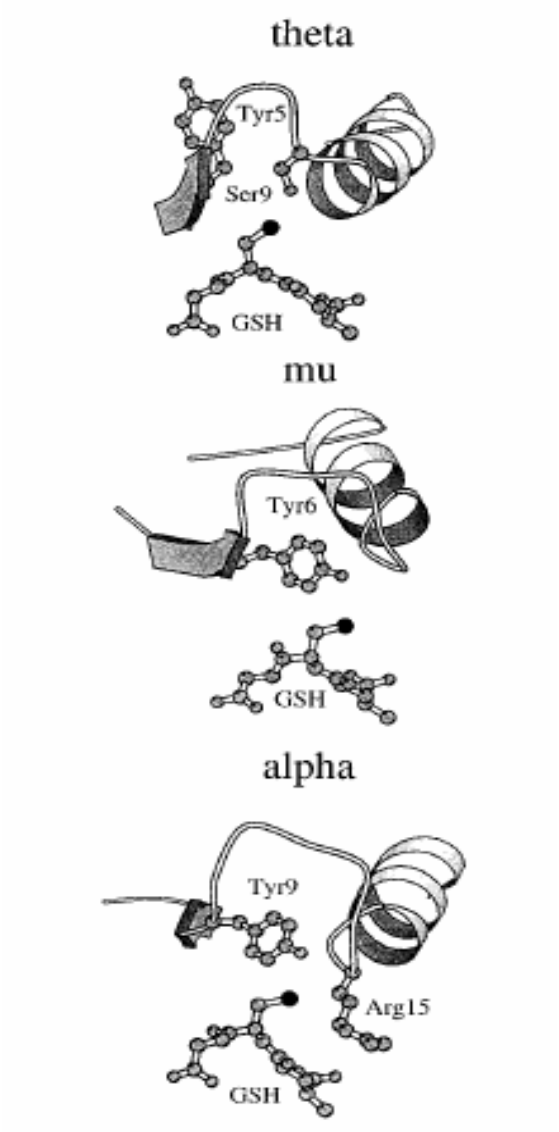


Figure 10 Evolution of first-sphere interactions between the active sites of class theta, mu, and alpha enzymes and the sulfur of GSH (Armstrong, 1997)

It is possible that the tyrosine residue often, but not always, found near the N-terminus of the class theta enzymes was recruited in the evolution of the protein to the other classes. The most recently evolved enzyme, class alpha, has an additional residue (Arg 15) conscripted into the first sphere of the sulfur of GSH.

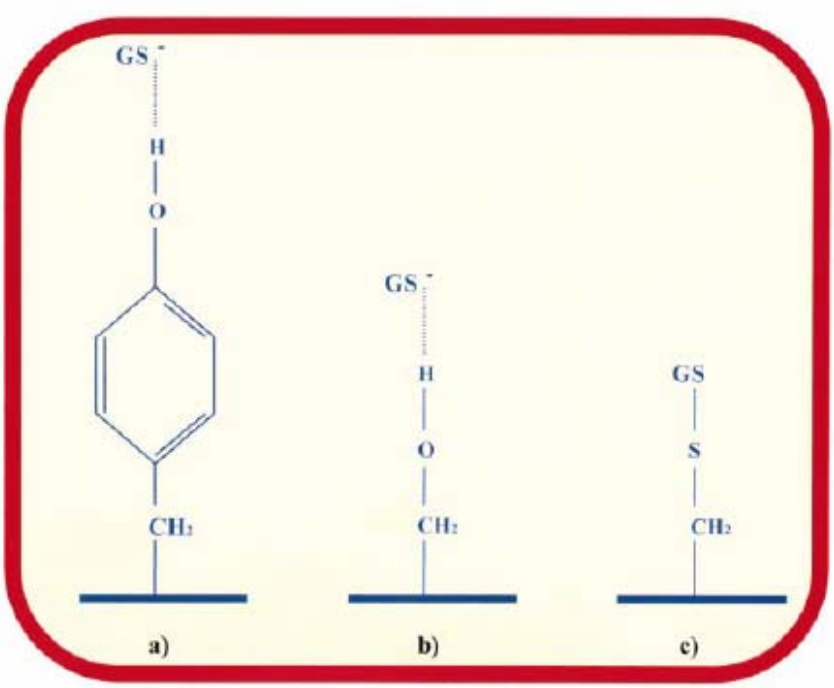


Figure 11 Active-site residues in GSTs (Sheehan *et al*., 2001)

In most GST classes, an N-terminal tyrosine residue (**a**) interacts with GSH to stabilize the thiolate anion, with a consequent decrease in p*K*a. In the Theta and possibly the Zeta classes, this role is carried out by a serine residue (**b**), while in the Omega and Beta classes a mixed disulphide is formed with a cysteine residue (**c**).

A crystal structure for Zeta-class GST (maleylacetoacetate isomerase) is now available from *A.thaliana* (Thom *et al*., 2001). While the overall fold of this protein is generally similar to the other GST classes, major differences are found in and around the α-2 helix which result in particular in a very small, almost buried, polar active site. This explains the poor catalytic activity of this enzyme with most GST substrates. The V-shaped dimer interface is lacking; in this regard, Zeta resembles the Theta and Beta classes. This interface is dominated by hydrophobic interactions between small aliphatic residues, and a methionine residue acts as a key in a 'lock and key' motif linking the dimer together. This

contrasts with other classes, which either lack this motif altogether or else feature an aromatic residue as a key. It is unclear from this structure which N-terminal residue is catalytically essential. However, serine-14 is close enough to the GSH thiol to interact with it (**Figure 11**), although its hydroxy group is pointing away from this group in the structure. It is possible that the protein undergoes a conformational change to allow this residue to interact with GSH.

In all plant GSTs the N-terminal domain and mainly hydrophobic inter-subunit interface are quite similar, and differences are concentrated in the C-terminal domain where a hydrophobic substrate is likely to bind. The GSTs from plants were found to have a larger cleft for binding hydrophobic co-substrates than mammalian GSTs and could potentially accept larger and more varied substrates. The structure for maize GST I (Neuefeind *et al*., 1997a), when crystallised with the ligand S-lactoylglutathione, as compared with that for maize GST III, crystallised without ligand (Neuefeind *et al*., 1997b), showed differences in the relative position of a l0-residue loop at the active site. This has been interpreted as evidence for an induced-fit mechanism, where binding of substrate to the GST induces a change in enzyme conformation to better accommodate the substrate. Also, GST III was found to have a hydrophobic, highly flexible carboxy-terminal region which may form a lid over the active site on substrate binding to maintain a hydrophobic environment.

Interestingly, the crystal structure of the plant GSTs has revealed some interesting differences as compared with mammalian GSTs. Firstly, the plant GSTs use a serine residue in place of a tyrosine at the active site to activate glutathione for conjugation reactions. Secondly, in the plant GSTs the G-site of each subunit is discreet and self-contained and thus thiol binding is possible without the strict co-alignment of subunits required with mammalian GSTs, where the G-site for each subunit requires an additional residue from the dimer's other subunit (Prade *et al*., 1997). Thus, much more variation should be tolerated at the dimer interface in plant GSTs and this indeed seems to be the case, with

considerable sequence diversity found between the amino acid residues which form the dimer interface.

Three membrane-bound glutathione transferases are known, one of which appears to be involved in xenobiotic mechanism. Microsomal GSH transferase I is an integral membrane protein that has been characterized from both rats and humans where it is found in large amounts in liver and is distributed in both microsomal and outer mitochondrial membrane. This protein bears no discernible relationship to any of the known cytosolic enzymes with respect to sequence. However, it does appear to be vaguely similar to two other membrane-bound GSH transferases, leukotrienes C_4 synthase and microsomal GSH transferase II, in which the three proteins are about the same size, share a small amount of sequence identity and are all membrane-bound. Considerably less is known about the xenobiotic mechanisms of membrane-bound GSTs.

A proposed membrane topology for the microsomal enzyme is illustrated in **Figure 12** (Armstrong, 1997)**.** Each subunit consists of five membrane spanning regions, at least two of which are likely to be α-helices perpendicular to the membrane surface. The N-terminus of the enzyme appears to be located on the luminal side of the endoplasmic reticulum while the C- terminus and the active site face the cytosol. The overall topology is most simply described as an N terminal in, C-terminal out orientation with five membrane spanning regions. Although it is unclear what the GSH-binding motif looks like, the enzyme lowers the pKa of the thiol of bound GSH, similar to those found in the cytosolic GSTs.

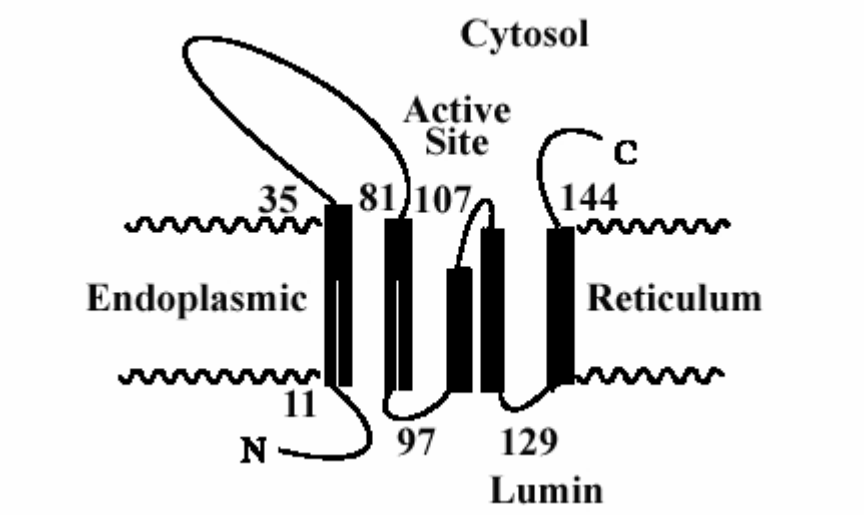


Figure 12 A proposed membrane topology for the microsomal GSH transferase subunit (Armstrong, 1997)

Derived from sites of proteolysis, chemical modification and mutagenesis (Andersson *et al*., 1994). Experimental evidence and hydropathy analysis indicate that there are at least three membrane spanning reagions: residues 11-35, 81-97, and 129-144. Two of the membrane spanning reagions are thought to be α-helices (Hebert *et al*., 1995).

1.6.3 Subcellular localization and Regulation of GSTs in Plants

Biochemical and immunological investigations point to a largely cytosolic localization for soluble GSTs in plants. For example, GSTs collectively constitute >1% of the soluble protein in maize leaves (Marrs, 1996; Edwards *et al.,* 2000). This is borne out by genomic analysis of the Arabidopsis GSTs: only one phi GST and one lambda GST show evidence of subcellular targeting to the plastid or mitochondria, and all the other GSTs contain no putative targeting sequence and would be anticipated to be in the cytoplasm. There is a limited number of accounts reporting expression of specific GSTs in the nucleus as well as extracellularly, however (Edwards *et al.,* 2000).

GSTs are present at every stage of plant development from early embryogenesis to senescence and in every tissue type examined. Although GSTs in major cereal crops are very highly expressed, representing up to 2% of the total protein in the foliage, relatively few studies have addressed their tissue-specific expression in plants. In one study carried out in in-bred maize lines, different GST isoenzymes were seen to be expressed in different tissues (Sari-Gorla *et al.,* 1993). Tissue-specific expression can be overridden by exposing plants to chemical treatments: maize (*Zea mays*) ZmGSTF2, for example, is normally expressed only in the roots, appearing in the foliage only after exposure to herbicide safeners or chemical treatments (Dixon *et al.,* 1997).

The inducibility of phi and tau GSTs following exposure of plants to biotic and abiotic stresses is a characteristic feature of these genes. In the course of biotic stress, both tau and phi GSTs are known to be induced by infection or by treatments that invoke plant defense reactions, as well as by osmotic stress and extreme temperatures. Several tau GSTs are also known to be strongly induced during cell division or when plants are exposed to auxin or cytokinin plant hormones (Marrs, 1996; Gonneau *et al.,* 1998). Expression of GSTs is also enhanced following exposure to a range of xenobiotics: again, GSTs may be induced in response to the general cellular injury and oxidative stress caused by herbicides and chemical toxins (Marrs, 1996). Some other chemicals can induce the expression of specific phi and tau GSTs without imposing any discernible chemical stress on the plant. The best example of this is seen in cereals treated with herbicide safeners, chemicals that enhance herbicide tolerance by increasing the expression of detoxifying enzymes, including GSTs (Davies and Caseley, 1999).

The expression of GSTs is regulated predominantly at the level of transcription (Marrs, 1996; Davies and Caseley, 1999). The transcriptional regulation of individual subunits ultimately influences the range of GST homodimers and heterodimers formed. For example, after treatment with

herbicide safeners there is an increased synthesis of specific subunits and novel heterodimers are observed. In maize, safeners induce the synthesis of the ZmGSTF2 subunit, which then associates with the constitutively expressed ZmGSTF1 subunit to form the ZmGSTF1-2 heterodimer, one of the major GST isoenzymes in safener-treated tissue (Dixon *et al.,* 1997). In wheat, the three tau GST subunits, TaGSTU2, TaGSTU3 and TaGSTU4, are induced by safeners and this results in their dimerization with the constitutively produced TaGSTU1 subunit to form the TaGSTU1-2, TaGSTU1-3 and TaGSTU1-4 heterodimers, respectively (Cummins *et al.,* 1997).

1.6.4 Catalytic Mechanism and Cellular Functions of GSTs in Plants

1.6.4.1 Catalytic Mechanism of GSTs in Plants

GSTs catalyze the nucleophilic addition of the thiol of reduced glutathione to electrophilic centers in endogenous and exogenous compounds. In plants, GSH conjugation by nucleophilic addition has been reported less frequently than GSH congugation by nucleophilic displacement, but this may be a consequence of the types of xenobiotics that have been studied rather than a true indication of the ability of plants to catalyze these various reactions.

$$\mathbf{GSH + RX \longrightarrow GSR + HX}$$

As a result of the conjugation reaction between electrophiles and GSH catalyzed by GSTs a conjugate that is less reactive than the parental compound is formed and the solubility of hydrophobic xenobiotics is increased. However, in some cases toxic products can be produced as a result of GSH conjugation. In spite of such exceptions, GSH conjugation usually results in the production of relatively nontoxic products.

From what is known of the enzyme kinetics of the GSH conjugation of model xenobiotic substrates, the majority of investigations suggest the reactions would be anticipated to undergo a random sequential two-substrate, two-product mechanism. Under physiological conditions, the reaction would be ordered with GSH adding first, given that the concentration of GSH in normal cells (1-10mM) is about three orders of magnitude higher than the dissociation constant between GSH and enzyme. The overall reaction rate being determined by the rate of release of reaction product from the active site (Labrou *et al.,* 2001).

The conserved nature of the G site suggests that the binding and correct orientation of glutathione is of central importance. Therefore, the main aspect of the catalytic mechanism is the lowering of the p*K*a of the glutathione thiol group from 9 in aqueous solution to between 6 and 7 when bound to the protein (Graminski *et al.,* 1989). For example, Z*m*GSTF1-1 the effect of this hydrogen-bonding activation is to lower the dissociation constant of the thiol from 8.7 to 6.2 (Labrou *et al.,* 2001). The more variable H site is responsible for accepting a wide range of hydrophobic co-substrates of diverse chemistries, with the powerful thiolate anion then driving a range of reactions.

1.6.4.2 Cellular Functions of GSTs in Plants

1.6.4.2.1 Detoxification and Toxification Reactions of GSTs with xenobiotics in Plants

1.6.4.2.1.1 Detoxification Reactions of GSTs with xenobiotics in Plants

Xenobiotics usually contain strong electrophilic centers and that electrophilic functional center of the substrates can be provided by a carbon, a nitrogen or a sulfur atom. A typical GST reaction involving xenobiotics results in the conjugation of the toxic substrate to form an *S*-glutathionylated reaction product (**Figure 13a**). The GSH conjugates so-formed are rendered less reactive

and more water-soluble, thus facilitating their eventual elimination. This reaction is one of the early steps along the mercapturic acid pathway in which hydrophobic xenobiotics are inactivated and eliminated from the organism (Habig *et al*., 1974). Therefore, GSTs are usually detoxification reactions.

In plants, the most commonly observed GSH conjugation reaction has been the nucleophilic displacement of a halogen from an electrophilic site on an aromatic ring, a heterocyclic ring, or an alkyl group. Among the pesticides metabolized in this manner are such as propachlor, chlorimuron ethyl (Lamoureux and Rusness, 1986 ; Brown, 1990). Nucleophilic displacements of phenols from fluorodifen, the displacement of a nitro group from PCNB, and the displacement of an alkyl group from insecticide, methidathion, also have been reported (Lamoureux and Rusness, 1986) (**Figure 14**). Some other pesticides which were also detoxified by GSTs are presented in **Figure 15**.

In plants, the S-glutathionylated conjugate is then rapidly transported from the cytosol into the vacuole for further processing through the action of specific transporters of the ATP-binding cassette (ABC) class (Edwards *et al*., 2000).

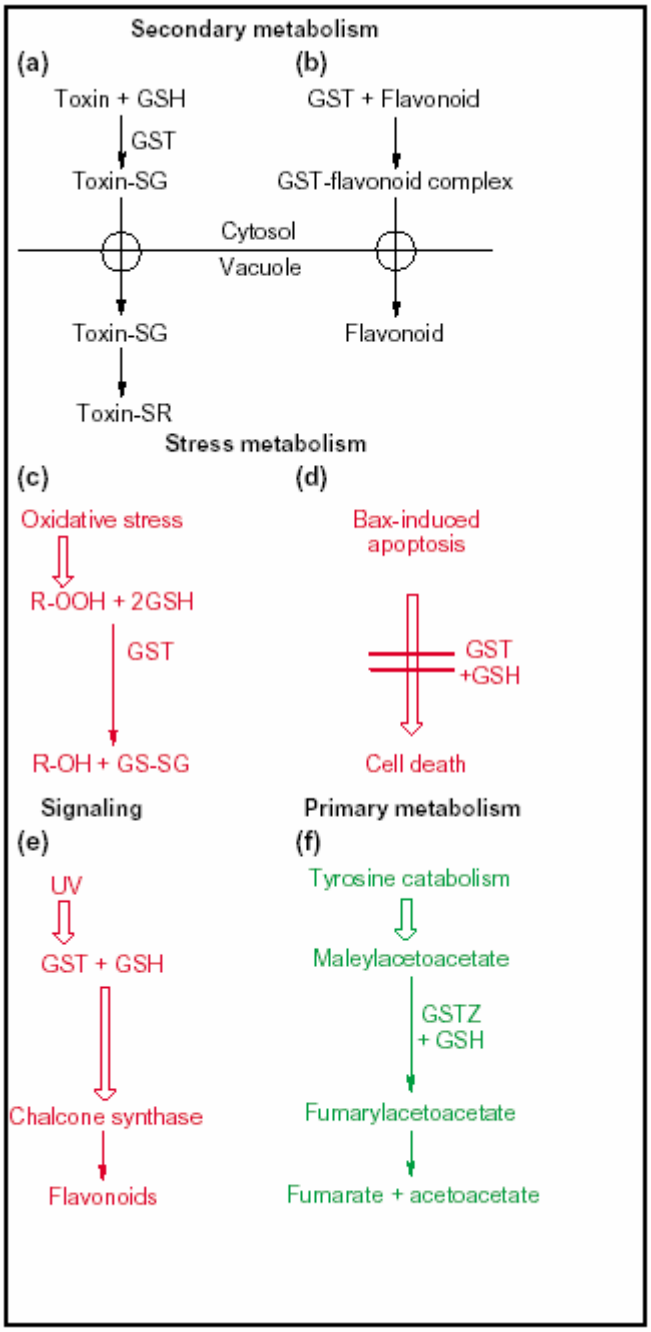


Figure 13 Overview of known GST functions in plants (Dixon *et al.*, 2002a)

Overview of known GST functions in plants. **(a)** In secondary metabolism, GSTs detoxify toxins by conjugation with GSH; the conjugates (toxin-SG) are then transported into the vacuole by ABC transporters (shown as circles) prior to proteolytic processing. **(b)** Some phi and tau class enzymes are also required for transport of flavonoid pigments to the vacuole. **(c-e)** Roles of GSTs in stress metabolism include acting as (c) glutathione peroxidases that can reduce cytotoxic DNA and lipid hydroperoxides; (**d**) in an antioxidant capacity, protecting against Bax-induced cell death; and (**e**) in stress signaling, playing a role in the induction of chalcone synthase following exposure to ultraviolet light. Finally, zeta GSTs (GSTZ) have a role in (f) primary metabolism as maleylacetoacetate isomerases. Wide arrows denote an induction process; narrow arrows denote enzymatic reactions; thick lines denote inhibition of a reaction; R, an alkyl group.

