RESEARCH ARTICLE

Molecular characterization of zeta class glutathione *S*-transferases from *Pinus brutia* Ten.

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Abstract

Glutathione transferases (GSTs; EC 2.5.1.18) play important roles in stress tolerance and metabolic detoxification in plants. In higher plants, studies on GSTs have focussed largely on agricultural plants. There is restricted information about molecular characterization of GSTs in gymnosperms. To date, only tau class GST enzymes have been characterized from some pinus species. For the first time, the present study reports cloning and molecular characterization of two zeta class GST genes, namely *PbGSTZ1* and *PbGSTZ2* from *Pinus brutia* Ten., which is an economically important pine native to the eastern Mediterranean region and have to cope with several environmental stress conditions. The *PbGSTZ1* gene was isolated from cDNA, whereas *PbGSTZ2* was isolated from genomic DNA. Sequence analysis of *PbGSTZ1* and *PbGSTZ2* revealed the presence of an open reading frame of 226 amino acids with typical consensus sequences of the zeta class plant GSTs. Protein and secondary structure prediction analysis of two zeta class *PbGSTZ*s have shared common features of other plant zeta class GSTs. Genomic clone, *PbGSTZ2* gene, is unexpectedly intronless. Extensive sequence analysis of *PbGSTZ2*, with cDNA clone, *PbGSTZ1*, revealed 87% identity at nucleotide and 81% identity at amino acid levels with 41 amino acids differences suggesting that genomic *PbGSTZ2* gene might be an allelic or a paralogue version of *PbGSTZ1*.

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Introduction

Glutathione *S*-transferases (GSTs, EC.2.5.1.18) detoxify endobiotic and xenobiotic compounds by conjugating glutathione (GSH) to a hydrophobic substrate (Wang *et al*. 2012; Kang *et al*. 2013). In addition to their enzymatic activities, the plant GSTs are involved in both GSH-dependent peroxidation or isomerization reactions and several other noncatalytic functions like binding of nonsubstrate ligands stress-induced signalling processes and preventing apoptosis (Barling *et al*. 1993; Roxas *et al*. 1997; Cummins *et al*. 1998; Dixon *et al*. 2000; Kampranis *et al*. 2000; Mueller *et al*. 2000; Thom *et al*. 2001; Naliwajski and Sklodowska 2014). To date, seven distinct classes of soluble GSTs have been identified. While phi, tau, lambda and dehydroascorbate reductase (DHAR) GSTs are specific to plants (Edwards and Dixon 2005), zeta class genes present in both plants and humans (Board *et al*. 1997; Sheehan *et al*. 2001). Zeta class GSTs are GSH-dependent isomerases (Dixon *et al.* 2000; Oztetik 2008). In plants, the first zeta class GST has been identified from carnation petals (*Dianthus caryophyllus*) (Meyer *et al*. 1991). To date, zeta class GST enzymes have been identified from carnation petals (*Dianthus caryophyllus*) (Meyer *et al*. 1991). *Arabidopsis thaliana* (Dixon *et al*. 2000), *Zea mays*, *Glycine max* (McGonigle *et al*. 2000), *Brassica napus* (L.) and *Oryza sativa* (L.) (Tsuchiya *et al*. 2004) in plants. Multiple copies of zeta class GST genes have been characterized in *A. thaliana*, *O. sativa*, *Z. mays*, *Glycine max* and *D. caryophyllus* (Meyer *et al.* 1991; McGonigle *et al.* 2000; Dixon *et al.* 2002; Wagner *et al.* 2002). *Pinus brutia* Ten. (Turkish red pine, East Mediterranean pine) is a member of family pinaceae, genus *Pinus* L. It is the most important forest tree in the northeastern Mediterranean region. During its life span, the conifers have to cope with severe stresses, including temperature fluctuations, drought, elevated ozone and ultra violet levels, halogenated hydrocarbons and other air pollutants. The presence of enzymes

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that can detoxify these compounds and respond to natural stresses is clearly beneficial for the adaptation. To date, zeta class GST studies on higher plants have mainly concentrated on *Arabidopsis* and agricultural crops, such as rice, wheat, maize and soybean (Basantini and Srivastava 2007). On the contrary, there is almost no information about molecular characteristics of zeta class GSTs in pinus.

