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Original Research

Association between herbivore stress and glutathione S-transferase expression in *Pinus brutia* Ten.

A. Semiz[1*], G. Çelik-Turgut[2], G. Semiz[2], Ö. Özgün[2], A. Şen[2]

[1] Pamukkale University, Vocational School of Health Services, Medical Laboratory Techniques, 20070, Kinikli-Denizli, Turkey
[2] Pamukkale University, Faculty of Science and Arts, Department of Biology, 20070, Kinikli-Denizli, Turkey

Abstract: Plants have developed mechanisms to defend themselves against many factors including biotic stress such as herbivores and pathogens. Glutathione S-transferase (GST) is a glutathione-dependent detoxifying enzyme and plays critical roles in stress tolerance and detoxification metabolism in plants. *Pinus brutia* Ten. is a prominent native forest tree species in Turkey, due to both its economic and ecological assets. One of the problems faced by *P. brutia* afforestation sites is the attacks by pine processionary moth (*Thaumetopoea wilkinsoni* Tams.). In this study, we investigated the changes in activity and mRNA expression of GST in pine samples taken from both resistant and susceptible clones against *T. wilkinsoni* over a nine month period in a clonal seed orchard. It was found that the average cytosolic GST activities of trees in March and July were significantly higher than the values obtained in November. November was considered to be the control since trees were not under stress yet. In addition, RT-PCR results clearly showed that levels of GST transcripts in March and July samples were significantly higher as compared to the level seen in November. These findings strongly suggest that GST activity from *P. brutia* would be a valuable marker for exposure to herbivory stress.

Key words: Glutathione S-transferase, *Pinus brutia*, *Thaumetopoea wilkinsoni,* stress physiology.

Introduction

Plants and insect herbivores have a long history of coevolution. In particular, insects develop ability to remove nutrients efficiently from plant tissues, and the plants in turn develop toxins and other deterrents to help protect against herbivore damage (1-3). Exposure of plants to unfavourable environmental conditions such as temperature extremes, freezing, salinity, wounding, drought and herbivore attack can increase the production of reactive oxygen species (ROS). Under normal conditions, the ROS molecules are scavenged by various antioxidative defense systems (4).Various biotic and abiotic stress factors lead to the excessive production of ROS in plants which are highly reactive and toxic, and cause oxidative damage to biomolecules (lipids, proteins, DNA) (5). Plants counteract ROS accumulation enzymatic antioxidant systems that include a variety of scavengers, such as superoxide dismutase, glutathione peroxidase (GPX), glutathione S-transferase (GST) and catalase (6, 7). Therefore, tolerance to stress may be improved by the enhancement of *in vivo* levels of antioxidant enzymes (8). One of the essential catalytic enzymes that play a crucial role in these defence mechanisms is the GST (9).

Glutathione S-transferase (EC 2.5.1.18) is a large and diverse group of enzymes that mainly catalyze the conjugation of intracellular glutathione (GSH) to a wide variety of substrates. GSTs are mostly found in the cytosol and widely distributed in most forms of life; bacteria, fungi, parasites, yeast, insects, mammals, and higher plants (10). Plant GSTs are divided into four classes (phi, tau, zeta and theta) based on amino acid identity and substrate specificity (11). Several stress-inducible GSTs have GPX activity; thereby protecting cells from oxidative injury by organic peroxides are created in plants during processes such as a pathogen attack (12), detoxification of microbial toxins (11). This reduction plays a vital role in preventing the degradation of organic peroxides to cytotoxic derivatives (13).