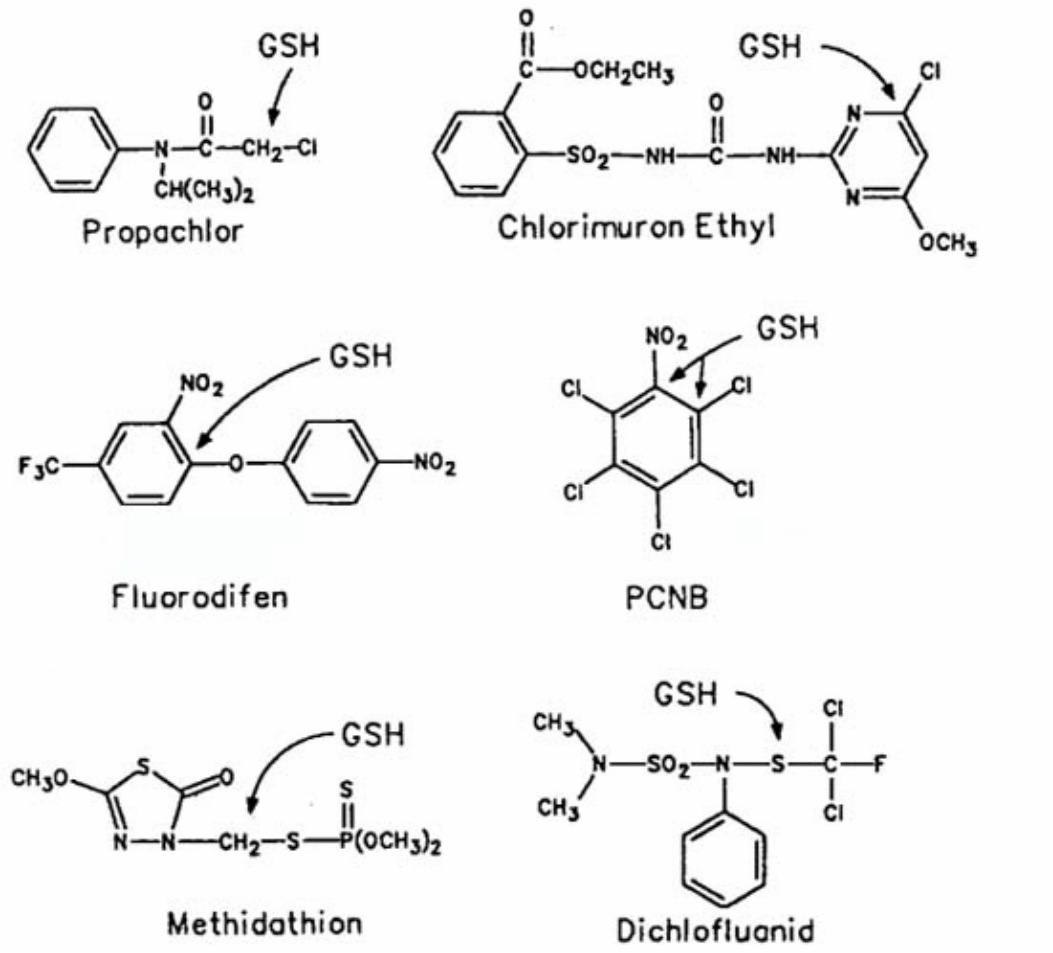


Figure 14 Structures of compounds known to be metabolized by conjugation with GSH in plants (Lamoureux and Rusness, 1993).

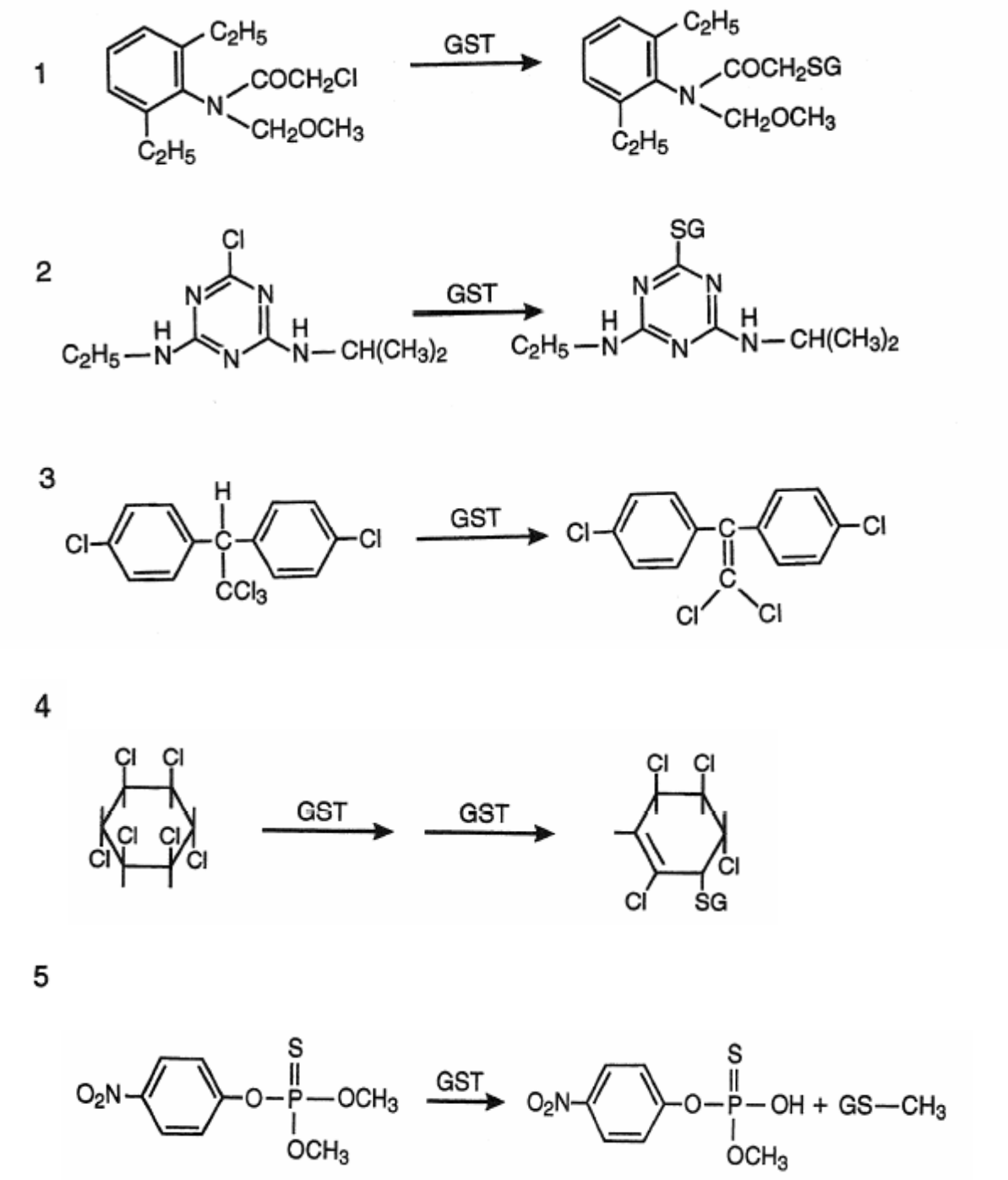


Figure 15 Metabolism of pesticides by GSTs (Hayes and Pulford, 1995)

(1) alachlor; (2) atrazine; (3) DDT; (4) lindane; (5) methyl parathion.

1.6.4.2.1.2 Toxification Reactions of GSTs with xenobiotics in Plants

Generally most of the GSH conjugates turn into detoxification products during the reaction catalyzed by GSTs. However, in some cases the toxic products can be produced as result of GSH conjugation. More serious situation can arise with a small number of GST substrates which yield a GSH conjugate, or a metabolite of the conjugate that is more reactive than the parental compound. For certain haloalka(e)nes, including ethylene dibromide and methylene chloride, GST-mediated conjugation with GSH may result in formation of highly reactive episulfonium ion intermediates, and thus catalyze activation reactions (Eaton and Bammler, 1999) (**Figure 16**). As in the rat, a highly reactive nephrotoxic GSH conjugate is produced in the metabolism of dibromoethane (Inskeep *et al*., 1986).

In strawberry, dichlofluanid is metabolized to a thiophosgene derivative in a process that appears to involve two GSH conjugation steps (Schuphan *et al*., 1981) (**Figure 14)**.

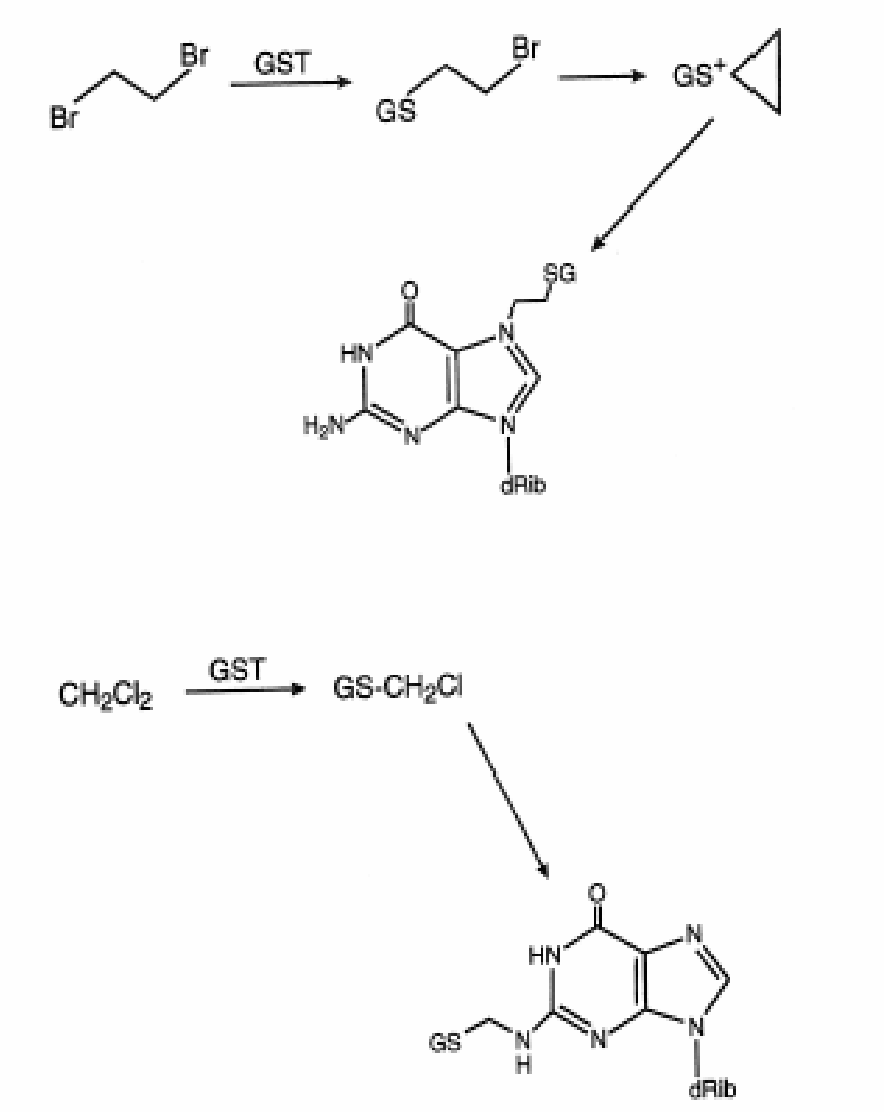


Figure 16 Reaction between GS conjugates formed between (1) ethylene dibromide and DNA and (2) methylene chloride and DNA.

1.6.4.2.2 GSTs in the conjugation of endogenous products in Plants

Although the role of GSTs in the conjugation of xenobiotics in plants is well established, with the S-linked peptide glutathione derivative and related metabolites accumulating in the vacuole (Wolf *et al*., 1996; Coleman *et al*., 1997), a similar role in the detoxification of endogenous metabolites is little emprical.

Very few natural products have been described which show evidence of glutathione conjugation in the course of their metabolism, like the GSH conjugate of caftaric acid identified in wine must and a sulphur containing metabolite of gibberellic acid, termed gibberthione (Lamoureux and Rusness, 1993), cinnamic acid in legumes (Edwards *et al*., 1991) and the isoflavonoid medicarpin, a legume phytoalexin (Li *et al*., 1997) (**Figure 17**). This suggests that either natural products are not glutathione conjugated, or that such conjugation is reversible.

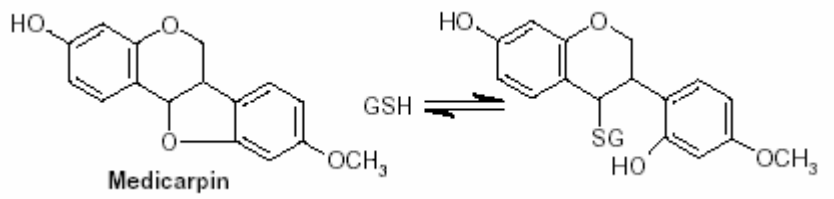


Figure 17 GSH conjugation reaction with the pyhtoalexin medicarpin (Edwards *et al*., 2000).

This addition reaction occurs spontaneously under basic conditions and can be readily reversed by reducing the pH.

GSH conjugation 'tags' numerous endogenous substrates for recognition by the glutathione pump. In plants, many secondary metabolites are phytotoxic, even to the cells that produce them, and thus targeting to the appropriate cellular localization, usually the vacuole, is crucial (Matern *et al*., 1986; Sandermann, 1992; 1994). Anthocyanins are blue-red flavonoid pigments, and Marrs *et al* (1995) have shown that they are also endogenous GST substrates. Stable anthocyanin pigmentation occurs when the molecules are transferred to the vacuole. In the presence of a functional *Bronze2* (*Bz2*) gene, maize anthocyanins accumulate exclusively within the vacuole. Marrs *et al* (1995) demonstrated that cyanidin 3-glucoside (C3G), the cytoplasmic product of the anthocyanin pathway, is a substrate for glutathionation by the GST encoded by *Bz2*. Genetically, the comparable step in the petunia anthocyanin pathway is controlled by the *Anthocyanin9* (*An9*) gene, which encodes a functional GST (Alfenito *et al*., 1998). *Bz2* and *An9* have evolved independently from distinct types of GSTs, but each is regulated by the conserved transcriptional activators of the anthocyanin pathway (Alfenito *et al*.,1998). In the absence of the GST-mediated step, anthocyanin accumulates in the cytoplasm, suggesting that conjugate formation was a prerequisite for vacuolar sequestration. Recent studies have shown that these *Bz2* and *An9* GSTs are flavonoid-binding proteins which involved in the intracellular binding and stabilization of flavonoids (Mueller *et al*., 2000), rather than in catalyzing their glutathionylation (**Figure 13b**).

1.6.4.2.3 Ligandin functions of GSTs in Plants

The concept of GSTs as carrier proteins or 'ligandins' was first proposed in the early 1970s, based on the finding that a similar protein was identified as the cellular binding factor for diverse steroids, bilirubin, heme, and bile salts in animal cells (Litwack *et al*., 1971; Ketley *et al*., 1975; Listowski *et al*., 1988). Compounds that bind GSTs as nonsubstrate ligands do so at a site other than the catalytic site of the enzyme.

In plants, some GSTs apparently serve as carriers of the natural auxin indole- 3-acetic acid (IAA). Bilang and Sturm (1995) and Jones (1994) have identified active GSTs as auxin-binding protein without detecting the formation of IAA-GSH conjugates. This nonenzymatic binding may allow temporary storage or modulation of IAA activity or IAA uptake from membranes and trafficking to receptors (Bilang and Sturm, 1995; Jones,1994). It has been proposed that the ligandin function of GSTs prevents cytotoxic events that could result from the excessive accumulation of molecules at membranes or within cells (Listowski *et al*., 1988).

Similarly, both phi and tau GSTs have high affinities for tetrapyrroles and porphyrin metabolites (Dixon *et al*., 1999; Lamoureux and Rusness, 1993). With both tetrapyrroles and plant hormones, binding inhibits GST activity toward xenobiotics, but the inhibitory ligands do not undergo conjugation. Similarly, the CDNB conjugating activity of petunia *An9*, a phi GST, is inhibited by flavonols, flavones and anthocyanins (Alfenito *et al*., 1998). There is ample evidence that specific plant GSTs that bind defined plant metabolites fit the definition of ligandins.

1.6.4.2.4 GSTs catalysing Peroxidase Reactions in Plants

Several plant GSTs have recently been demonstrated to have additional activities as glutathione peroxidases (GPOXs), by catalysing the nucleophilic attack of GSH on electrophilic oxygen and reduction of cytotoxic hydroperoxides to the less-toxic monohydroxy alcohols, the resulting sulfenic acid derivative of GSH then spontaneously forming a disulfide with another GSH molecule (**Figure 13c**), an activity characteristic of mammalian theta class and zeta class GSTs (Board *et al*., 1997).

GSTs with GPOX activities have been identified in purified isoenzymes from *A. thaliana* (Eshdat *et al*., 1997), wheat (Cummins *et al*., 1997), peas

(Edwards, 1996), maize (Dixon *et al*., 1997; 1998) and soybean (Skipsey *et al*., 1997). Tobacco seedlings overexpressing a tobacco tau GST with a high glutathione peroxidase activity are more tolerant to chilling and salt than wild type plants (Roxas *et al*., 1997). A further link between GSTs functioning as glutathione peroxidases and oxidative-stress tolerance was discovered in black grass. Herbicide-resistant weeds that are cross-resistant to multiple classes of herbicides express a phi GST that is a highly active glutathione peroxidase; this GST is barely detectable in herbicide-sensitive black grass (Cummins *et al*., 1999). Therefore, the expression of these GST-GPOXs is strongly enhanced by a variety of chemical treatments, particularly those associated with the formation of active oxygen species, so these GST-GPOXs responding to oxidative stress. Recently, this hypothesis has gained further support from the observation that expression of a tomato GST in yeast suppresses Bax-controlled apoptosis induced by oxidative stress (Kampranis *et al.*, 2000) (**Figure 13d**).

GSTs also have roles in stress signalling in plants (**Figure 13e**), following the observation that the induction of the genes encoding enzymes of flavonoid biosynthesis (chalcone synthase) and flavonoid accumulation in parsley upon exposure to UV light requires GSH and the expression of a specific tau GST (Loyall *et al*., 2000).

1.6.4.2.5 Isomerase Activity of GSTs in Plants

In addition to their role as detoxification enyzmes, GSTs are also essential for the isomerization of specific metabolites. The proposed mechanism involves the transient formation of a GSH adduct, spontaneous isomerization of the compound and finally the release of the isomer and GSH.

GST-mediated isomerase reactions recently identified in animals include prostaglandin-H E-isomerase activity (Meyer *et al*., 1996), and the isomerization of 13-*cis* retinoic acid to all-*trans* retinoic acid (Chen and Juchau, 1998). In the

latter case, the enzyme catalysing the reaction is clearly a GST, yet reaction does not require GSH and these enzymes appear to have recruited other proteinaceous thiols to catalyse the reaction.