In the present study, two zeta class *GST* genes were isolated and characterized from *P. brutia* Ten. One of them, *PbGSTZ1*, was amplified and cloned using *P. brutia* cDNA and the other, *PbGSTZ2*, was amplified and cloned using *P. brutia* genomic DNA. Most interestingly, genomic *PbGSTZ2* which was obtained from the genomic PCR had the same fragment size as cDNA clone *PbGSTZ1*. Sequence comparison of two zeta class *PbGST zeta* genes indicated 87% identity at the nucleotide and 81% identity at amino acid level with 42 amino acids differences suggesting that *PbGSTZ2* might be an allelic or a paralogue version of *PbGSTZ1*. *PbGSTZ2* has no intron structure displaying an uncommon exon/intron structure with other zeta class GST enzymes in plants. This was confirmed by performing genomic PCR with two different primer pairs (443 and 681 bp). Protein and secondary structure prediction analysis showed that the presence of an open reading frame (ORF) of 226 amino acids with the typical consensus sequences of zeta class plant GSTs. mRNA expression levels of *PbGST zeta* from needles were also investigated and compared among individual *P. brutia* trees in the forest area.

Materials and methods

EST identification

Needles from *P. brutia* trees (mature trees, more than 10 years old) were harvested from Yalıncak area within the METU Campus in Ankara/Turkey and stored at −80°C immediately after collection. The GenBank® EST database was searched using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) with *A. thaliana* GST cDNA (GenBank accession no. AY087032), as the query sequence. Two *P. taeda* EST sequences (GenBank accession no. CF667317 and CF667231) encoding an unknown protein with high similarity to zeta class GSTs were identified. Based on these two EST sequences, two primers, PbGSTZ Frw: 5′-AGG ATC CAT GGC GTC CGT GAG TCA GG-3′ and PbGSTZ Rev1: 5′-GGA GCT CCA TGC TTT TGC ATC AGG TT-3′, were designed to amplify *P. brutia GST zeta* cDNA. *Bam*HI and *Sac*I restriction sites were integrated and underlined, respectively (figure 1).

Molecular cloning of zeta class genes from P. brutia

Total RNA was isolated from *P. brutia* Ten. needles using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), then reverse transcribed into cDNA using RT-PCR Kit (Sigma, Munich, Germany). Genomic DNA was isolated from *P. brutia* Ten. needles using DNeasy Plant Mini Kit (Qiagen). PCR was performed in a total volume of 50 μL, containing

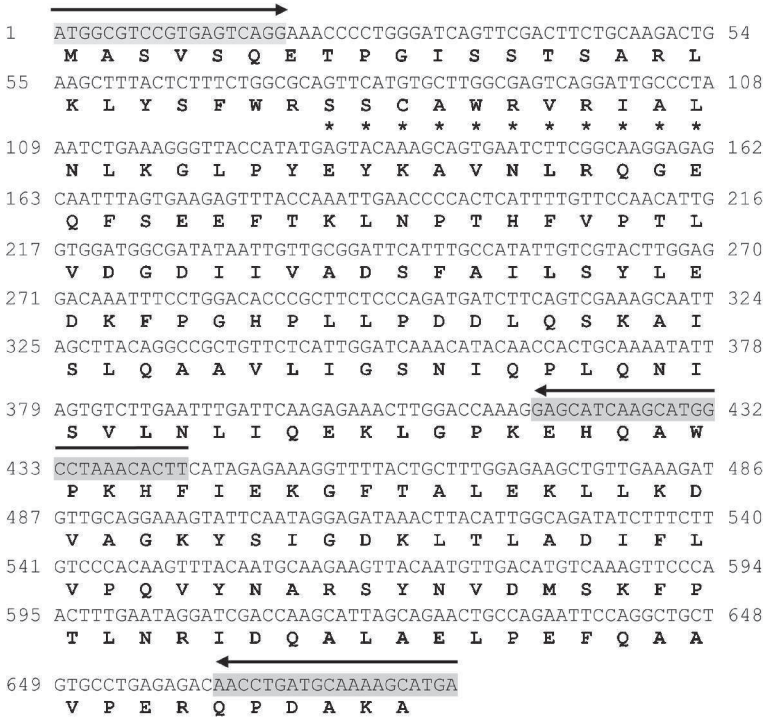

Figure 1. Nucleotide and deduced amino acid sequence of PbGSTZ1. *Conserved domains.