Gymnosperms represent a large group of plants and are important forest-forming species (14). During their lifetime, the conifers have to cope with several stresses including temperature extremes, salinity, wounding, drought, air pollutants and herbivore attack. The presence of enzymes that can respond to natural stresses is beneficial for the adaptation of conifer trees. However, data on GSTs in conifer tree species under herbivore attack are very scarce. Turkish red pine (*Pinus brutia* Ten.) is a prominent native forest tree species in Turkey, due to both its economic and ecological assets. One of the problems faced by *P. brutia* afforestation sites is the attacks by pine processionary moth (*Thaumetopoea wilkinsoni* Tams.). Larvae of *T. wilkinsoni* feed on the needles from late autumn to mid-spring. *T. wilkinsoni* builds its permanent winter nest starting from October-December depending on temperature. Defoliation by this pest removes photosynthetic material, and it affects many vital functions (15). The highest defoliation by larvae occurs from March to May depending on temperature. In this period, larvae are generally in biggest larval size and their daily needle consumption is also correlated with their morphology. After May, the new shoots of trees emerge from May to late June. It is well

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*** Corresponding author:** Asli Semiz, Pamukkale University, Vocational School of Health Services, Medical Laboratory Techniques, 20070, Kinikli-Denizli, Turkey. Email: akirikbakan@pau.edu.tr

known that defoliation on trees, though repeated over consecutive years, seldom causes death (16), but increases susceptibility to secondary pest attacks (17, 18). In spite of the capability of defoliated trees to re-foliate and survive, the effects of defoliation are very significant (19, 20) with losses in volume, radial growth (15, 18, 21) and biomass production (17).

The purpose of this study was to investigate the changes in the enzyme activity and expression of GST in *P. brutia* with respect to herbivore attack in a clonal seed orchard. The reason why this seed orchard was selected as a sampling area is to study the relationships between the genetic diversity and GST expression since we have known all genetic background of the trees (clone numbers and their replication within seed orchard). Furthermore, it was aimed to collect data for the management of the present and future seed orchards.

Materials and Methods

Plant material

The plant materials were collected from a clonal seed orchard located in Ciglik, Antalya, in southern Turkey (320 m a.s.l., lat 37°01′33″N, long 30°32′59″E). The orchard consists of 30 selected clones originating from a seed stand situated in Eskibag-Gundogmus, near Manavgat region of Antalya in Turkey. Each clone in the orchard was represented by about 70 ramets (range: 50-90). The grafts were planted with 8 × 8-m spacing in the seed orchard in 1992. Twenty *P. brutia* trees were selected to be used in this study. Ten ramets per resistant (clone #9268) and susceptible clones (clone #9294) (thus, 2 × 10 = 20 trees) in the seed orchard were sampled for this study. The site was observed throughout three following years (started from 2005), and needle material collected in 2013 from 10 grafts (ramets) per clone, each distributed randomly in the seed orchard. The sampling area was mapped (Figure 1) and trees were labelled on their branches. At three months (March, July, and November of 2013), needles were collected from those same branches at the same time. Each month was compared to November considering November as a control since trees were not under damage yet. Trees were selected based on their susceptibility to *T. wilkinsoni* in terms of needle terpene contents (22). As a result, totally 60 samples were collected from susceptible and resistant clones from related seed orchard. Fresh needles were transported in LN_2 (liquid nitrogen) from sampling sites to the laboratory in Pamukkale University and stored at -80 °C until analysed.

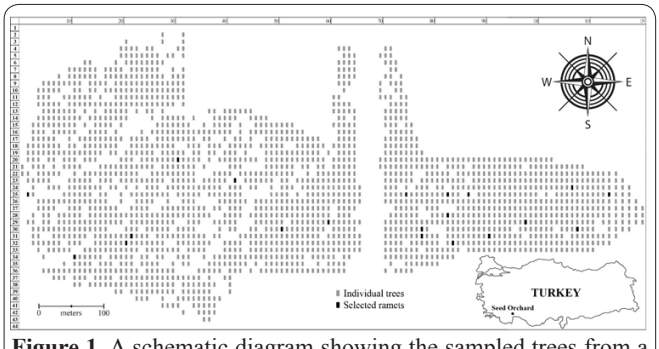

Figure 1. A schematic diagram showing the sampled trees from a clonal seed orchard located in Ciglik, Antalya, in southern Turkey.