It has recently been reported that the Zeta GSTs in *Aspergillus nidulans* and human have maleylacetoacetate *cis-trans* isomerase (MAAI) activity (Fernandez-Canon and Penalva, 1998). This elusive enzyme, the last to be identified in the catabolic pathway leading to the degradation of tyrosine and phenylalanine, catalyzes the conversion of maleylacetoacetate to fumarylacetoacetate (**Figure 13f**). This GST mediated *cis–trans* isomerization reaction involves the reversible addition of GSH to the *cis* double bond; after rotation, GSH is eliminated and the *trans* isomer is formed (**Figure 18**). In view of their sequence similarity, plant zeta GSTs probably have a similar activity. The carnation (*Dianthus caryophyllus*) zeta genes are induced during senescence (Marrs, 1996; Dixon *et al*., 1998), which is consistent with a role in the degradation of aromatic amino acids.

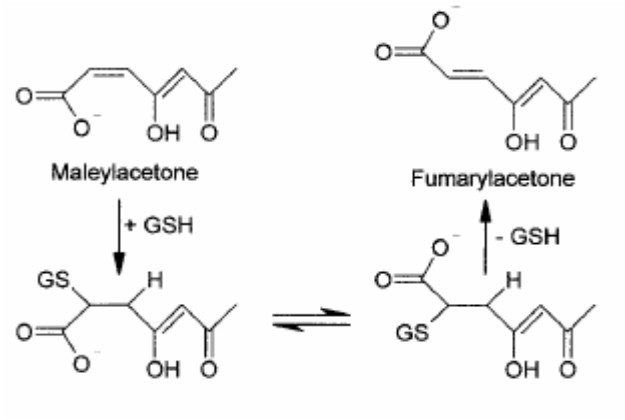


Figure 18 Isomerization reactions catalyzed by recombinant *Arabidopsis* zeta GST (Dixon *et al*., 2000)

Isomerisation of maleylacetone to fumarylacetone with the formation of the GSH intermediate shown.

All above mentioned roles of plant GSTs in xenobiotic detoxification and endogenous metabolism is summarized in **Figure 19**.

1.6.5 Model Substrates for the Characterization of GST Isoenzymes

A large number of diverse chemicals serve as substrates for GSTs. However, 1-chloro-2,4-dinitrobenzene (CDNB) is known as the general substrate for nearly all GSTs since it is used for the demonstration of multiple forms of GSTs in all biological organisms from animals to plants. When conjugated with glutathione it gives *S*-(2,4-dinitrophenyl) glutathione (σ-complex formation) (Armstrong, 1991), which has an absorbance spectrum that allow a simple spectrophotometric assay at 340 nm., is different from the parental compound CDNB (Habig *et al*., 1974).

Although the presence of activity towards CDNB or other substrates is suggestive of the presence of enzymes from certain GST classes, it can by no means be regarded as proof of the presence of that specific class of GSTs. Likewise, the absence of any detectable CDNB activity does not provide proof of the absence of GSTs, as some GST isoenzymes have very little activity toward CDNB, but very high activity toward other substrates. For example, both class theta 1-1 and theta 2-2 GST isoenzymes were shown not to display any activity with CDNB, but they were characterized by other substrates, namely; 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP), 4-nitrophenylbromide (4-NPB) and 4-nitrobenzyl-chloride (4-NBC) for class theta 1-1 (Meyer *et al*., 1991b) and 1-menaphthyl sulfate (MS) for class theta 2-2 (Hussey and Hayes, 1992).

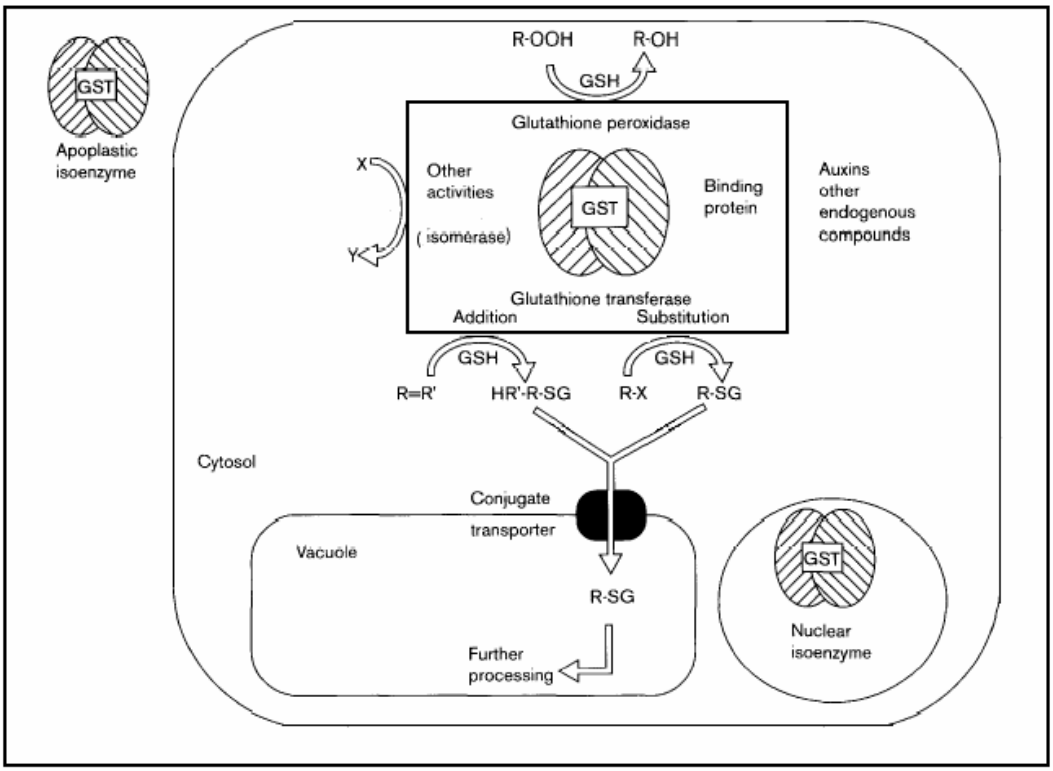


Figure 19 Overview of the plant GSTs in xenobiotic detoxification and endogenous metabolism (Dixon *et al*., 1998)

Most plant GSTs are assumed to be cytosolic, although there is evidence for apoplastic and nuclear isoenzymes. The primary transferase activity of GSTs results in glutathione conjugation of the substrate, usually in a substitution reaction but occasionally as an addition reaction; conjugated molecules (R-SG) are then transported into the vacuole for further processing. There is evidence for alternative activities of GSTs; these include glutathione peroxidase, isomerase and binding activities, which may play additional roles in endogenous metabolism.

The GST isoenzymes show marked differences in their abilities to conjugate GSH with various electrophiles. The model GST substrates that display selectivity for particular subunits are often used in a 'diagnostic'sense to identify isoenzymes (**Figure 20**). For example, 1,2-dichloro-4-nitrobenzene (DCNB) is selective for M class GSTs and used as a selective marker for this class.

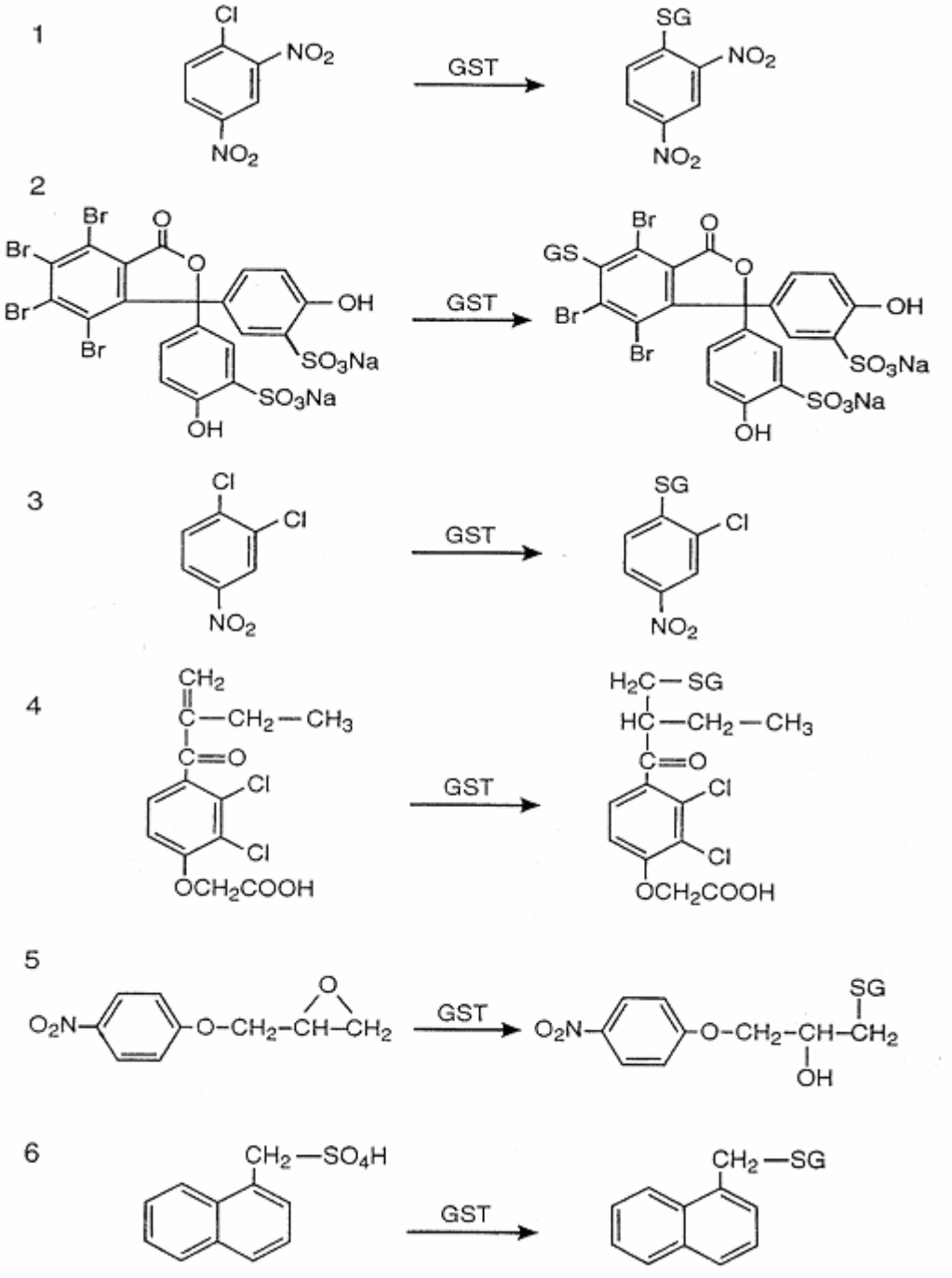
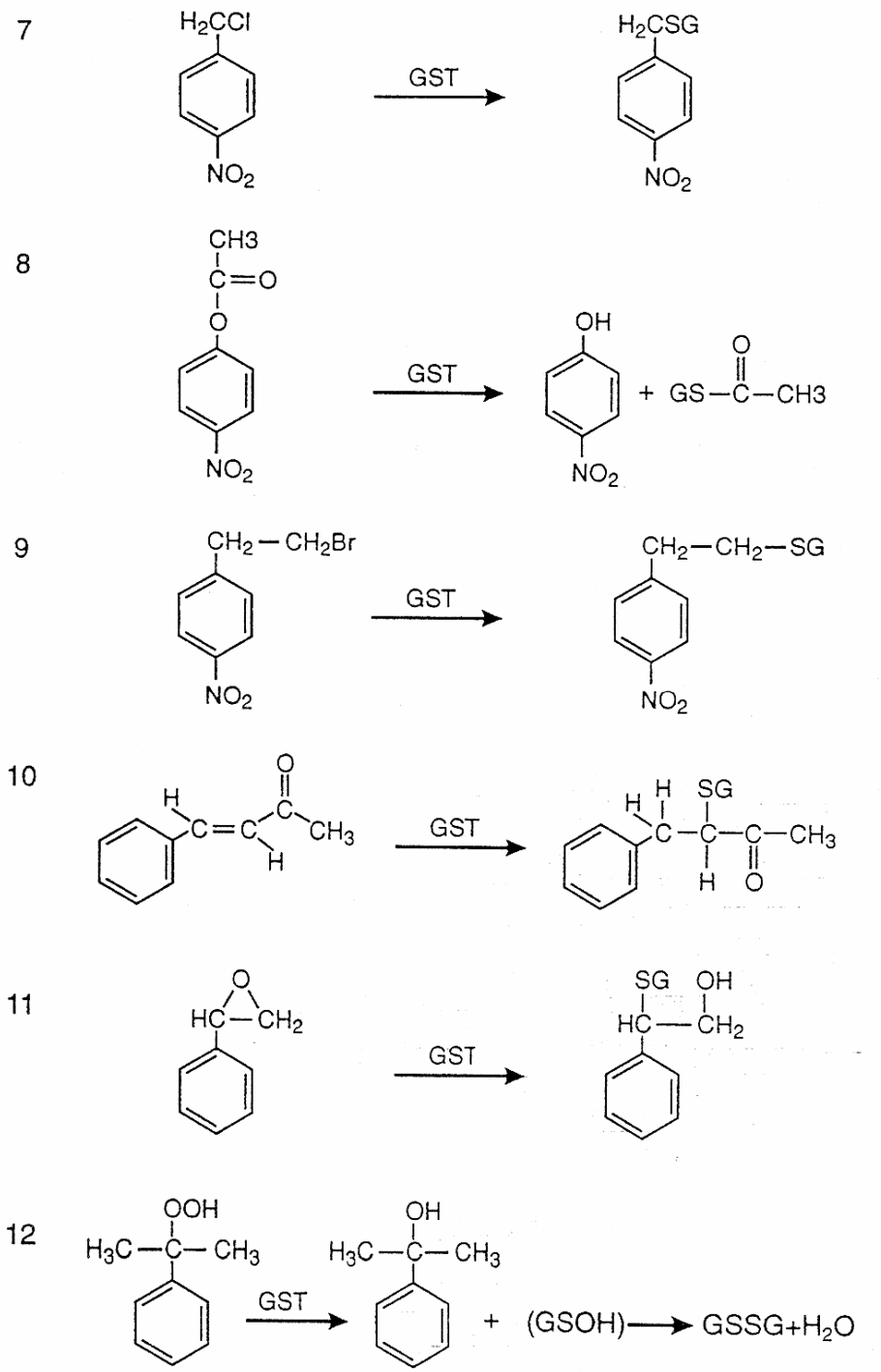


Figure 20 Model substrates used for characterization of GSTs

(Hayes and Pulford, 1995)

(1) CDNB; (2) bromosulfophthalein; (3) DCNB; (4) ethacrynic acid; (5) EPNP; (6) 1-menaphthyl sulfate; (7) 4-NBC; (8) 4-nitrophenylacetate; (9) 4-NPB; (10) trans-4-phenyl-3-buten-2-one; (11) styrene-7,8-oxide; (12) cumen hydroperoxide

Figure 20 (continued)



1.7 Chromosomal Location and Evolution of GSTs in Plants

1.7.1 Chromosomal Location

The completion of the *Arabidopsis* genome project has provided a complete overview of the GST gene family, which in this model plant is composed of 47 members divided into four distinct classes. The two larger classes, phi and tau, are presented by 14 and 28 members, respectively, are predominating, while the two smaller classes, zeta and theta are presented by only 2 and 3 members, respectively, which have also close homologues in animals.

The *At*GST family shows considerable sequence divergence with less than 25% identity at the amino acid level between different classes. **Figure 21** shows an amino acid sequence alignment based on a representative GSTs from each class. By aligning all sequences of a GST class, a triplet of amino acids at positions 66 to 68 relative to the sequence of *At*GST2 was identified as a distinguishing feature of the different classes (**Figure 21**).

The majority of *At*GSTs are present in the genome in clusters of two to seven genes, presumably as a result of multiple duplication events. All zeta-GSTs and theta-GSTs are located in single clusters and are well conserved. In contrast, the phi- and tau-class GSTs are present in a series of clusters which resulted in the generation of considerable sequence variability. Within the larger clusters, the amino acid sequence of the most divergent phi and tau GSTs shows only between 50 and 60% identity within each group (Wagner *et al* ., 2002).

Besides sequence similarity, different *At*GSTs classes are also characterized by conserved gene structures: the phi class GSTs have 2 introns, the tau-class GSTs have 1 intron, the zeta class GSTs have 9 introns and the theta-class GSTs have 6 introns.

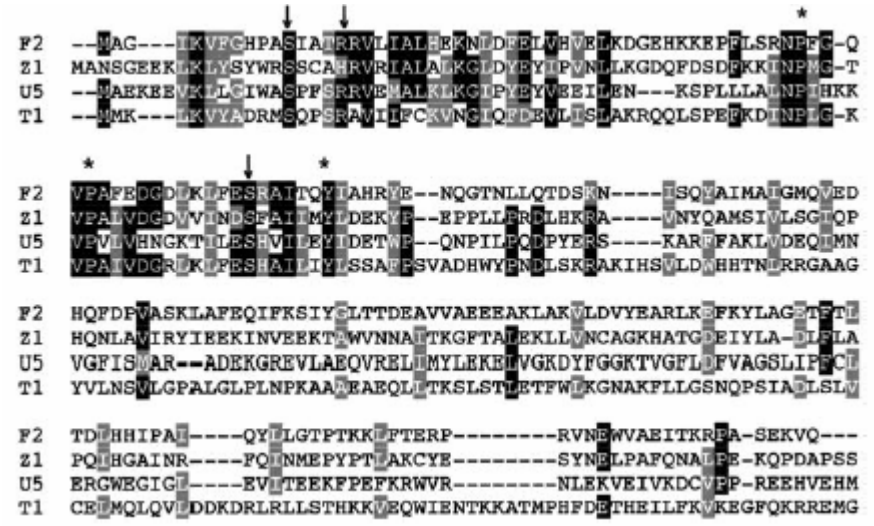


Figure 21 Sequence alignment of one *At*GST from each class

(Wagner *et al*., 2002)

With the last 11 amino acids of *At*GSTT1 omitted. The vertical arrows indicate positions that are conserved in nearly all *At*GSTs and form part of the catalytic site. The star indicates additional positions that are conserved in all 47 *At*GSTs. The composition of the amino acid triplett at the relative positions 66–68 is diagnostic for each GST class. Black background, identical amino acids; grey background, conserved substitutions.

1.7.2 Evolution of the GSTs

'Drug-metabolizing enzyme' (DME) have existed on this planet for more than 2.5 billion years. Genes encoding DMEs have functioned in many fundamental processes in prokaryotes and, more recently, in countless critical life processes in plants and animals.

GSTs constitute a very ancient protein superfamily that is thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (Martin, 1995). Other GSH- and cysteine-binding proteins share the thioredoxin-like fold, and it is increasingly becoming clear that GSTs share sequence and structural similiarities with several stress-related proteins in a wide range of organisms (Rossjohn *et al*., 1996). The multiple GST classes arose by a process of gene amplification followed by divergence which resulting in novel catalytic activities (Armstrong, 1998).

The three-dimensional structures indicate clear evolutionary distinctions between the various enzyme classes. The most pronounced structural distinctions involve the catalytic residue (tyrosine or serine) and the dimer interface. The theta and sigma class enzymes have in common a relatively flat and more hydrophilic interface that lacks the ball-and-socket interaction characteristic of the alpha, mu, and pi classes. However, the sigma class enzyme uses the same catalytic residue (tyrosine) as do the class alpha, mu and pi enzymes.