Preparation of cytosolic fractions from *P. brutia* needles

Approximately 1 g of needles were cut into small pieces and crushed in LN_2 by mortar and pestle. Powdered needles were weighed (0.2 g) into plastic tubes with 2 ml of 0.1 M Tris-HCl buffer, pH 7.8, containing 20 mM of β-ME (β-mercaptoethanol), 2 mM of EDTA (ethylenediaminetetraacetic acid), 5 mM GSH, 0.5% Nonidet P40, 3 µg/ml of Pepstatin A, 5% PVP (polyvinylpyrrolidone). Then, the mixture was homogenized for 1 min with 15 sec intervals for four times by Ultra-Turrax at 13,500 rpm on ice. The homogenate was centrifuged at 12,000 *xg* for 30 min at 4 °C (23). A calculated volume of glycerol was added as a stabilizer to the supernatant (cytosol) fractions to obtain 10% final concentration. All fractions were stored at -80 °C until use. The protein amount in the *P. brutia* needles were measured with BCA (Bicinchoninic acid) as described by Smith et al. (24) using crystalline bovine serum albumin as a standard.

Enzyme assays

GST activities using CDNB [1-chloro-2,4-dinitrobenzene; measured in 0.1 M potassium phosphate buffer, pH 7.5 containing 1 mM CDNB, 1 mM GSH, and 25 µg cytosolic protein], DCNB [1,2-dichloro-4-nitrobenzene; measured in 0.1 M potassium phosphate buffer, pH 7.5 containing 1 mM DCNB, 1 mM GSH, and 25 µg cytosolic protein], and EA [ethacrynic acid; carried out in 0.1 M Tris-HCl, pH 7.8 containing 0.2 mM EA, 0.5 mM GSH, and 25 µg cytosolic protein] as substrates were determined at room temperature spectrophotometrically by following the change in absorbencies at 340 nm and 270 nm, respectively (25) as optimized by Sen and Kirikbakan (23).

RNA isolation and reverse transcription

Total RNA was isolated using plant RNA isolation mini kit (Agilent Technologies) as described by Semiz and Sen (26). Needles (60 mg) were ground to a fine powder in LN_2 and RNA was extracted with 60 µl of extraction solution/β-ME mixture. The homogenate was centrifuged through the mini prefiltration column at 16,000 *xg* for 3 min at 4 °C. Equal volume of isopropanol was added to the filtrate and it was incubated for 5 min at 4 °C. Then the mixture was applied to mini isolation column and centrifuge at 16,000 *xg* for 30 sec at 4 °C. After washed with 500 µl of wash solution (two times), RNA was purified in 50 µl of nuclease-free water. The extracted RNA amount was measured on a Nanodrop, and the integrity was checked using 1% agarose gel. For cDNA synthesis, 2 µg of total RNA was mixed with 0.5 µg of oligo(dT), 1 mM dNTPs, 5X reaction buffer, 200 U RTase (Applied Biological Materials Inc., Canada), and incubated at 50 °C for 60 min. The reaction was stopped by heating at 85 °C for 5 min, and the cDNA was stored at -80 °C for further use.

Semi-quantitative two-step RT-PCR assay was performed by using gene specific primers: upstream, 5′-ATGGAGAATCAGGTTAAGGT-3′; downstream, 5′-ATCTGCATAGCAAATTCTG-3′ (27). 25-µl PCR reaction mixture containing 2.5 µM forward and reverse primers, 0.3 mM dNTPs, 2.5 units of Taq DNA polymerase, and 2 mM $MgCl_2$ in the reaction buffer [200

Table 1. Mean values (nmol/min/mg protein ± SE) of three enzyme activities in the needles of susceptible (#9294) and resistant (#9268) clones of *Pinus brutia* in different months.