An analysis of both the sequences and exon-intron boundaries of several eukaryotic GST genes provides some interesting clues as to the evolutionary relationship of the gene families. Pemble and Taylor (1992) have recently compared the cDNA and gene sequences of class alpha, mu, pi, and theta GST from eukaryotes and prokaryotes and have argued that the alpha/mu/pi precursor gene arose from the duplication of the theta gene, leading to the suggestion that this class might represent the ancestral class. The highly conserved 3'-noncoding

sequences of the mu and theta genes also suggest that the mu gene diverged from this precursor before the pi or alpha gene. The progenitor of the theta class may be kappa class gene which encodes the mitochondrial enzyme (Pemble *et al*., 1996).

Alignments of the non-mammalian GST sequences suggest that there might be additional classses that are as distinct from each other as they are from the well-established mammalian alpha, mu, pi and theta classes. Clearly, the sigma class, which is well represented in cephalopods, the phi and tau classes in plants and the delta class in insects are examples of the additional diversity of GSTs (Buetler and Eaton, 1992; Blocki *et al*., 1993; Toung *et al*., 1993).

Many of the workers have used sequence comparisons to generate phylogenetic trees to identify likely patterns of divergence, as it is explained above (Pemble and Taylor, 1992; Pemble *et al*., 1996). Ideally, it should be possible to compare all full-length sequences known to code for GSTs, but in practice a subset of sequences is usually used to avoid misleading results (Synder and Maddison, 1997). **Figure 22** summarizing a possible pattern of divergence in GST superfamily.

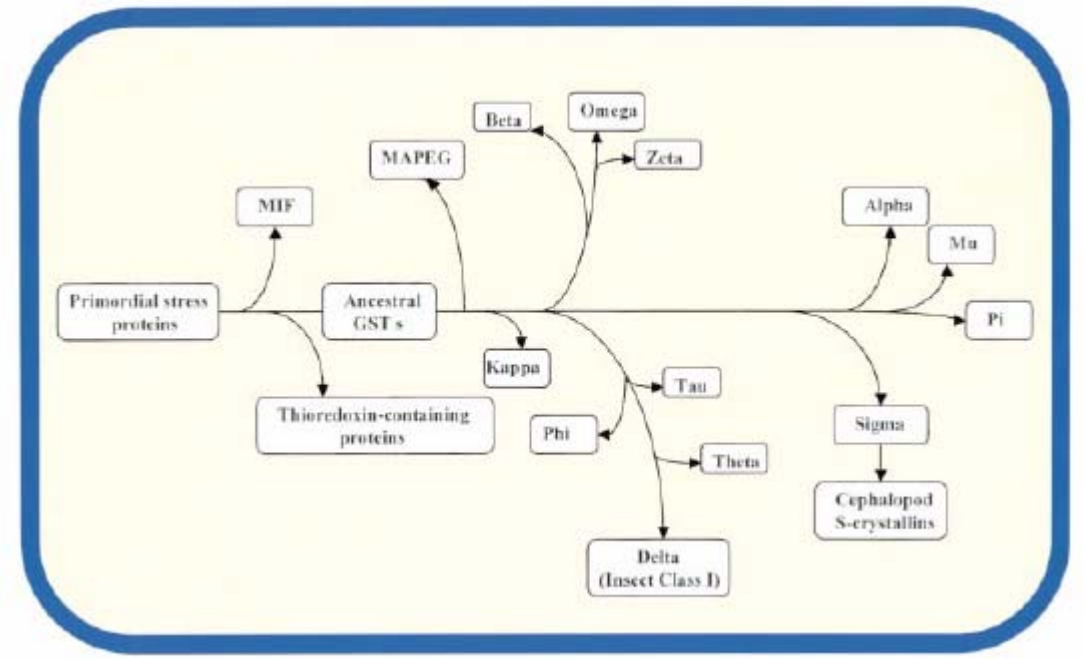


Figure 22 Possible pattern of divergence in the GST superfamily
(Sheehan *et al*., 2001)

Today, most of the data are consistent with that of the kappa class is being the progenitor of theta class and the other soluble GSTs. When solved, the three-dimensional structure of the kappa class enzyme will probably reveal more details about it is evolutionary relationship to the other soluble GST enzymes (Armstrong, 1997).

1.8 Isolation and Purification of GSTs and Identification of Their Subunits

The first report of GST activity in plants was published in 1970 by Frear and Swanson. The characterization of cytosolic GST has been greatly facilitated by the availability of affinity chromatography gels to which these enzymes bind. The multifunctional nature of GST has allowed a variety of affinity gels to be

designed that can be used to isolate GST, like agarose containing immobilized bromosulfopthalein, cholic acid, glutathione, S-hexylglutathione, S-octyglutathione, thyroxine, and triazine dye (Hayes, 1990). Among these affinity gels two matrices in particular, glutathione-agarose (Simons and Vander Jagt, 1977) and *S*-hexylglutathione-agarose (Guthenberg and Mannervik, 1979) have been widely used to purify alpha, mu, pi, and sigma class GSTs (**Figure 23**). Class mu, pi, and sigma GST are absorbed efficiently by both glutathione agarose and s-hexylglutathione agarose. By contrast, the class alpha GST do not display strong affinity for *S*-hexylglutathione agarose but most isoenzymes of this class are efficiently adsorbed by glutathione-agarose (Hayes, 1986).

The class theta GSTs have proven to be more difficult to purify than the other classes of GST because they are labile and are the least abundant family. Class theta GSTs are not retained neither by glutathione-agarose nor *S*-hexylglutathione agarose, but can be purified by affinity chromatography on the triazinyl dye gels, Orange A matrix and Blue Sepharose (Hiratsuka *et al*., 1990; Meyer *et al*., 1991; Mainwaring *et al*., 1996a). The reason for the failure of currently used glutathione-affinity chromatography matrices to purify theta class GSTs may be because this class possesses a much deeper active site than the other GST isoenzymes (Wilce *et al.,* 1995). Therefore, GSH immobilized to an agarose support via a longer spacer arm may provide an effective affinity gel for class theta GST (Lopez *et al.,* 1994). Following affinity purification of GSTs, the individual isoenzymes are normally resolved by exploiting differences in their charge using either ion-exchange chromatography, chromatofocusing, or isoelectric focusing (Jakoby, 1978; Mannervik, 1985). Alternatively, adsorbtion chromatography on hydroxyapatite can be a highly effective purification step when ion-exchange chromatography not provide homogeneous protein (Hayes *et al*., 1987).

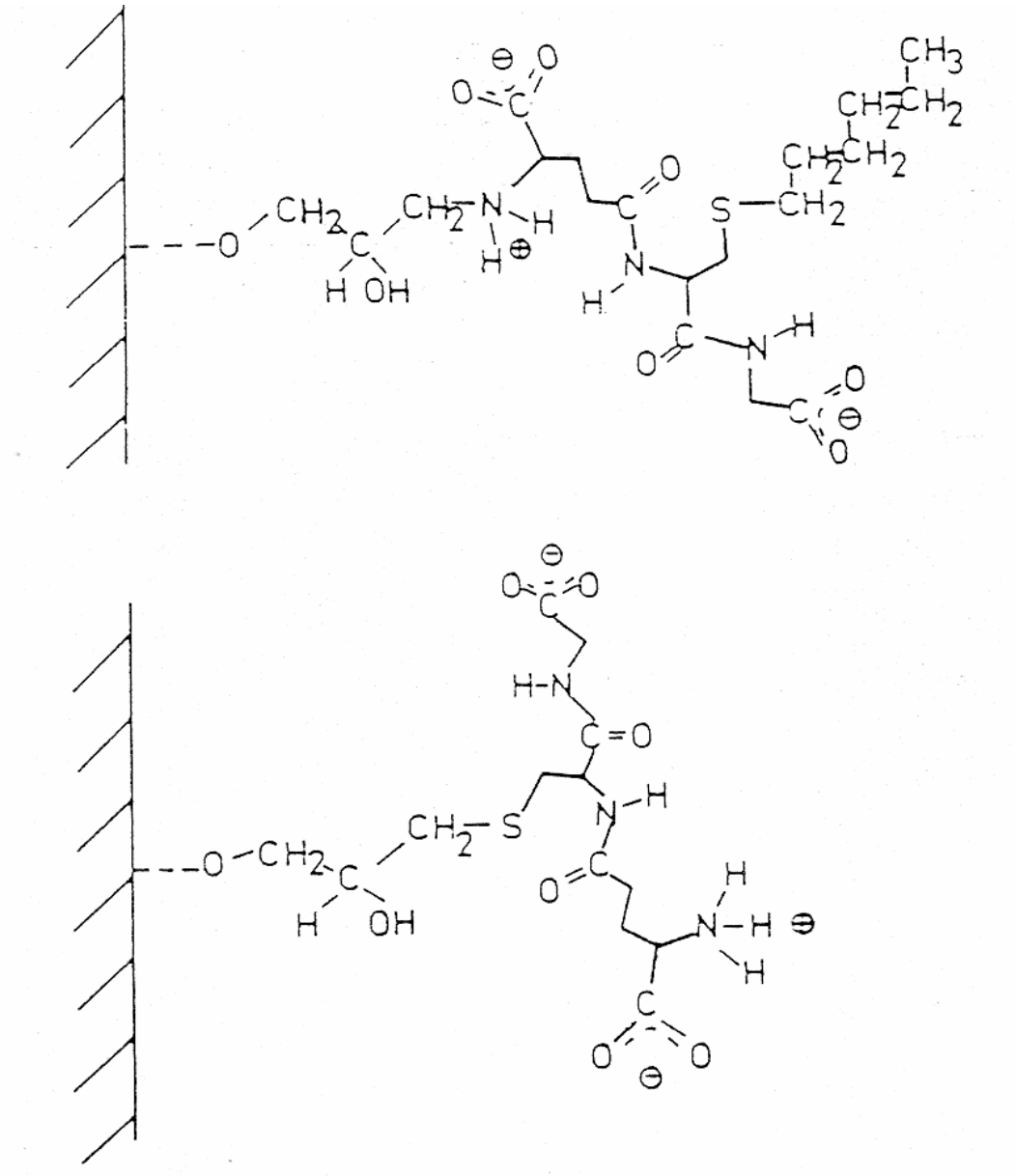


Figure 23 Structure and possible conformation of affinity ligands used for purification of GSTs

(a) Immobilized S-hexylglutathione; (b) Immobilized glutathione. In both cases the ligand is attached by coupling with the oxirane group of epoxy-activated Sepharose. It is proposed that the conformations of the ligands are complementary to the glutathione binding site (G-site) of a GST subunit.

The analytical methods that have proved valuable in the identification of the GST isoenzymes and the subunits they comprise include SDS-PAGE (Hayes and Mantle, 1986a), isoelectric focusing (Hales *et al.,* 1978; Aceto *et al.,* 1989), reversed-phase high-pressure liquid chromatography (HPLC) (Ostlund Farrants *et al.,* 1987), electrospray mass spectrophotometry (Yeh *et al.,* 1995), Western blotting (Hayes and Mantle, 1986b), and immunoassay with either polyclonal antibody against purified GST, specific GST peptides, or monoclonal antibodies (McCusker *et al.,*1989; Peters *et al.,* 1990; Juronen *et al.,* 1994, 1996a and 1996b, Sherratt *et al.,* 1997). Recently, reversed-phase HPLC has been increasingly employed to identify GST subunits. Satisfactory resolution of GST subunits can be obtained using a μ-Bondapak C_{18} column (particle size less than 10μm) developed with a 40 to 60% gradient of acetonitrile in 0.1% trifluoroacetic (Johnson *et al*., 1992).

In plants, as in the studies of mammalians after dialysis or gel filtration, the enzyme preparation frequently is subject to procedures such as DEAE chromatography (Mozer *et al*., 1983), affinity chromatography (O'Connel *et al*., 1988), phenyl Sepharose CL - 4B chromatography (Williamson and Beverely, 1988), chromatography on Mono-Q (Dean *et al*., 1991) or electrophoresis (O'Connell *et al*., 1988).

For example, glutathione S-transferase has been purified from leaves of sugarcane using isoelectic focusing, Sephadex G-100 gel filtration, DEAE cellulose ion-exchange chromatography, and affinity chromatography over GSH-linked to epoxy-activated Sepharose 6B (Singhal *et al*., 1991).

In 1993, glutathione S-transferases has been purified from needles of Norway spruce (*Picea abies* L. Karst.). Two isoforms of the enzyme which exhibit different physico-chemical and catalytic properties were separated by affinity chromatography on epoxy-activeted 4% linked- beaded agarose, using

GSH as the ligand, ion-exchange chromatography and isoelectric focusing (Schröder and Berkau, 1993).

Schröder and Wolf (1996) have shown the large difference in GST activity between healthy and damaged trees. To obtain more detailed information about the kinetic properties of GST in healthy and damaged trees, the enzyme was purified from needles collected from the Norway spruce stand in the valley (750 m above see level) where the largest differences in GST activity between damaged and healthy trees were observed. After the affinity chromatography and Mono-Q anion-exchange chromatography steps, the enzyme in needle extracts from healthy trees was purified 307-fold and recovery was about 3% of the total GST activity. The corresponding values for the enzyme in needle extracts from damaged trees were 255-fold and 3%. Anion exchange chromatography on a Mono-Q bed was indicated as the most effective step of the purification procedure in this study.

In 1998, the GST of wheat (*Triticum aestivum* L.) was isolated by glutathione-agarose affinity chromatography. The protein bound to the affinity column were analysed by SDS-PAGE. Reverse phase-HPLC analysis revealed 6 major peaks, which were isolated and their relative molecular masses determined by electrospray ionization mass spectrometry. As a result of this study, wheat GST subunits can be assigned to two groups after also determination of their N-terminal amino acids; constitutive subunits with identical N-termini and MW around 23.2 kDa, and constitutive and inducible subunits with some differences in N-terminal sequences, and MW around 24.9 kDa (Pascal *et al*., 1998).

The other study related with the cloning, characterization and regulation of a family of phi class glutathione transferases from wheat performed by Cummins and co-workers in 2003. In this study affinity columns were prepared by coupling either glutathione (Simons and Vander Jagt., 1981), S-hexyl-glutathione (Cummins *et al*., 1997), or S-sulphobromophtalein glutathione

(Mozer et al., 1983) to epoxy-activated Sepharose. In each case, after washing away unbound protein, affinity-bound GSTs were recovered by addition of 5mM glutathione (glutathione and S-sulphobromophtalein glutathione matrices) or 5mM S-hexyl glutathione to the loading buffer. The GSTs were then further purified by anion exchange chromatography using Q-Sepharose and their purity confirmed by SDS-PAGE (Thom *et al*., 2002).

1.9 Scope of This Work

Since 60's GSTs in mammalian and other species have been intensively studied. Plant GSTs had this chance after 70's, as their role discovered in herbicide detoxification. But, there is only a limited number of studies considering the isolation, characterization and purification of GSTs from forest trees, especially not in *Pinus brutia,* Ten (Turkish Red Pine, Kızılçam). As *Pinus brutia* Ten. is one of the most important forest trees of the Mediterranean Region in Turkey and considering the role of GST's under stress conditions, it was decided to examine the GST enzyme composition of this economically important tree.

Our first aim was to purify class Zeta enzymes from *Pinus brutia*. But, because of the contraversary results about the substrate availability for this class from the present literature between different research groups, our direction has been changed from Zeta class GSTs to the general investigations of GSTs in *Pinus brutia*. Therefore, only the molecular genetic studies were carried out on conserning the gene sequence of class Zeta GSTs isoenzyme, while purification studies focused on showing generally the presence of GST isozymes in *Pinus brutia*.

The substrate 1-chloro-2,4-dinitrobenzene (CDNB) is used in characterization of the purified enzyme as a common substrate, although it is not the substrate of all GST isozymes.

In this work, GST Zeta cDNA was cloned and characterized from *Pinus brutia* needles by using of RT-PCR and 5'RACE methods sequentially. The genomic DNA was isolated according to the method of Stange *et al*., 1998. The amplification of GST Zeta gene was obtained by PCR method. Thiol amount determinations and Northern Blot analysis were carried out on *Pinus brutia* needles.

Following the molecular studies, GSTs from *Pinus brutia* needles were purified. The purification protocol was modified from the method of Schröder and Wolf (1996). In this method the purification performed by the sequential use of dialysis or Sephadex G-25 column, Anion exchange DEAE cellulose column, *S*-hexylglutathione agarose affinity column chromatography.

After each of the purification step the purity of the fractions was checked on silver stained SDS-PAGE including the crude extract.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

RNA isolation kit and Mini prep for plasmid isolation were from QIAGEN Inc., Valencia,CA, U.S.A., Superscript reverse transcriptase were from GIBCO-BRL Life Technologies, Grand Island, NY, U.S.A., Jetsorb (DNA extraction kit from agarose gel) was from GENOMED Inc., St. Louis, MO, U.S.A., TA Ligation kit was from Invitrogen Corporation, Carlsbad, CA, U.S.A., monobromobimane were from Thiolyte MB, Calbiochem, Bad Soden, Germany, Turboblotter Rapid Downward Transfer Systems from Schleicker and Schuell Inc., Keene, New Hampshire, U.K., Hybond -XL membrane and G50 column were from Amersham Pharmacia Biotech, U.K. Limited, Herrings Sperm DNA, StripEZ DNA kit was from Ambion Company, x-ray film were from Eastman Kodak Company, Dallas, TX, U.S.A.

1-chloro-2,4-dinitrobenzene (CDNB), Ethacrynic Acid ([2,3-dichloro-4-(2-methylene butyryl)-phenoxy]acetic acid), reduced glutathione (GSH), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), Nonidet P-40, sodium dodecyl sulphate (SDS), SDS-PAGE molecular weight markers, ammonium persulphate (APS), bromophenol blue, diethylaminoethyl cellulose (DE-52), coomassie brilliant blue R-250, coomassie brilliant blue G, sucrose, N,N'-methylene-bisacrylamide (Bis), hydroxymethyl aminomethane (Tris), N,N,N',N'-tetramethylenediamine (TEMED), acrylamide, silver nitrate, glycerol, sodium carbonate, sodium thiosulfate, Tween-20, guanidine hydrochloride, dithiothreitol (DTT), nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl

phosphate (BCIP) premixed solution, *S*-hexylglutathione agarose, formaldehyde, sodium azide, and cellulose membrane dialysis tubing were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

PVP-K30 and Pepstatin A were obtained from Fluka Chemical Company, Neu-Ulm, FRG.

2-mercaptoethanol, ultra pure methanol, glacial acetic acid, dimethyl sulfoxide (DMSO) and Ciocalteu's Folin phenol reagent were from Merck, Darmstadt, Germany.

All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2 Sample Site and Plant Material

Needles from *Pinus brutia* trees (mature trees, more than 10 years old) of healthy appearance were harvested from Yalıncak area within the METU Campus in Ankara / TURKEY. Needles were either stored at –80 ° C immediately after collection or were crushed in liquid nitrogen and the powder was stored as aliquots at –80 ° C.

2.3 Methods

2.3.1 Molecular cloning of GST-Zeta genes from *Pinus brutia*

2.3.1.1 Isolation of Total RNAs from *Pinus brutia* needles

Needles of *Pinus brutia* were homogenized under liquid nitrogen in porcelain mortar and kept at –80°C. 100 mg were used for RNA isolation with the QIAGEN RNAeasy kit according to the manufacturer`s instruction. After the isolation, the concentration of RNA was determined spectrophotometrically by

taking the difference in absorbance at 260 nm and 320 nm, and calculated according to the formula given below:

(Abs λ 260 - Abs λ 320) x D.F. = μg / μl amount of RNA

where, D.F. is dilution factor.