Clone No	Enzyme	November	March	July	*F*	*P*
9268	CDNB	13.15 ± 0.63 a	37.20 ± 0.94 b	27.81 ± 1.38 c	138.332	<0.001
	DCNB	14.44 ± 0.53 a	43.19 ± 1.01 b	39.28 ± 1.74 b	168.529	<0.001
	EA	3.04 ± 0.70 a	13.02 ± 2.84 b	5.08 ± 0.73 c	91.828	<0.001
9294	CDNB	19.97 ± 2.06 a	41.75 ± 1.95 b	34.65 ± 2.91 b	22.382	<0.001
	DCNB	22.59 ± 1.63 a	61.60 ± 5.02 b	48.70 ± 4.86 b	23.020	<0.001
	EA	3.04 ± 0.17 a	15.89 ± 0.81 b	10.28 ± 1.06 c	69.162	<0.001

F (variance ratio) and *P* (significance) values from one-way analysis of variance (ANOVA) are included. Abbreviations: CDNB: 1-chloro-2,4-dinitrobenzene, DCNB: 1,2-dichloro-4-nitrobenzene, EA: ethacrynic acid. Those with the same letter are not significantly different at the $P < 0.05$ level by the Tukey's test (n= 10).

mM Tris-HCl, pH 8.3, 200 mM KCl (potassium chloride), and 50 mM $(NH_4)_2SO_4$ (ammonium sulfate)]. Amplification conditions were 95 °C for 5 min, followed by 30 cycles of 94 °C/51.7 °C/72 °C for 30 sec/60 sec/60 sec, respectively. The final extension was 72 °C for 5 min. The PCR products were analyzed on 1.5% agarose gels containing ethidium bromide. The intensity of the bands was measured using Scion Image Version Beta 4.0.2 software. Levels of mRNA for CYP genes were determined by measuring the band intensity of the RT-PCR product on each agarose gel and are reported relative to β-actin expression.

Western blot analysis

Needles were ground to a fine powder in LN_2 and 1 g needle powder with the addition of 0.1 g of PVPP (polyvinylpolypyrrolidone) was extracted with 10 ml extraction buffer [5% sucrose, 4% SDS (sodium dodecyl sulfate), and 5% β-ME] for 10 min at room temperature with gentle stirring. The lysate was centrifuged at 10,000 x*g* for 20 min. The supernatant was heated at 100 ºC for 3 min and then cooled to room temperature. Proteins were precipitated with eight volumes of cold acetone and incubated for 60 min at -20 ºC. Then the mixture was centrifuged at 10,000 x*g* for 20 min. The pellet was re-suspended in 5 ml of extraction buffer and centrifuged at 10,000 x*g* for 20 min. After washed with 80% cold acetone, the pellet was precipitated by adding four volumes of cold acetone (28). The pellet was dissolved in cold solubilization buffer [7 M urea, 2 M thiourea, 2% CHAPS (3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), 0.5% polyampholyte, protease inhibitor cocktail, and 2 mM DTT (dithiothreitol)]. Protein concentration was measured using BCA reagent.

SDS-PAGE and Western blotting were performed as described previously (29). Briefly, protein samples (100 µg protein) were separated on 8.5% polyacrylamide gels using the discontinuous buffer system of Laemmli (30). The proteins were transferred onto nitrocellulose membrane with the Hoefer blotting system (90 V, 90 min). Following transfer, the membranes were blocked using 5% non-fat dry milk in TBST [20 mM Tris-HCl, pH 7.4, 400 mM NaCl (sodium chloride) and 0.1% (v/v) Tween 20] for 60 min and incubated with rabbit polyclonal anti-plant GST antibody (diluted 1:1000 in blocking solution) for overnight at 4 °C. The membranes were then washed with TBST (tris-buffered saline and tween 20) (3 × 5 min), incubated with the secondary antibody (ALP-conjugated anti-rabbit IgG at a 1:10,000 dilution) for 60 min and again washed with TBST (3 × 5 min). Visualization of the bands was carried out using the NBT (nitrobluetetrazolium) / BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrate system. The final images were photographed by using computer-based gel imaging instrument (DNR LightBIS Pro Image Analysis System, Israel).