2.3.1.2 Cloning of Zeta-GST cDNA from *Pinus brutia*

Sequences of Zeta-GST from *A. thaliana* were retrieved from the GenBank and from the EST bank of *Pinus sp.* (www.workbench.sdsc.edu). These sequences were aligned with CLUSTAL software and used to derive 4 PCR primers from conserved domains:

GSTN1 5'-GT(N*) CC(N) AC(N) (CT)T(N) GT(N) GA(CT) GG-3'
GSTN2 5'-TA(CT) (CT) T(N) GA(AG) GA(CT) AA (AG) TT(CT) CC-3'
GSTC1 5'-AA(N) AC(CT) TG(N) GG(N) AC(N) A(AG)(AG) AA -3'
GSTC2 5'-TC (N) G(AG)(N) GC(N) GT(AG) AA(N) CC-3'
(N* could be one of any four nucleotides)

2 μg RNA were reverse transcribed with the Superscript reverse transcriptase and polyT primer. PCR was run 32 cycles and the conditions were given in the Appendix. The cDNA was used for PCR reactions with following primer combinations:

1 GSTN1 and GSTC1,
2 GSTN1 and GSTC2,
3 GSTN2 and GSTC1.

A nested PCR reaction was run with 1 μl of the first PCR according to the following scheme:

from the reaction 1 ; **A** GSTN1 and GSTC2,
B GSTN2 and GSTC1,

C GSTN2 and GSTC2;
reaction 2 ; **D** GSTN2 and GSTC2;
reaction 3 ; **E** GSTN2 and GSTC2.

After the nested PCR, the products were resolved on 1% agarose-TBE gel and the DNA was visualised with ethidium bromide (Maniatis *et al*., 1982) The DNA bands were cut from the agarose gel and the DNA was extracted with the Jet Sorb. The fragments were cloned into pCRII plasmid by the TA cloning kit. The ligation mixtures were transformed into DH5α *E.coli* strain and plated on LB/ampicilline plates with X-gal for the selection of recombinant plasmids. The white colonies were subjected to a colony PCR with reverse and universal primers in order to identify plasmids containing the insert of the correct length. From those colonies that possessed inserts, overnight cultures in LB/ampicilline were set up. Plasmids were isolated from these cultures by the Plasmid mini Kit according to the manufacturer`s instruction. The length of the inserts was checked by the restriction endonuclease digests with *Eco*RI at 37 °C, which cuts the insert out of the pCRII plasmid vector (**Figure 24**). The identity of the inserts was checked by sequencing (MWG Company).

2.3.1.3 5'RACE for Rapid Amplification of cDNA ends

Because the insert obtained by PCR (A-1 combination) was not complete at 5' end, RACE (Rapid Amplification for cDNA Ends); an effective method to obtain full-length cDNA from mRNA, was utilized. mRNA was reverse-transcribed with a gene-specific primer; GSTZ2 (5'- CCT GCA ACA TCT TTC AAC AG-3'). Terminal transferase was used to add a homopolymeric A tail to the 3'end of the cDNA. Tailed cDNA was amplified by PCR using a gene specific primer GSTC3 (5'- GGC CTG TAA GCT AAT TGC-3') and the Poly T primer. The obtained cDNA was further amplified by a second PCR using specific primer GSTC4 (5'- AGA TCA TCT GGG AGA AGC-3') and Poly T primer. As a result the RACE products were cloned into the pCRII plasmid vector for sequencing process (**Figure 25**).

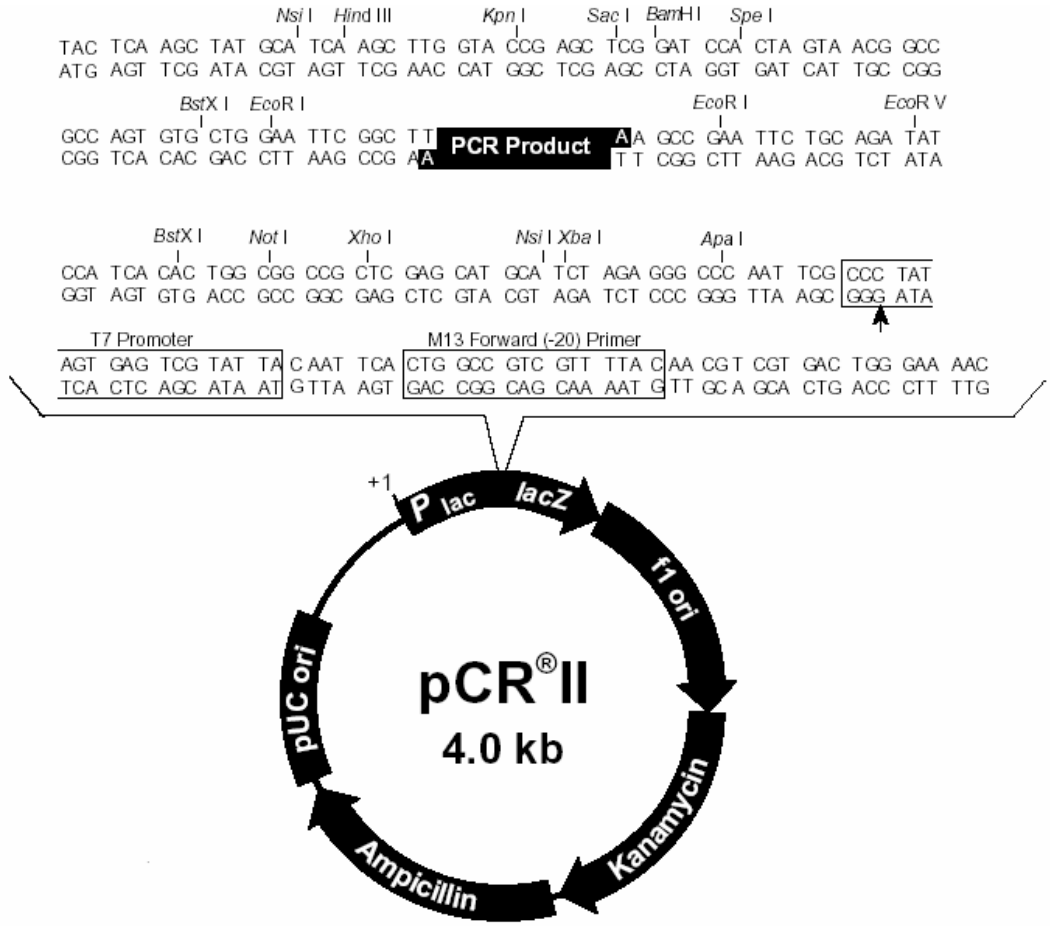


Figure 24 The partial map of pCRII plasmid vector

(from http://www.invitrogen.com/content/sfs/vectors/pcrii_map.pdf)

The sequence of the multiple cloning site is shown with a PCR product inserted by TA cloning. *Eco*R I sites flank the inserted PCR product on each side. The arrow indicates the start of transcription for the T7 RNA polymerase.

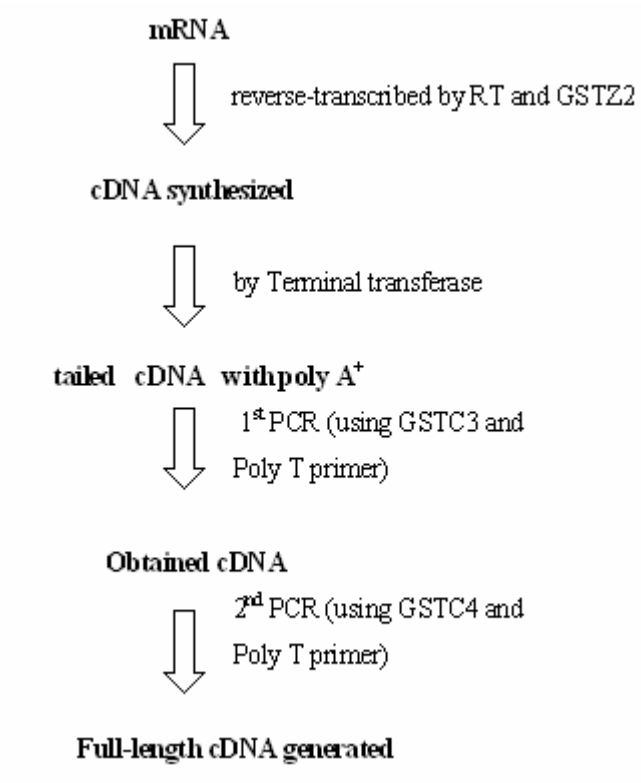


Figure 25 Flow Chart of 5' RACE (Rapid Amplification of cDNA ends)

RT: Reverse Transcriptase, GSTZ2, GSTC3 and GSTC4 are all Gene Specific Primers (GSP)

2.3.1.4 Isolation of Genomic DNA and amplification of GST Zeta gene by PCR from *Pinus brutia*

In order to investigate the heterogenocity in 30 different *Pinus brutia* trees, genomic DNA was isolated from needles. The aim was to amplify a part of the GST-Zeta gene, which contains many introns in *A. thaliana*, and try to detect a polymorphism in the introns of *Pinus brutia*. To isolate genomic DNA, needles of *Pinus brutia* were homogenized under liquid nitrogen and kept at –80ºC. 100mg of powder were used for genomic DNA isolation according to the Stange *et al*., (1998). CTAB buffer were used as extraction buffer containing 2% CTAB, 1.42 M NaCl, 20mM EDTA, 100mM Tris-HCl pH: 8.0, 2% w/v PVP-K30, 5 mM ascorbic acid and 4 mM DIECA (diethyldithiocarbamic acid). During the isolation procedure RNase A was also added (0.05 %v/v) to the samples for further purification. After genomic DNA isolation, DNA concentrations were measured using a UV-160B spectrophotometer (Shimadzu), according to Maniatis *et a*l., (1982). To check the quality, DNA (1μg) was digested for 1 hour with 1 unit *Eco*RI and subjected to agarose gel electrophoresis with undigested DNA as control.

The DNA was used for PCR reactions with the primer combinations of GSTZN (5'-GCA TGG CCT AAA CAC TTC-3') and $GSTZC_2$ (5'-CCT GCA ACA TCT TTC AAC AG-3'). After PCR, the products were resolved on 1 % agarose gel and the DNA was visualised with EtBr under UV light. The DNA bands were cut from the agarose gel and the DNA was extracted with the Jet Sorb. The fragments were cloned into pCRII plasmid by the TA cloning kit. The ligation mixtures were transformed into DH5α *E.coli* strain and plated on LB/ampicilline plates with X-gal for the selection of recombinant plasmids. The white colonies were subjected to a colony PCR with reverse and universal primers in order to identify plasmids containing the insert of the correct length. From those colonies, that possessed inserts overnight cultures in LB/ampicilline were set up. Plasmids were isolated from these cultures by the Plasmid mini Kit

according to the manufacturer`s instruction. The length of the inserts was checked by the restriction with *Eco*RI, which cuts the insert out of the pCRII plasmid. The identity of the inserts was checked by sequencing (MWG Company).

2.3.1.5 Determination of thiols

Determinations of thiols were carried out according to Schupp and Rennenberg (1988). 30 mg of powder was extracted in 750 µl 0.1 N HCl. After centrifugation 120 µl supernatant were mixed with 180 µl 0.2 M CHES-buffer (2-(*N*-cyclohexylamino)-ethane-2- sulphonic acid), pH 9.3, and 30 µl 5 mM DTT were added to reduce disulfides. After reduction, 20 µl of 30 mM monobromobimane was added and derivatization of thiols allowed to proceed for 15 min in the dark. The reaction was stopped and the conjugates stabilized by adding 240 µl 10% acetic acid. Bimane conjugates were separated by HPLC (SUPELCOSIL^{TM} LC-18, 25 cm x 4.6 mm, 5 µm, Sigma-Aldrich, Deisenhofen, Germany) using 10% (v/v) methanol/0.25% (v/v) acetic acid (pH 3.9) as solvent A and 90% (v/v) methanol/0.25% (v/v) acetic acid (pH 3.9) as solvent B. The elution protocol employed a linear gradient from 96% A to 82% A in 20 min and the flow rate was kept constant at 1 ml/min. Bimane derivatives were detected fluorimetrically (RF535, Shimadzu, Kyoto, Japan) with excitation at 390 nm and emission at 480 nm.

The thiol concentrations in the plant extracts were calculated from a standard calibration curve that was constructed from the corresponding nmol / injected volume values of thiol standards (1 mM GSH, 0.1 mM cystein and 0.1 mM γ-EC; 0 - 30 µl) (**Figure 26**) and the fluorimetrically detected values were estimated from this standard curve.

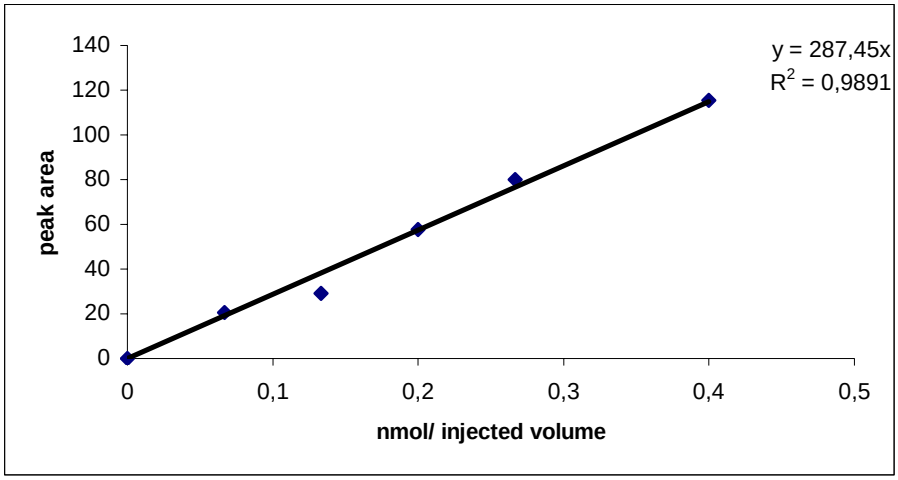


Figure 26 Calibration curve for standards used in GSH amount estimation

2.3.1.6 Northern Blot Analysis

2.3.1.6.1 Gel preparation and treatment

RNA was isolated with the QIAGEN RNAeasy kit as mentioned above. After isolation, the concentrations of RNAs were determined spectophotometrically at 260 nm. The agarose gel was prepared by using 1X MAE and 2 % formaldehyde. RNA samples were prepared for loading into the gel by the additions of necessary amounts of RNA loading buffer. Samples placed on 65°C thermoshaker for 10 min to denature. After denaturation samples were kept on ice for 5 minutes and loaded onto the agarose gel. RNA samples were separated by using 1X MAE buffer as the electrophoresis buffer. Following electrophoresis, RNAs visualized within the gel with UV light and photographed.

The capillary blot was set up by using Turboblotter Rapid Downward Transfer Systems from Schleicker and Schuell according to the manufacturer's instruction. 20X SSC were used as the transfer buffer. Transfer allowed to

proceed overnight. The Hybond -XL membrane was used from Amersham Pharmacia Biotech UK Limited.

After blotting, the transfer apparatus carefully dismantled, membrane was marked with a pencil to allow identification of the tracks. To fix the nucleic acids to the membrane, membrane was baked at 80°C for 2 hours.

2.3.1.6.2 Hybridization protocol (in tube)

Membrane was settled in hybridization tube and Church buffer (0.5 M Na_2PO_4, 1% SDS pH: 8.0) was used as hybridization buffer. Herrings Sperm DNA was added into the prehybridization buffer. The tube was kept at 65 °C for 90 minutes in hybridization oven. Then radiolabelled probe was prepared by using the StripEZ DNA protocol. The GST Zeta cDNA from *Pinus brutia* was denatured at 95°C for 5 minutes, snap cooled on ice, and briefly centrifuged to draw the contents to the bottom of the tube. After addition of buffer, dNTPs, Klenow fragment of DNA Polymerase, and [α-^{32} P] dATP (3000Ci/mmol, 10mCi/ml), the reaction was incubated at 37°C for 10 minutes. Afterwards this mixture was cleaned with G50 column and denatured in the boiling water bath for 3 minutes. This mixture was added into the hybridization tube and hybridized at 65°C overnight.

After hybridization, the blot was washed as follows with low and high stringency wash solutions; a) twice, 10 minutes in 2X SSC, 0.1 % SDS,

b) once, 30 minutes in 1X SSC, 0.1 % SDS

(1X SSC is 0.15M NaCl and 15 mM sodium citrate, pH 7.0)

After last wash, the blot was wrapped in SaranWrap and exposed to X-ray film. The cassette was kept at -80°C for 2 weeks and the film was developed.

2.3.2 Preparation of Cytosolic extracts from *Pinus brutia* needles

Crude enzyme extracts were prepared of the procedure described by Schröder and Wolf (1996). Briefly, 5 gr. of frozen needles first cut into small pieces by scissors then were homogenized in liquid N_2 in precooled porcelain mortars and extracted at 4 ° C with 10 vol. (w/v) of 0.1 M Tris-HCl buffer, pH: 7.8, containing 20 mM 2- mercaptoethanol, 5% PVP K-30, 2 mM EDTA, 0.5% Nonidet P-40, 5mM GSH and 3µg/ml Pepstatin A. After homogenization for 90 sec. at 13,500 rpm with an UltraTurrax, the crude extract was centrifuged at 15,000 rpm (Hettich INC, USA) using 1112 rotor for 30 min., at 4°C with table top centrifuge. The pellet was discarded and supernatant fraction was passed through a layer of filter paper. The filtrate thus obtained was referred as cytosol **(Figure 27)**. Most frequently, the protein determination, enzyme activity measurement and purification process were started immediately after the preparation of the cytosol without any further storage. Otherwise, the cytosol in small aliquots of 0.5 ml was stored at -80°C to be used later in SDS-PAGE analysis.

2.3.3 Protein Determinations

The protein concentrations in the cytosol prepared from *Pinus brutia* needles were determined by the method of Lowry and co-workers (Lowry *et al.,* 1951) with crystalline bovine serum albumin (BSA) as a standard. Aliquots of 0.1 to 0.5 ml of 1:50 diluted cytosol were taken into test tubes and were completed to a final volume of 0.5 ml with distilled water. Then, alkaline copper reagent was prepared by mixing 2% copper sulfate, 2% sodium potassium tartarate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100, respectively. Afterwards, 2.5 ml of the alkaline copper reagent was added to each tube, mixed by vortex and allowed to stand undisturbed for 10 minutes at room temperature. Finally, 0.25 ml of 1 N Folin Phenol reagent was added to each test tube, mixed immediately within 8 seconds by vortex and incubated 30 minutes at room

temperature. The intensity of color developed in each tube was measured at 660 nm.

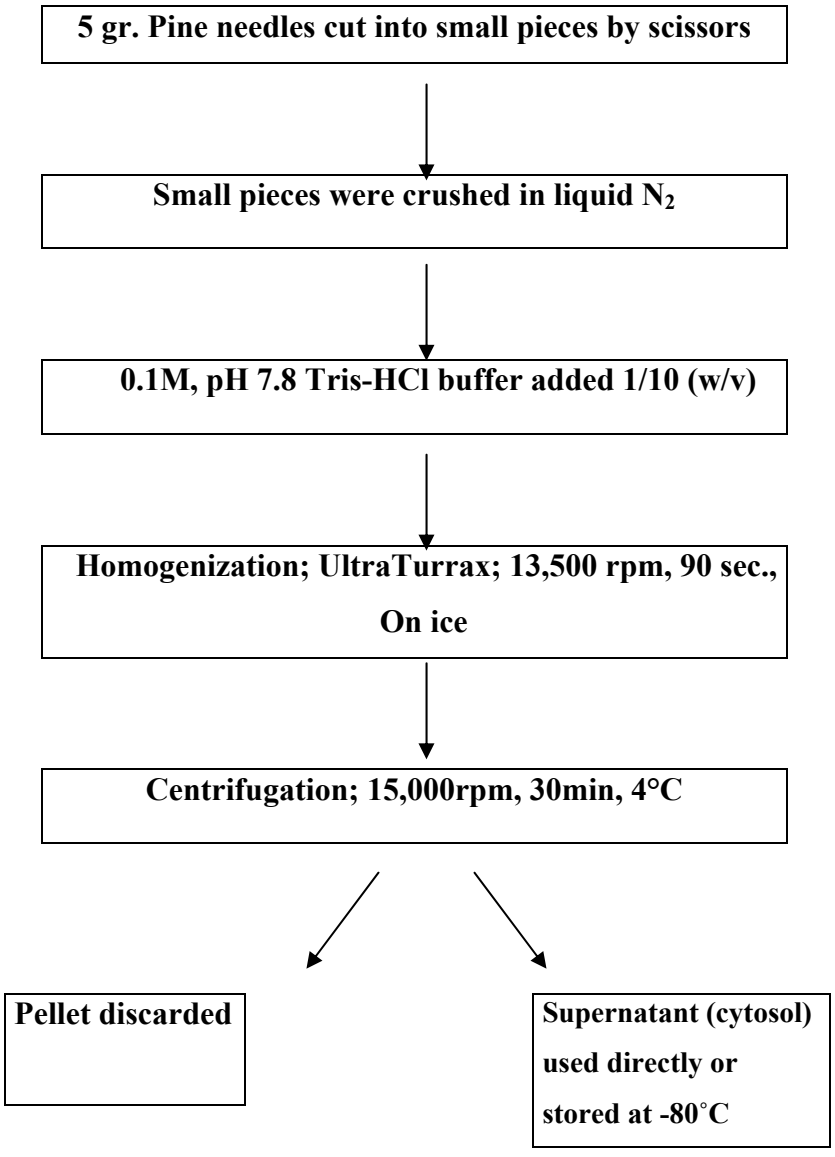


Figure 27 Outline of cytosol preparation from *Pinus brutia* needles

The protein concentrations in the crude extracts were calculated from a standard calibration curve that was constructed from the corresponding $O.D_{660nm}$ values of BSA standards (0 to 200 μg). The average protein amount of *Pinus brutia* cytosols used throughout this study was found as 3.5 μ 1 mg/ml. The protein concentrations in the column chromatography fractions were also determined spectrophotometrically according to the method of Warburg and Christian (1941).

2.3.4 Determination of Cytosolic GSTs Activities towards CDNB

GSTs activities against the substrate CDNB (1-chloro-2,4 dinitrobenzene) were determined spectrophotometrically by monitoring the formation of the conjugation product under the conditions given in **Table 2** (Gillham, 1971; Habig *et al*., 1974; Habig and Jakoby, 1981). The *Pinus brutia* needles cytosolic fractions were used as the enzyme source. All enzyme activity measurements were carried out at 25˚C using a spectrophotometer equipped with thermoregulated cell holder.

A typical reaction mixture included 20 mM phosphate buffer, pH 8.0, 1 mM CDNB, 1 mM GSH, and 0.2 mg ± 0.05 *Pinus brutia* cytosolic protein in a final volume of 1 ml as shown in **Table 2**.

Table 2 Conditions for spectrophotometric GSTs enzyme assays with CDNB

Substrate	Stock [Substrate] (Mm)	Final [Substrate] (mM)	Final [GSH] (mM)	Final [Buffer] (M) "pH" *	λ_{max} (nm)	ε ($\mu M^{-1}cm^{-1}$)
CDNB	20.0	1.0	1.0	0.1 "8.0"	340	0.0096

* 0.20 M Potassium phosphate buffer was used with CDNB

The constituents of the incubation mixture for GSTs enzyme assays are shown in **Table 3**. The activity measurements were performed using UV 160 A Schimadzu double beam spectrophotometer at 340 nm. The reactions were followed for 2 minutes which is in the linear period of the reaction. The reactions were started by the addition of cytosol. Incubation mixtures without the enzyme source were used as blanks (nonenzymatic reactions), and concentrations of the formed conjugation products were determined from the slopes of initial reaction rates. The reaction rate was calculated using the ε values of CDNB as 0,0096 μM^{-1} cm^{-1} (Habig and Jakoby, 1981). The GSTs activities were expressed as unit/mg protein. One unit of enzyme activity is defined as the amount of enzyme that forms one nmole of product per minute under defined assay conditions.

Table 3 The constituents of the incubation mixture for GSTs enzyme assays with the substrate (CDNB) given in the previous table

Constituents of The Reaction Mixture	**Added volume (µl)**
Substrate Solution[a]	50
Combination Solution - Buffer[b] - 50.0 mM GSH[c], - H_2O,	900
Enzyme Source[d] - *Pinus brutia* Cytosol (3.5 µ 1 mg/ml), or purified protein fraction	50

[a] The substrate is first dissolved in pure ethanol then H_2O is added in a ratio of 3:2, respectively to have a final 3%(v/v) ethanol in the reaction mixture.

[b] The proper buffer volume is added to have the final [buff.] indicated in Table 2

[c] The proper volume is added to have the final [GSH] indicated in Table 2

[d] The proper dilution is made to have 20 µg cytosolic protein for GSTs assay with CDNB

2.3.5 Purification of GSTs from *Pinus brutia* Cytosols

The purification scheme, for obtaining GST from *Pinus brutia* cytosolic fractions, consisted of a sequential gel filtration (Sephadex G-25), DEAE cellulose anion exchange, *S*-hexylglutathione agarose affinity column chromatography using CDNB as a substrate to follow the activity in the eluted fractions.

The pooled fractions of last step with activity against CDNB were stored at -80°C after checking the purity and determining the molecular weight of the obtained GST isoenzymes using SDS-PAGE.

2.3.5.1 Gel Filtration Column Chromatography on Sephadex G-25

The column (2.5cm X 45.0cm) packed with Sephadex G-25 was equilibrated in the cold room with 10 mM potassium phosphate buffer, pH 7.8 containing 2 mM EDTA, 20 mM 2-mercaptoethanol and 10%(v/v) glycerol. The freshly prepared cytosolic samples from *P.brutia* containing 4.53 mg protein and 130.84 unit /mg of GSTs activity towards CDNB was applied to the column with the flow rate of 60 ml / min. Afterwards, the column was washed with the equilibration buffer until no absorption of effluent at 280nm was detected. The absorbance at 280 nm as well as the activity against CDNB in the collected fractions (5ml each) was determined and the fractions having activity were combined in one fraction of which the activity against CDNB and the protein content were determined. The combined fraction was then applied to the DEAE Anion Exchange column chromatography.

The Sephadex G-25 gel was regenerated in the column, without repacking, by washing with 0.2 M NaOH (about 5 bed volumes). The column was then washed extensively with distilled water (more than 10 bed volumes) and equilibrated again with the equilibration buffer. The resin is stored at 4°C in distilled water or neutral buffer containing 0.02 % sodium azide to avoid microbial growth.

2.3.5.2 DEAE Anion Exchange Column Chromatography

The column (4.0cm X 30.0 cm) packed with DEAE cellulose and equilibrated in the cold room with 25 mM potassium phosphate buffer pH: 7.8 containing 2mM EDTA, 20mM 2-mercaptoethanol and 10% (v/v) glycerol. The combined fraction, from the previous step, containing 2.41mg protein and 184.93 unit /mg of GSTs activity towards CDNB was applied to the column with the flow rate of 60 ml/hour. The column was then washed with the equilibration buffer until no absorption of effluent at 280 nm was detected. The bound proteins were eluted from the column with a linear NaCl gradient (0 - 1.0 M) consisting of

100ml of the equilibration buffer and 100 ml of the same buffer containing 1.0 M NaCl.

The elution fractions with the highest activity against CDNB were pooled collected and applied to *S*-hexylglutathione affinity column. The washing unbound fractions with the highest activity against CDNB were also collected and stored at -80°C.

The DEAE anion-exchanger was regenerated by repacking the column, in the beaker, by washing with 0.5 N HCl (about 5 bed volumes) for 40 min., following 0.5 N NaOH (about 5 bed volumes) for 40 min and then with 0.5 N NaOH in 96 % of ethanol (about 5 bed volumes) for 15 min., and finally with 0.5 N NaOH (about 5 bed volumes) for 30 min. by repeated washes with deionized water (more than 10 bed volumes) between the each step and at the end. The column was then equilibrated again with the equilibration buffer. The resin is stored at 4°C in 10X neutral buffer.

2.3.5.3 Affinity Column Chromatography on S-Hexylglutathione Agarose

The column (1.0 cm X 5.0 cm) packed with *S*-hexylglutathione agarose was equilibrated in the cold room with 10 mM potassium phosphate buffer, pH 7.8 containing 2 mM EDTA, 20 mM 2-mercaptoethanol and 10% (v/v) glycerol. The combined fraction, from the previous step, containing a total of 0.92 mg protein with 545.94 unit/mg of GSTs activity towards CDNB was applied to the column at a flow rate of 8 ml/hour. Afterwards, the column was washed with one bed volume of the equilibration buffer. The column was further washed with the same buffer containing 0.2 M NaCl until no absorption of effluent at 280nm was detected. Afterwards, the bound proteins were eluted with 20 ml of equilibration buffer containing 0.2 M NaCl and 15 mM GSH at a flow rate of 12 ml/hr.

GSTs activities against CDNB were measured in the fractions collected from the column. The elution fractions with the highest activity against CDNB were pooled collected in small aliquots of 0.5ml were stored at -80°C to be used later in SDS-PAGE analysis. The washing unbound fractions with the highest activity against CDNB were also combined in one fraction and stored at -80°C.

The *S*-hexylglutathione agarose affinity gel was regenerated in the column, without repacking, by washing with 1.0 – 2.0 M NaCl or 3.0 – 6.0 M guanidine – HCl (about 5 bed volumes) to remove the bound substances. The column was then washed extensively with distilled water (more than 10 bed volumes) and equilibrated again with the equilibration buffer. The resin is stored at 4°C in neutral buffer containing 0.02 % sodium azide to avoid microbial growth.

2.3.6 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis, in the presence of the anionic detergent sodium dodecyl sulfate (SDS), was performed on 4 % stacking gel and 12 % or 15% separating gel in a discontinuous buffer system as described by Laemmli (1970). The seven proteins given below were used as molecular weight standards.

- Bovine Albumin	(M_r 66000)
- Egg Albumin	(M_r 45000)
- Glyceraldehyde-3-Phosphate Dehydrogenase	(M_r 36000)
- Carbonic Anhydrase	(M_r 29000)
- Trypsinogen	(M_r 24000)
- Trypsin Inhibitor	(M_r 20100)
- α-Lactalbumin	(M_r 14200)

2.3.6.1 Preparation of Reagents

(A) Stock Separating Gel Buffer: (1.5 M Tris-HCl, pH 8.8)

36.3 gr Tris base were dissolved in about 100 ml distilled water and pH 8.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

(B) Stock Stacking Gel Buffer : (0.5 M Tris-HCl, pH 6.8)

12.1 gr Tris base were dissolved in about 100 ml distilled water and pH 6.8 was adjusted with 1 M HCl. Finally completed to 200 ml.

(C) Stock Gel Solution: (Acrylamide-BIS, 30 % A, 2.67 % C)

60.0 gr acrylamide were dissolved in about 175 ml distilled water and then 1.6 gr BIS (Bis-acrylamide) were added and solution was completed to 200 ml with distilled water. Finally, the solution was filtered through course filter paper.

Note: % A represents acrylamide monomer percent concentration and % C indicates the crosslinking monomer concentration, which were calculated as below:

% A = [(gr acrylamide/total volume)] X 100

% C = [gr BIS/(gr acrylamide + gr BIS)] X 100

(D) 10 % SDS Solution:

10 gr SDS were dissolved in water with gentle stirring and completed to a final volume of 100 ml.

(E) Catalyst: (10 % Ammonium Persulfate "APS")

Prepared freshly by dissolving 100 mg ammonium persulfate (APS) in a final volume of 1 ml distilled water.

(F) Tracking Dye: (0.05 % Bromophenol Blue)

Tracking dye solution was prepared by dissolving 5 mg solid bromophenol blue in a final volume of 10 ml.

(G) 5 X Electrode (Running) Buffer: (25 mM Tris, 192 mM Glycine, pH 8.3)

Stock running buffer solution was prepared by dissolving and completing 15 gr Tris base, 72 gr glycine to 1 liter distilled water. The pH of the buffer was not adjusted with acid or with base. This buffer was diluted 1:5 and 1 gr solid SDS was added to 1 liter of buffer before use.

(H) 4 X Sample Dilution Buffer: (SDS Reducing Buffer)

0.25 M Tris-HCl buffer, pH 6.8 containing 8 % SDS, 40 % glycerol, 20 % 2-mercaptoethanol, 0.004 % bromophenol blue. It was prepared by mixing the following volumes of given solutions:

2.5 ml	1 M Tris-HCl, pH 6.8
4.0 ml	Glycerol
2.0 ml	2-mercaptoethanol
0.4 ml	Tracking Dye
0.8 gr	SDS
Distilled water to 10.0 ml	

2.3.6.2 Electrophoresis Procedure

Vertical slab gel electrophoresis was carried out using the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, USA) that can be used to run two gels simultaneously. The assembly of the glass plate cassettes (8.3 X 7.4 cm) and the process of gel casting were done according to instruction manual provided with the apparatus. Once the cassettes were properly assembled and mounted, the preparation of the separating and stacking gels was started.

The 12 % separating gel and 4 % stacking gel polymerizing solutions were prepared just before use by mixing the given volumes of stock solutions in the written order as given in **Table 4**. The separating gel solution was first prepared with the TEMED added just before casting the gel into the glass assembly from the edge of one of the spacers until the desired height of the solution (about 5 cm) was obtained. Then, the liquid gel was overlaid with distilled water (about 0.5 ml), without disturbing the gel surface, to obtain an even interface between the separating gel and the stacking gel. The gel was then allowed to polymerize at room temperature for a minimum of 30 minutes. After polymerization, the layer of water was removed completely using filter paper without hurting the gel surface. The stacking gel was then poured on the top of the resolving gel and the comb was inserted into the layer of the stacking gel without trapping air bubbles under the teeth of the comb. The gel was then allowed to polymerize for a minimum of 30 minutes. After the gel was polymerized, the comb was removed carefully and the wells were washed with distilled water and filled with electrode buffer. At this point, the gel cassettes were removed from the casting stand, mounted and clamped onto the running frame with the notched glass plate of each cassette facing inside. When running only one gel, the blank plastic plate, provided with the system, was mounted in the place of the second cassette in the casting stand and in the running frame.

Aliquots from the protein samples to be analyzed and from the standards mixture were diluted 3:1 with the 4X sample buffer (3 parts sample and 1 part

sample buffer), to have the samples in 62.5 mM Tris-HCl buffer, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue. Then the samples and standards were placed in a boiling water bath for 5 minutes. Afterwards, protein samples and molecular weight standards (5 – 25 µl) were loaded into different wells using a 25 µl Hamilton syringe with a tipped needle.

Table 4 Formulations for SDS-PAGE separating and stacking gels

	Separating Gel		**Stacking Gel**
Monomer Concentration	**12 %**	**15 %**	**4 %**
Acrylamide/bis	12.0 ml	15.0 ml	1.3 ml
Distelled water	10.0 ml	7.0 ml	6.1 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	----
0.5 M Tris-HCl, pH 6.8	----	----	2.5 ml
10% (w/v) SDS	300 µl	300 µl	100 µl
10 % APS	185 µl	185 µl	50 µl
TEMED	15 µl	15 µl	10 µl
Total monomer	**30 ml**	**30 ml**	**10 ml**

After loading the samples, the running buffer (135 ml) was added to the compartment formed by the running frame and the cassettes (the upper buffer compartment) and the system was checked for leakage. The running buffer (250ml) was then also added to the outer tank (the lower buffer compartment). Thereafter, the running frame was inserted into the outer tank, the safety cover was replaced and the leads were plugged into the EC250-90 electrophoresis power supply. The power supply was adjusted to give a constant current of 15

mA when the samples were in the stacking gel and 30 mA when the samples passed to the separating gel. Under these conditions the voltage was about 50 V at the beginning and elevated up to 150 V at the end of the run that took a total of about 1.5 hours.

The power supply was switched off, when the dye front is just 0.5 cm from the lower end of the glass plates, the running frame was taken out and the buffer was removed from the upper buffer compartment. Afterwards, the clamps were detached and the cassettes were removed from the running frame. To gain access to the gels in the cassette, the glass plates were pried apart using a spatula taking care not to chip the edges of the glass plates. The left-top corner of each gel was cut to indicate the order of wells. The gels, usually adhered to one of the glass plates, were taken carefully using gloves and placed in the previously prepared appropriate solutions to stain the samples which have been resolved on the gels, or to prepare the gels for subsequent blotting.

2.3.6.3 Silver Staining of the SDS-PAGE Gel

The silver staining of the SDS-PAGE gels was carried out according to the method of Blum *et al.* (1987) as explained in **Table 5**.

The relative mobility (R_f) of each protein was determined by dividing its migration distance from the top of the separating gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel.

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The R_f values (abscissa) were plotted against the known molecular weights (logarithmic scale ordinate) and standard line was drawn and its slope was used in the calculation of the molecular weight of proteins.

Table 5 Procedure for Silver Staining of Proteins in Polyacrylamide Gels

Steps	Solution[a]	Time of Treatment[b]
1) Fix	50 % Methanol; 12 % Acetic acid; 0.5 ml 37 % HCOH /liter	≥ 1 h
2) Wash	50 % Ethanol	3 X 20 min
3) Pretreat	$Na_2S_2O_3.5H_2O$ (0.2 g/liter)	1 min[c]
4) Rinse	H_2O	3 X 20 sec[c]
5) Impregnate	$AgNO_3$ (2 g/liter)	20 min
6) Rinse	H_2O	2 X 20 sec[c]
7) Develop	Na_2CO_3 (60 g/liter); 0.5 ml 37 % HCOH /liter; $Na_2S_2O_3.5H_2O$ (4 mg/liter)[d]	10 min
8) Wash	H_2O	2 X 2 min
9) Stop	50 % Methanol; 12 % Acetic acid	10 min
10) Wash	50 % Methanol	≥ 20 min[e]

[a] Solutions freshly prepared in a quantity that is 10-fold larger than the volume of the gel.

[b] Steps 1-10 were carried out on a shaker at room temperature (20-25³C).

[c] The times indicated here should be observed exactly in order to ensure a reproducible image development.

[d] (20 ml/ liter) from the solution prepared in step 3 are added instead.

[e] After step 10, gel is transferred to 4°C for storage.

CHAPTER III

RESULTS AND DISCUSSION

In this study, the total RNAs from *Pinus brutia* needles were isolated, GST Zeta cDNA was cloned by RT-PCR from those mRNAs isolated and the identity of the inserts was checked by sequencing. Later, the length of the insert was elongated by applying 5' RACE (Rapid Amplification for cDNA ends) method and the identity of the insert was checked by sequencing again. The genomic DNA was also isolated from *Pinus brutia* needles, amplified by PCR and sequenced, and compared to the sequence of cDNA. Afterwards, the GST isozyme was purified from *Pinus brutia* needles by applying the cytosol sequentially to the Sephadex G-25, the anion exchange, and *S*-hexylglutathione affinity chromatography columns.

The needle samples were collected from Yalıncak area closer to the METU Campus in Ankara. The GST activities in those samples were determined by using CDNB as substrate.

3.1 Molecular Cloning of GST-Zeta genes from *Pinus brutia*

3.1.1 Cloning of Zeta-GST cDNA from *Pinus brutia*

In order to characterize GST Zeta gene form a higher plant, we cloned the corresponding cDNA from *Pinus brutia*. Using *A.thaliana* GST-Zeta sequence available from GenBank and GST-like EST from *Pinus* sp., degenerate primers were designed by using of the CLUSTAL software and synthesized against conserved domains. Part of GST-Zeta coding sequence was amplified

from *Pinus brutia* by RT-PCR. After the nested PCR, the products were resolved on 1% agarose gel and the DNA was visualised with ethidium bromide (**Figure 28**). The length of the fragment was estimated as about 670-680 base pairs, by the usage of PCR markers which are ranged in size from 150-1000 base pairs. These markers are convenient for sizing PCR and restriction fragments, as seen in **Figure 28.** Since the obtained cDNA sequence was not in full length due to the degenerate nature of the primers, the missing 5'terminus was obtained by application of 5'-RACE method which is effective in obtaining full length cDNA from mRNA (**Figure 29 and 30**). The results of the sequence analysis of the cDNA obtained was converted to amino acid information as shown below and the GST protein of *Pinus brutia* was estimated to be containing 226 amino acids.