Statistical analysis

The data were analyzed with one-way analyses of variance (ANOVA), and if the main effects were significant, Tukey's multiple range tests were used for comparison of means. The t-test was used to show clonal differences between susceptible and resistant clones. Differences were considered to be statistically significant at $P < 0.05$ (*). These statistical analyses were done with the SPSS 15.0 for Windows statistical software package (31).

Results

We determined that average cytosolic GST activities (using CDNB, DCNB, and EA as the substrates) of samples collected in March and July of resistant clone showed a statistically significant increase with respect to the samples collected in November (Table 1). Similarly, average cytosolic GST activities of the susceptible clone gathered in March and July showed significantly higher values as compared to the samples collected in November (Table 1). The statistical analysis pointed out a significant effect of months and enzyme-month interactions (November, March and July) on enzyme activity (Table 1). GST activities were strongly affected by herbivore pressure. In particular, CDNB, DCNB and EA activities in resistant clone were the lowest in November and the highest in March. For susceptible clone, these three activities also showed a similar trend. Additionally, it was found that there was a significant difference in the enzyme activity between two clones except CDNB and DCNB in July, and EA in November. Further analysis with t-test showed that there was also clonal variation in terms of enzyme activity under stress conditions (CDNB-November: F=8.140, t=-3.170, df=18, P<0.05; CDNB-March: F=1.614, t=-2.10, df=18, P<0.05; DCNB-November: F=2.118, t=-4.743, df=18, P<0.001; DCNB-March: F=4.029, t=-3.596, df=18, P<0.02; EA-March: F=0.359, t=-2.375, df=18, P<0.029; EA-July F=6.474, t=-4.802, df=18, P<0.001).

The mRNA level of GST in *P. brutia* was also determined. The relative transcript level of GST in susceptible clone was increased by ca. 11-fold in the March as compared to the November (Figure 2). Similarly,

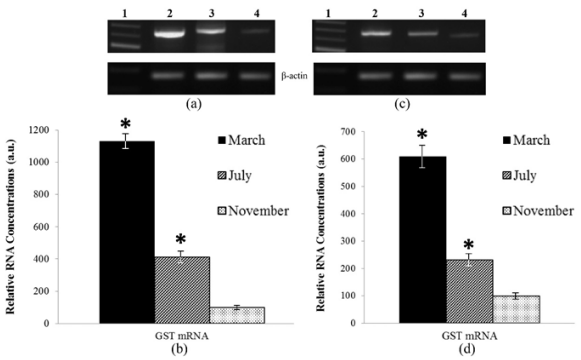

Figure 2. The expression levels of cytosolic GST mRNAs in *Pinus brutia*. Assays were carried out as described in Materials and Methods. (a) Representative agarose gel showing GST mRNA expressions (clone #9294), analyzed by RT-PCR. Lane 1, 1 KB DNA ladder; Lane 2, March; Lane 3, July, Lane 4, November. (b) Comparison of GST mRNA levels of clone #9294. The bar graphs represent the mean intensity of the bands obtained from RT-PCR results. (c) Representative agarose gel showing GST mRNA expressions (clone #9268), analyzed by RT-PCR. Lane 1, 1 KB DNA ladder; Lane 2, March; Lane 3, July, Lane 4, November. (d) Comparison of GST mRNA levels of clone #9268. The bar graphs represent the mean intensity of the bands obtained from RT-PCR results. Results are presented as the mean from three independent experiments and expressed as relative mean ± standard deviation, the asterisks denote significant differences between the months regarding the November sampling within each clone ($P < 0.05$) (n=10).

the level of GST mRNA was increased significantly by ca. 4-fold in July with respect to November (Figure 2). As presented in Figure 2, the transcript level of GST in resistant clone was increased by ca. 6-fold in March as compared to the November. Also, the level of GST transcript in July was also increased significantly by ca. 2-fold with respect to November (Figure 2).

Western blot analyses were performed to investigate whether the observed activation of catalytic activities was consistent with the protein levels of GST isoforms in *P. brutia*. But unfortunately, the primary antibody that is specific for *Nicotiana tabacum* did not recognize the GST protein in the pine being studied.

Discussion

Stress by biotic and abiotic agents elevates the amount and specific activities of some enzymes (32, 33). GST is one of those enzymes and can protect cells from a broad range of biotic and abiotic stress. GST activities in pine needles collected in March and July from both resistant and susceptible clones increased relative to that samples collected in November (Table 1). We found highest enzyme activity in March for both susceptible and resistant clones, suggesting that these enzymes are induced by herbivore feeding occurred in March. Although to the lesser extent, GST enzyme activities in *P. brutia* were also induced in July. This situation might be associated with the drought stress due to hot summer in the study area (34). Based on these findings, we propose that plants under herbivore attacks have the ability to elevate their GST levels, which may enable them to efficiently remove ROS that accumulate in response to insect feeding (35, 36). Increased levels of ROS may act as a signalling molecule for increased activation of ROS scavengers such as peroxidases and catalases as has been reported in other studies (37-39). There are so many studies representing an increase in the activity of various plant GST isozymes influenced by wide range of external factors (heavy metal toxins, herbicides, oxidative stress, drought, low and high temperatures, ultraviolet light, insect invasion and pathogen attack) (40-43). Ferry et al. (44) revealed that both catalase and a protein with high similarity to the GST from *Arabidopsis* sp., which is a typical antioxidant enzyme, were strongly up-regulated by aphid infestation.

Protein and mRNA levels of GST were further investigated to determine the underlying mechanism of increase in GST catalytic activity. Unfortunately, we could not detect any protein band because our primary antibody does not recognize pine GST protein. There is no commercially available anti-GST antibody for the gymnosperm species. However, similar to the activity levels, the mRNA levels of GST in samples collected in March and July were increased significantly relative to the levels of samples taken in November for both clones (Figure 2). Similar increase of mRNA coding a protein homologous to GST and alteration of gene expression under pathogen attack have been reported for wheat and other plant species such as *Arabidopsis* (45-49). Expression of GST transcripts has been represented in resistant barley as well as resistant sorghum in response to aphid feeding, further implicating the potential role of GST in the plant defense response to insect stress (36, 50, 51). Similarly, the up-regulation of GST, both at activity and mRNA level, in *P. brutia* in response to herbivore attack has demonstrated in the present study. The changes in the expression levels of GST mRNAs in *P. brutia* had a relationship with sampling time and also herbivore pressure level. Furthermore, there is the difference in the induction level of GST activities and mRNA levels between resistant and susceptible strains of *P. brutia*, which could be attributed the genetic factors affecting tree's physiologic structures. Therefore, further studies are required to clarify the underlying mechanism of GST regulation such as the mediator of induction, signalling molecules and cascades under pathogen attack.

Our study exemplified the effect of herbivore and drought stress on the activity, and mRNA expressions of GST in *P. brutia*. As a pioneer study, this investigation aimed to realize the effect of biotic and abiotic stresses on the GST in *P. brutia* that was not studied before in this aspect. In this context, measurement of GST activity as stress marker will remain an important role in assessing the stress response of plants. Our findings show that the induction of GST enzyme activity could be considered as a response to herbivore damage. The variation of GST expression in our study will indeed advance understanding of defense mechanisms against *T. wilkinsoni* at the transcriptional level in *Pinus* species. Since this work is the first study carried out in resistant and susceptible *P. brutia* regarding the GSTs, we have no means of comparing our results with the ones in the literature. In future, a further examination and purification of GST isozymes in *P. brutia* will clarify the isozyme composition in *P. brutia* and the particular role of isozyme(s) in herbivore defence mechanism. Finally, the present study has demonstrated that GST involved

in the stress/defense response to *T. wilkinsoni* damage in resistant trees differs from susceptible trees. In conclusion, GST is thought to be an important modulator of defences against chewing insects in plant species.

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