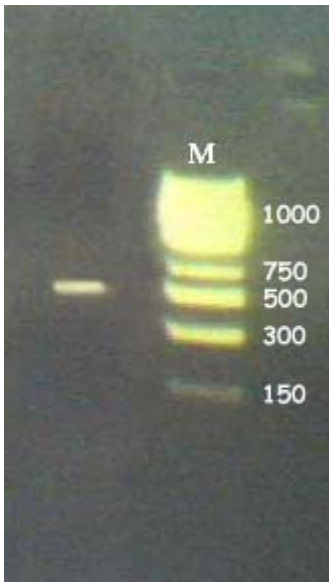


Figure 28 Nested PCR products were resolved on 1% Agarose gel

The DNA was visualized with EtBr, M is PCR markers

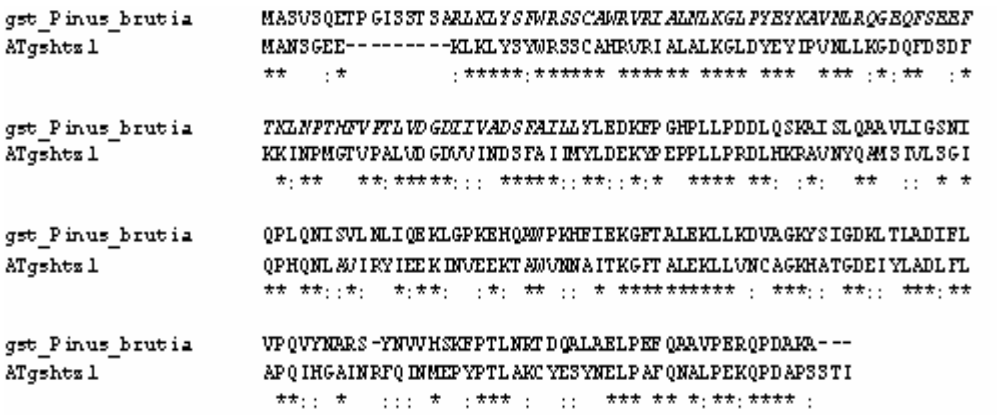


Figure 29 Comparison between the sequence of GST-Zeta from *Pinus brutia* and *Arabidopsis thaliana*

The sequences were aligned by CLUSTAL software. (*) indicates identical residues, (:) indicates similar amino acids substitutions. The *Pinus brutia* sequence printed in bald originated from the first RT-PCR experiment (section 2.3.1.2. in Materials and Methods), italic characters represent the sequence from 5' RACE experiments (section 2.3.1.3. in Materials and Methods), and normal script represents sequence derived from sequencing of the expression construct.

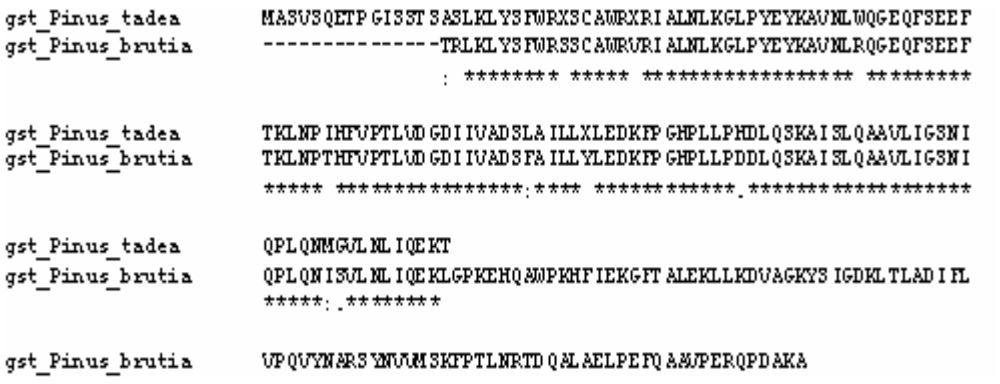


Figure 30 Comparison between the sequence of GST-Zeta from *Pinus brutia* and EST from *Pinus tadea*

The sequences were aligned by CLUSTAL software. (*) indicates identical residues, (:) indicates similar amino acids substitutions.

The resulting full length sequence was composed of 226 amino acids and show high sequence similarity to *A.thaliana* and *Pinus tadea* Zeta GSTs, as well as to some other distinct species. In plants, the first Zeta class GST was identified from carnation petals (*Dianthus caryophyllus*), which accumulated during senescence and in response to ethylene treatment (Meyer *et al*., 1991). In 1997, nucleotide and deduced amino acid sequence of GSTZ1 from human were reported by Board and his co-workers (Board *et al*., 1997). More recently a wheat Zeta GST cDNA was isolated from two wheat cultivars (Subramaniam *et al*., 1999) and from Arabidopsis (Wagner *et al*., 2002).

The sequence alignments of those Zeta class GSTs show that the TA-GSTZ1 (wheat) sequence shares about 49% similarity with the carnation GST gene. Both these plant sequences share about 40% similarity with the human and the nematode GSTs, while the latter two share 52% similarity between them. The N-terminal region has even higher similarity, which may reflect the biological importance of this region. The N-terminal region of the GSTs is believed to be functionally important in glutathione binding. The **SSCXWRVRIAL** motif present in this region is conserved in the Zeta class GSTs so far and may be important for certain activity of these enzymes (Board *et al*., 1997). This finding in the literature is consistent with our results presented in **Figure 29** and **30,** in the sequence of GST-Zeta from *Pinus brutia*, *A.thaliana*, and *P.tadea*.

3.1.2 Isolation of Genomic DNA from *Pinus brutia*

As explained in 2.3.1.4. (under Materials and Methods Section), *Pinus brutia* genomic DNAs were isolated (**Figure 31**) and PCR were applied to examine possible variation in intron lengths. The experimental design was set up to detect a polymorphism in the introns of *Pinus brutia* by an amplification of a part of the GST-Zeta gene, which normally contains many introns in *A.thaliana*.

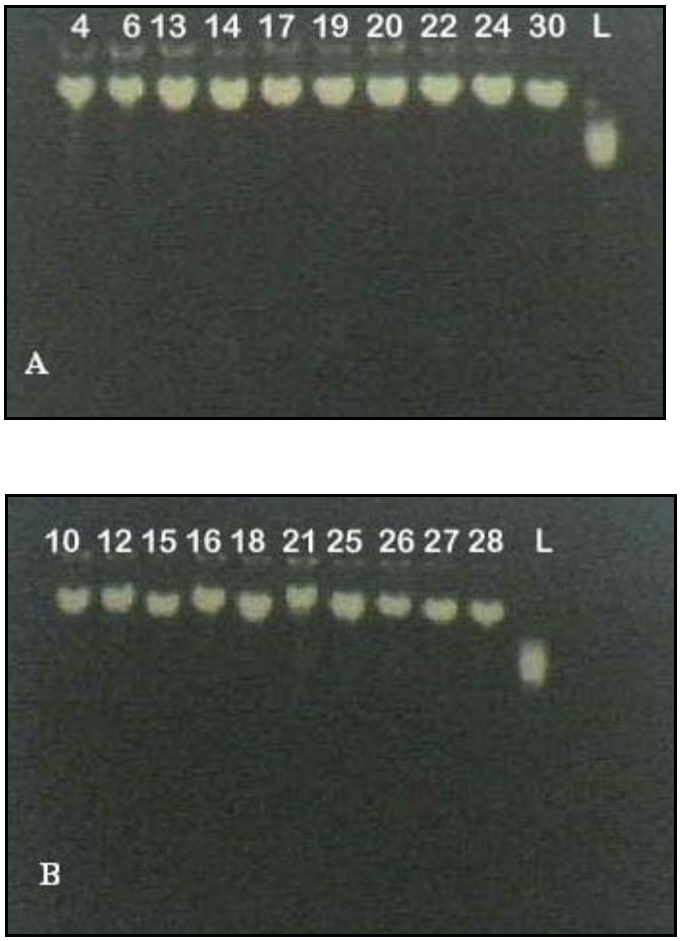


Figure 31 Isolated Genomic DNAs of 20 different samples from *Pinus brutia* L is 1kb DNA ladder

The reason for this experimental design of genomic DNA isolation was to investigate the heterogenocity in 30 different *Pinus brutia* trees in terms of possible different insert lenghts. As a result of this experimental design, only fragments with lengths corresponding to the cDNA were amplified. If the fragments amplified from genomic DNA (PCR products) and cDNA are of the same size, it means that nothing have been spliced out from the genomic DNA. Even if there would be one intron of 200 bp, we expect to see a fragment amplified from genomic DNA 200 bp longer than that from cDNA. By knowing

this and our results showing that 'only the fragments with lengths corresponding to the cDNA could be amplified' indicates that the GST-Zeta gene in *Pinus brutia* was most probably intronless, however this result needs to be further clarified. Therefore, only very little variability of GST-Zeta locus among the individual trees can be expected. On the other hand, genomic sequence shows that carnation has 10 exons: 9 introns (Itzhaki and Woodson, 1993), *C.elegans* has 6 exons: 5 introns, human has 8 exons : 7 introns (Board *et al*., 1997), TA-GSTZ1 has 9 exons : 8 introns (Subramaniam *et al*., 1999), and *Arabidopsis* has 10 exons : 9 introns (Wagner *et al*., 2002) (**Table 6**), which shows genomic organization of gst_Pinus_brutia is different from *A.thaliana* and other Zeta class GSTs.

Table 6 The difference of conserved gene structure and number of amino acid residues between different species

Source of GST Zeta Gene	Conserved Gene Structure (exons : introns)	Number of Amino Acid Residues
Dianthus caryophyllus	10 : 9	221
Caenorhabditis elegans	6 : 5	214
Human	8 : 7	216
Wheat	9 : 8	213
Arabidopsis thaliana	10 : 9	219
Pinus brutia	Intronless	226

3.2 Correlation of GST Zeta mRNA levels to thiol content

As explained in 2.3.1.5. (under Material and Methods Section), Bimane derivatives of Cys, γ-EC, and GSH were detected fluorimetrically (RF535, Shimadzu, Kyoto, Japan) with excitation at 390 nm and emission at 480 nm (Schupp and Rennenberg, 1988).

The amount of GSH in *Pinus brutia* needles have been found as changing between 233.14 - 435.96 nmole/g FW for 30 individual trees. This result indicates that the antioxidant GSH amounts is varying between individual trees approximately 1.8 fold.

The GSH amount found in the measurements were consistent with the literature, as Schupp and Rennenberg (1990) reported that GSH amounts in spruce (*P.abies* L.) were approximately 300 nmole/g FW. However, many environmental factors like, sulfur content of the soil and atmosphere, growth at high altitudes, temperature are known to affect the concentration of glutathione in plant cells.

As glutathione *S*-transferases use GSH as a co-substrate, GSH amount and the expression level of GST-Zeta in individual trees of *Pinus brutia* were compared in order to understand the correlation between them. The expression levels of GST-Zeta among individual trees of *Pinus brutia* were also compared in order to examine the presence of genetic variation. Therefore, the cDNA was utilized as the probe for the application of northern blot analysis.

From the blot analysis it was concluded that there were a substantial variation in the GST-Zeta steady state mRNA levels (**Figure 32**). Densitometric quantification revealed that the mRNA levels varied up to three-fold, whereas GSH amounts varied approximately 1.8 fold (**Figure 33**), and there were no

correlation between the GST-Zeta expression and GSH concentration in the needles of 20 individuals of *Pinus brutia*.

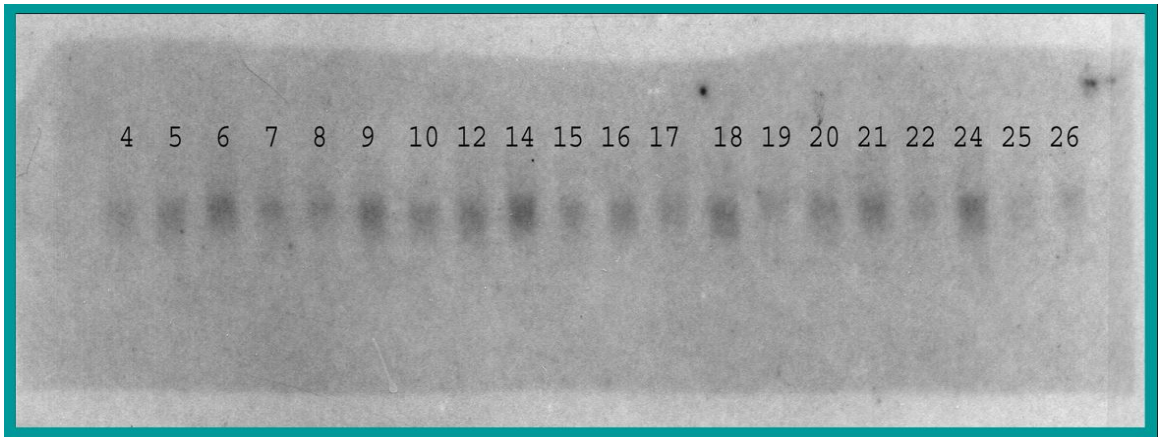


Figure 32 The gel photograph of Northern blot with 20 different RNA samples from *Pinus brutia*

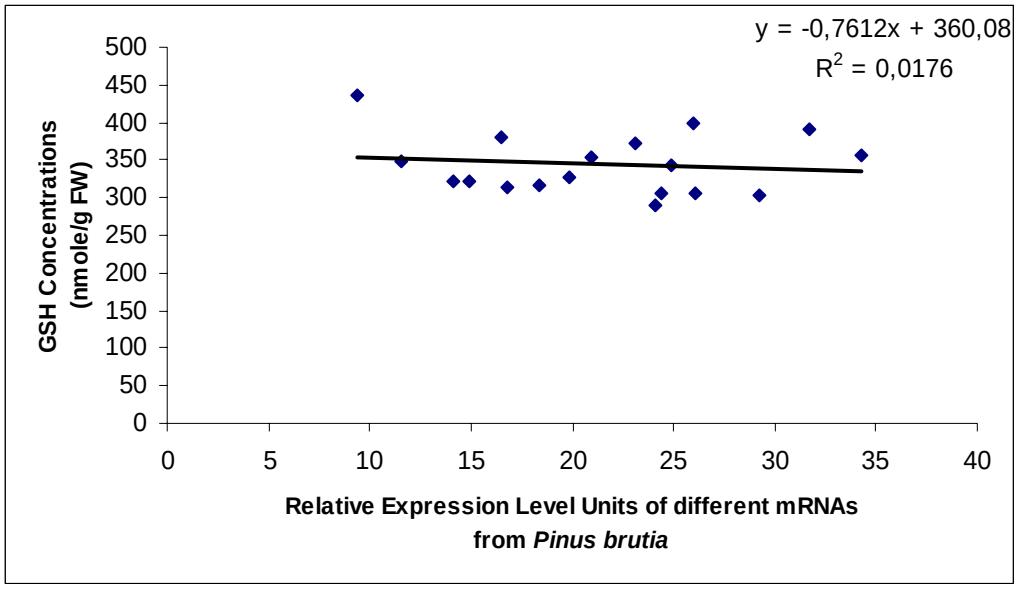


Figure 33 The relation between Expression Levels and Glutathione Concentrations of 20 different RNA samples from *Pinus brutia*
Relative Expression Level Unit is given according to the lowest level of expression.

3.3 The GSTs of *Pinus brutia* Cytosolic Fractions

Ethacrynic acid (EA) was used as a substrate to determine the activity of GST-Zeta isozyme specifically at the beginning of this work, since purification and characterization of Zeta isozyme was aimed. As seen in **Table 7**, ethacrynic acid was shown to be one of the substrates of human GSTZ1-1 isozyme during the initial discovery of Zeta class GSTs by the work of Board and his co-workers (Board *et al*., 1997). However, the attemps of using EA as the substrate to determine GST-Zeta activity of *Pinus brutia* was unsuccesful. In fact, in the literature, it was also reported that the EA was a GST inhibitor, *in vitro* (Morgan *et al*., 1996; van Iersel *et al*., 1996, 1997). Eventually, in this study, CDNB was used as a substrate to identify GSTs in *Pinus brutia*.

Table 7 Activity of recombinant human GSTZ1-1 with various substrates (Board *et al*., 1997)

Substrate	Specific activity (nmol/min per mg of protein)
Cumene hydroperoxide	160 ± 11
t-Butyl hydroperoxide	170 ± 4
1-Chloro-2,4-dinitrobenzene	n.d.
1,2-Dichloronitrobenzene	n.d.
4-Phenylbut-3-en-2-one	n.d.
Ethacrynic acid	45 ± 15
7-Chloro-4-nitrobenz-2-oxa-1,3-diazole	32 ± 5
1-Menaphthyl sulphate	n.d.
Dichloromethane	n.d.
p-Nitrobenzyl chloride	n.d.
p-Nitrophenyl acetate	14 ± 6
trans-Non-2-enal	n.d.
trans-Oct-2-enal	n.d.
Hexa-2,4-dienal	n.d.
trans,*trans*-Deca-2,4-dienal	n.d.

Glutathione S-transferase (GSTs) activities in the cytosolic fractions prepared from *Pinus brutia* needles were determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate by monitoring the thioether formation at 340 nm, as described by Habig and co-workers (Habig *et al*., 1974).

The average value of GST enzyme activity of cytosols prepared from *Pinus brutia* needles was calculated as 560.5 μ 30 unit/ml towards CDNB at 25°C. The effect of temperature on the GST activity against CDNB was shown in **Figure 34**. As it has apparently seen in the **Figure 34**, there was at about 68% decrease in the GST activity against CDNB after 25°C**.** With these results obtained it was decided to continue to perform all the enzyme assays at 25 ºC.

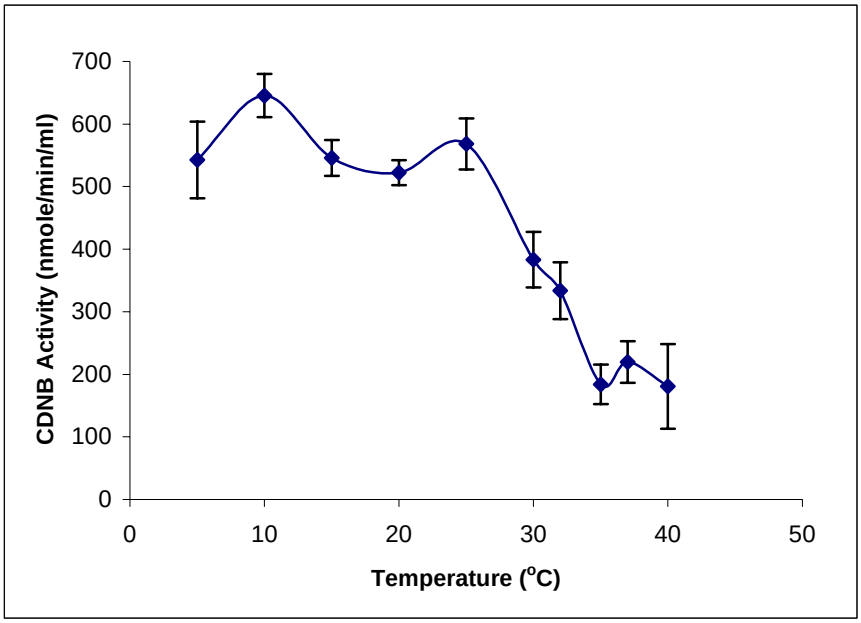


Figure 34 The effects of temperature on the GST activity against CDNB

3.4 Purification of GSTs

The purification of the GST class enzymes from *Pinus brutia* needles were carried out basically according to the purification protocols used by Schröder and Wolf (1996) with some modifications. This method included the sequential gel filtration column chromatography on Sephadex G-25, Anion exchange DEAE cellulose column, and *S*-hexylglutathione agarose affinity.

The cytosol prepared as described under "Methods" was subjected to dialysis prior to ion exchange chromatography on DEAE. However, it was noted that almost 50 % of GST activity towards CDNB was lost during overnight dialysis at 4°C. Therefore, the cytosol was immediately applied to Sephadex G-25 in order to get rid off PVP-K30 and Nonidet P-40. Schröder and Wolf (1996) were used PD-10 columns for the same purpose. The chromatography on Sephadex G-25 provided not only the removal of additives of homogenisation buffer, but also the exchange of buffer prior to next chromatographic step. Relevant literature clearly explains that a 0.1 M potassium phosphate buffer pH: 7.8, containing 1% PVP K30, 5 mM EDTA, and 0.25 % Nonidet K40 (Schröder *et al*., 1990a, 1990b; Schröder and Berkau, 1993) yielded 20 ± 2 % lower GST activity for the conjugation of CDNB than the Tris buffer system.

3.4.1 Gel Filtration Column Chromatography on Sephadex G-25

Cytosolic samples from *Pinus brutia* needles containing a total of 297.85 mg protein with 130.84 units / mg of GSTs activity towards CDNB was applied to the Sephadex G-25 column (2.5 cm X 45 cm).

The elution profile of Sephadex G-25 is shown in **Figure 35**. The flowthrough fractions from the column having high CDNB activity were combined. The combined fraction had a total of 160.27 mg protein with 184.93 unit/mg of GSTs activity towards CDNB. As shown in **Table 8**, almost 46 % of

cytosolic proteins were lost. Sephadex G-25 column provided about 1.4 purification fold with about 76 % recovery of the total enzyme activity.

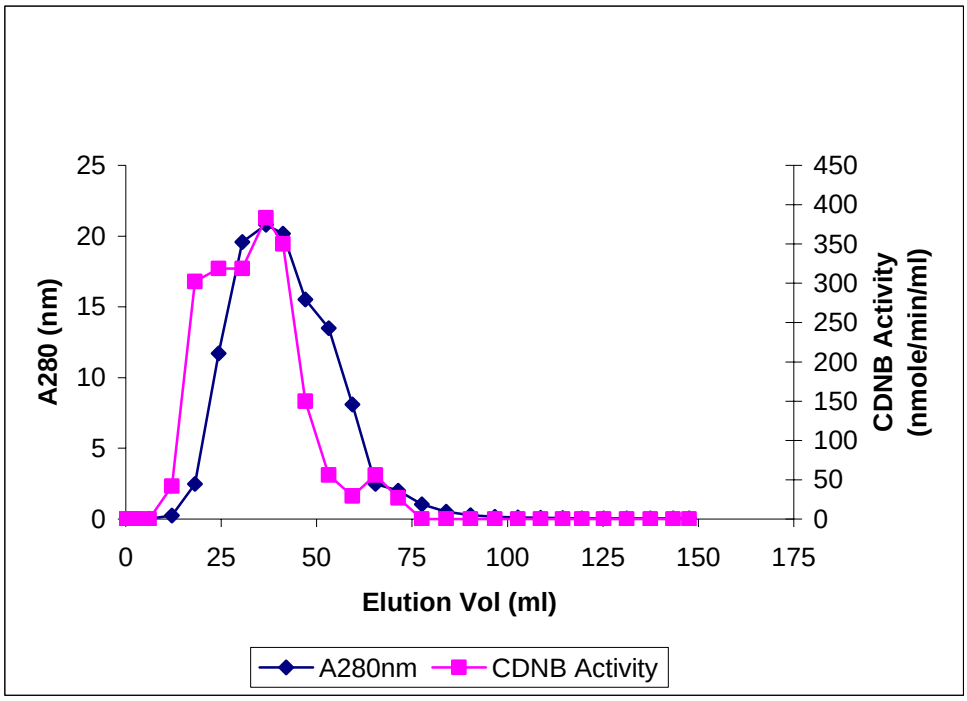


Figure 35 Purification profile for *Pinus brutia* needles cytosolic GSTs on the Gel Filtration Chromatography Column (2.5cm X 45cm) of Sephadex G-25

The freshly prepared cytosolic fraction from the needles having 4.53 mg/ml protein and 130.84 unit/mg of GST activity against CDNB were applied to the column. The GST containing fraction identified by their activity towards CDNB, were pooled for further purification. Activities of GSTs against CDNB as a substrate were measured at 340 nm, as described under 'Methods'.

3.4.2 DEAE Cellulose Anion Exchange Column Chromatography

As described under "Methods", the combined fraction, from the previous step, was applied to the DEAE column (4.0 cm X 30 cm). Afterwards, the column was washed with the equilibration buffer until no absorption of effluent at 280 nm was detected. Then, the bound proteins were eluted from the column with a linear NaCl gradient consisting of 100 ml of the equilibration buffer and 100 ml of the same buffer containing 1.0 M NaCl. The elution profile of the column is shown in **Figure 36.**

As seen in the figure, great amount of protein flowed through the column without binding, however, the amount of GSTs lost in the flowthrough fraction was only 30 % of the total in comparison to that of eluted at the salt gradient. All of the GSTs with activity against CDNB which was bound to the column were eluted at about 0.3 M NaCl concentration. The eluted fractions with the highest activity against CDNB were combined in one fraction (**Figure 36**) and applied immediately to the *S*-hexylglutathione agarose affinity column for further purification. The combined fraction had a total of 39.33 mg protein with 545.94 unit / mg of GSTs activity towards CDNB. According to these results, as shown in **Table 8**, the ion exchange column provided about 4.17 fold purification with about 55 % recovery of GSTs activity against CDNB.

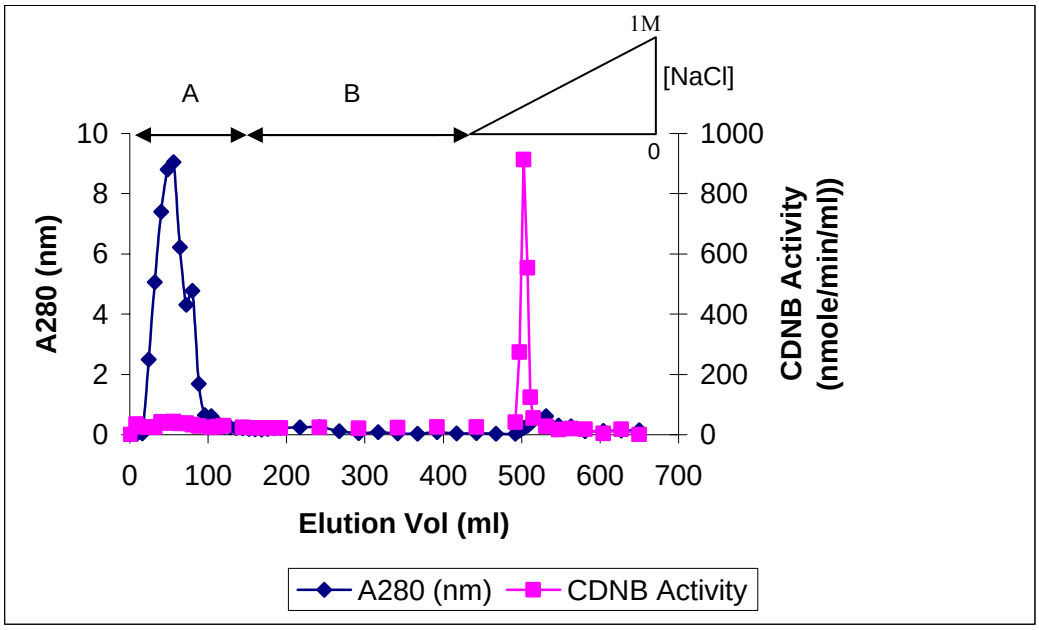


Figure 36 Purification profile for *Pinus brutia* needles GSTs on DEAE Cellulose anion exchange Chromatography Column (4.0cm X 30.0cm)

The combined fraction from previous step having 2.41 mg/ml protein and 184.93 unit/mg of GST activity against CDNB were applied to the column. Activities of GSTs against CDNB were measured at 340 nm, as described under 'Methods'. The fractions with activity towards CDNB that were bound by the column were eluted by a linear NaCl gradient. A: Sample Application, B: Washing with equilibration buffer, C: Gradient with 100ml equilibration buffer and 100 ml equilibration buffer containing 1M NaCl.

3.4.3 Affinity Column Chromatography on *S*-Hexylglutathione Agarose

As described under "Methods", the combined fraction, from the previous step, was applied to the *S*-hexylglutathione agarose affinity column (1.0 cm X 5.0 cm). Afterwards, the column was washed with one bed volume of the equilibration buffer at a flow rate of 10ml /hour. Nonspecifically bound proteins were further washed away with the same buffer containing 0.2 M NaCl until no absorption of effluent at 280nm was detected. Afterwards, the bound proteins were eluted with 20 ml of equilibration buffer containing 0.2 M NaCl and 15 mM GSH.

During the sample application and washing the eluted fractions exhibited some activity towards CDNB as substrate (**Figure 37**). This was due to the leakage from the column. The affinity column material has a tendancy to loose its affinity towards GSTs after several repeated chromatography. The lower flow rate during sample application and washing may overcome the leakage, however, elongation of the chromatographic procedure may result in the loss of enzyme activity.

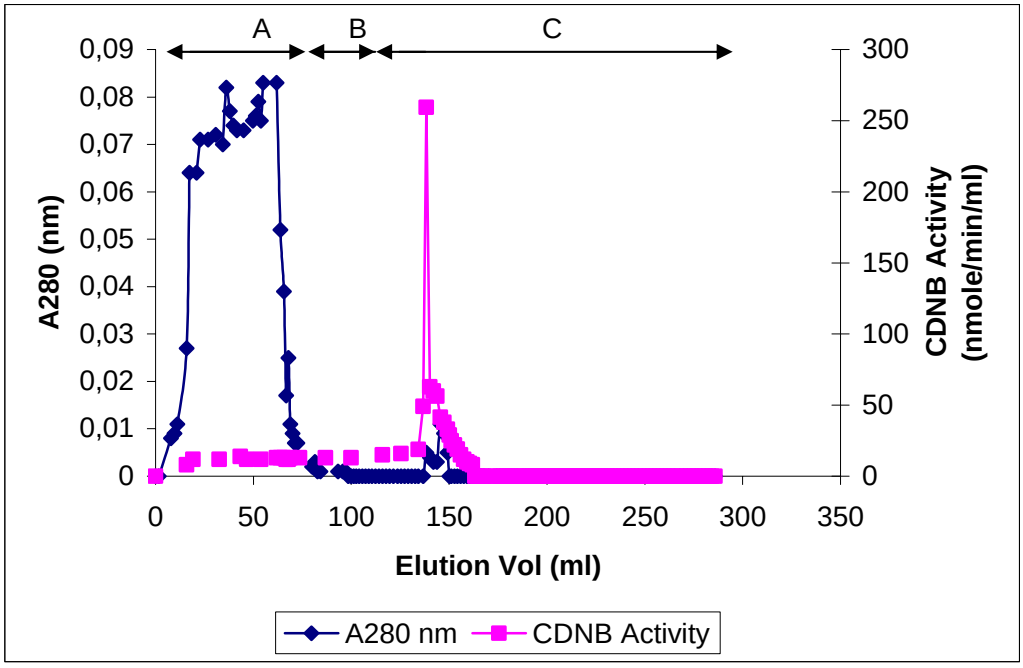


Figure 37 Purification profile for *Pinus brutia* needles GSTs on *S*-hexylglutathione affinity Chromatography Column (1 cm X 5 cm)

The pooled fraction from the previous anion-exchange chromatography step having activity towards CDNB were applied into the affinity column. Activities of GSTs against CDNB were measured at 340 nm, as decribed under 'Methods'. A: Sample application and washing, B: Washing with equilibration buffer containing 0.2 M NaCl, C: Elution of the bound GSTs with equilibration buffer containing 0.2 M NaCl, 15mM GSH.

As seen in **Figure 37,** the bound fractions with the highest activity against CDNB were eluted in one major peak with reinforced GSH concentration, but the protein content of the sample containing GSTs that was eluted at about 15 mM GSH from the affinity column was very low. The combined fraction had a total of 0.375 mg protein with 2022 unit/mg of GST enzyme activity towards CDNB. According to these results, the *S*-hexylglutathione agarose affinity column provided about 15.45 purification fold and 1.95 % recovery of the enzyme activity.

The affinity column eluate was immediately stored at - 80°C for SDS-PAGE analysis, protein and activity determinations.

All of the purification process results are summarized in **Table 8** for the purification of GSTs from *Pinus brutia* needles cytosolic fractions.

Table 8 Purification Table for GSTs from *Pinus brutia* needles

FRACTIONS	Vol (ml)	Protein (mg/ml)	Total Protein (mg)	Protein Recovery (%)	Activity Against CDNB (nmole/min/ml)	Total Activity (nmole/min)	Activity Recovery (%)	SA (nmole/min/mg)	Purification Fold
Pinus brutia **cytosol**	65.75	4.53	297.85	100	592.72	38971.34	100	130.84	'1'
Sephadex G-25	66.50	2.41	160.27	53.8	445.67	29637.10	76	184.93	1.4
DE52 Column	42.75	0.92	39.33	13.2	502.26	21471.62	55.1	545.94	4.17
Affinity Column	3.75	0.10	0.375	0.13	202.20	758.25	1.95	2022.00	15.45

3.5 SDS-PAGE Electrophoresis of the Purified GSTs

The fraction eluted from the purification columns and the purified GST, were analyzed by SDS-PAGE in order to resolve the subunit composition of GSTs from *Pinus brutia* cytosol. The molecular weight was calculated from the standard curve for molecular weight markers (**Figure 38**).

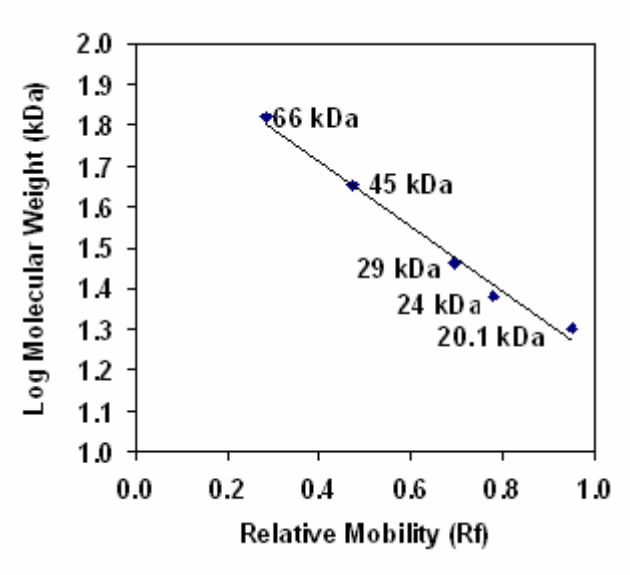


Figure 38 Typical molecular weight standard curve (12 % SDS-PAGE)

Bovine Albumin (M_r 66,000), Egg Albumin (M_r 45,000), Carbonic Anhydrase (M_r 29,000), Trypsinogen (M_r 24,000), Trypsin Inhibitor (M_r 20,100)

Figure 39 shows the photograph of one typical gel from SDS-PAGE after silver staining. The lanes contained molecular weight markers and fractions from the different purification steps. The SDS-PAGE revealed that the GST is a homodimer with a subunit molecular weight of about 24,000 Da.

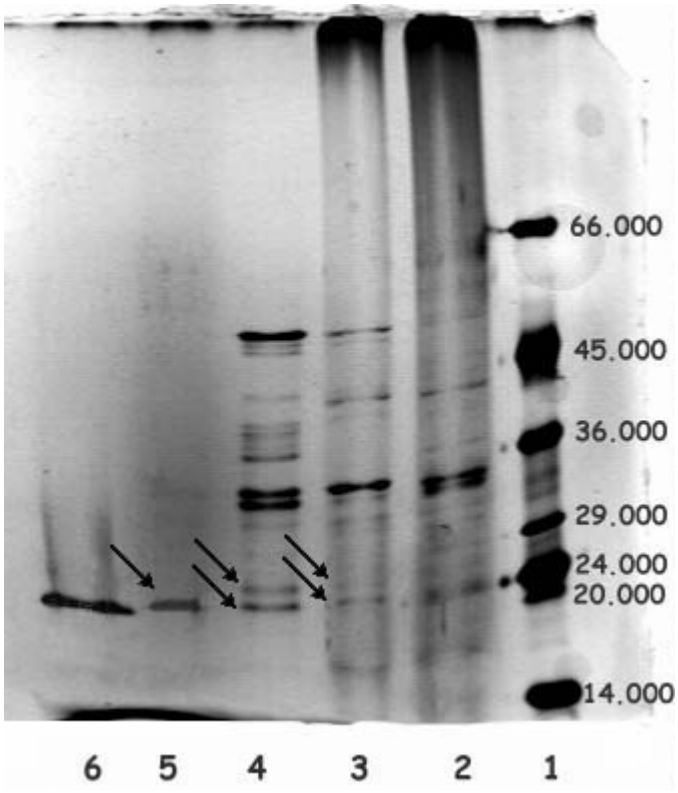


Figure 39 SDS-PAGE stained with silver (12 %), of the purification fractions and molecular weight markers Each well contains 20 µg of protein except for Affinity bound fraction which has 8 µg of protein

Lane 1	SDS-PAGE Standards
Lane 2	Cytosolic Fraction
Lane 3	G-25 Column Pooled Fraction
Lane 4	DE52 Column Pooled Fraction
Lane 5	S-Hexylglutathione Affinity Bound Fraction
Lane 6	Tripsinogen Weight Marker

The purified isoenzyme from the fraction of *S*-hexylglutathione agarose was migrated as a single band in SDS-PAGE with the estimated subunit Mr of around 24,000 using molecular marker proteins on the same gel which was similar to the calculated values 23.2 kDa and 24.9 kDa were obtained by Pascal *et al*., (1998) for two GST subunits from wheat (*Triticum aestivum* L.). Our result was also consisted with that of Schröder and Berkau (1993), who found two GST isozymes from Norway spruce trees (*Picea abies* L. Karst.) with molecular weight of 24 and 26 kDa.

CHAPTER IV

CONCLUSION

The *Pinus brutia* GST-Zeta cDNA was cloned and characterized. gst_Pinus_brutia**,** is composed of 226 amino acids, shares high sequence similarity with the Zeta class GSTs of *A.thaliana* (53%) and *Pinus tadea* (91%).

The gst_Pinus_brutia gene, most probably, is intronless, accordingly we can conclude that the genomic organization of gst_Pinus_brutia is different from *A.thaliana*.

The steady state mRNA levels of gst_Pinus_brutia and amount of GSH vary substantially among needles from different trees. The expression level does not correlate with the GSH content in the needles.

The enzyme purified with 1.95 % recovery with the fold of 15.45 at the end of three sequential chromatographic steps namely, gel filtration (Sephadex G-25), DEAE cellulose anion exchange, and *S*-hexylglutathione agarose affinity column.

The purified GST fraction was applied to the 12% SDS-PAGE. The silver stained gels exhibited a single band with the Mr of 24.000 Da.

APPENDIX

PCR CONDITIONS

Number of Cycles	Processes	^{0}C	Minute(s)
1st cycle			
Step 1	Denaturation of DNA	94	5
Step 2	Annealing	48	1
Step 3	Extention	72	1
2nd-31st cycle			
Step 1	Denaturation of DNA	94	1
Step 2	Annealing	48	1
Step 3	Extention	72	1
32nd cycle			
Step 1	Denaturation of DNA	94	1
Step 2	Annealing	48	1
Step 3	Extention	72	5

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