10 μL of the first strand cDNA or 100 ng/μL of genomic DNA, 0.5 μL (2.5 U/μL) of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1 μL (2 mM) of dNTP mix (Fermentas), 4 μL (4 mM) of $MgCl_2$ and 1 μL (50 mol/μL) of each primer (PbGSTZ Frw and PbGSTZ Rev1). PCR cycle parameters consist of an initial denaturation of 2 min at 94°C followed by 35 cycles of 15 s (1 min for genomic PCR) at 94°C, 30 s (45 s for genomic PCR) at 60°C and 1 min at 68°C and a final extension of 5 min (10 min for genomic PCR) at 68°C. PCR products about 681 bp were recovered from agarose gel using Qiaquick Gel Extraction Kit (Qiagen) and cloned into pGEM-T Easy Vector (Promega, Mannheim, Germany). The sequence of the constructs was confirmed by automated DNA sequencing (Lark Technologies, London, UK). The cDNA clone was named as *PbGSTZ1* and genomic clone was named as *PbGSTZ2*. For identifying exon/intron structure of *PbGSTZ2*, partial amplification of ORF, 443 bp, was carried out using PbGSTZ Rev2 5′-AAGTGTTTAGGCCATGCTTGATGCTC-3 and PbGSTZ Frw primers (figure 1).

Sequence and phylogenetic analyses

PbGSTZ1 and *PbGSTZ2* genes were searched for homologous via BLASTX in NCBI (National Center for Biotechnology Information). Protein sequence of PbGSTZ1 and PbGSTZ2 were deduced from cDNA, aligned with other plant GSTs using ClustalX software and further adjusted manually using BioEdit. Typical motifs that were conserved among PbGSTZ1 and other zeta class plant GSTs were analysed via ScanProsite (http://prosite.expasy.org/scanprosite). The secondary structure prediction was performed with JPred 4 program (http://www.compbio.dundee.ac.uk/www-jpred/). Phylogenetic tree reconstruction, using Bioedit ver. 7.3.0 (Hall 1999), was used to determine the genetic relationships among *PbGSTZ1* and the main classes of zeta class plant GSTs.

Northern blot analysis

Total RNA was isolated using RNeasy kit (Qiagen) as mentioned earlier. The fractionated RNA was blotted onto Hybond-XL membrane (Amersham, Munich, Germany). PbGSTZ cDNA probe labelled with [α-32 P] dATP (3000 Ci/mmol, 10 mCi/mL). Blots were prehybridized at 65°C with hybridization buffer containing 0.5 M Na_2PO_4, 1% SDS pH: 8.0, Herrings Sperm DNA (Ambion, Darmstadt, Germany). After 16 h of hybridization at 65°C, filters were washed at 65°C with 2× SSC, 0.1% SDS solution for 10 min and then washed twice with 1× SSC, 0.1% SDS solution for 20 min. The blot was wrapped in Saran Wrap and exposed to X-ray (Kodak) film and developed.

Results

Sequence characterization of the PbGSTZ1 and PbGSTZ2

The isolated full-length cDNA sequence of the *PbGSTZ1* gene was 884 bp and genomic sequence of the *PbGSTZ2* gene was 681 bp (GenBank accession no. GU270573.1 and JN711455). *PbGSTZ1* and *PbGSTZ2* deduced amino acid sequences encoded a peptide of 226 residues with a predicted molecular mass of 26.37 kDa (figure 1). Molecular weight of PbGSTZ1 and PbGSTZ2 peptides are well correlated with other cytosolic GSTs (*PbGSTZ2* nucleotide and deduced amino acid sequence are shown in figure 2). Putative *PbGSTZ1* had a conserved N-terminal domain including the GSH-binding site (G-site) and C-terminal domain which was supposed to accommodate a range of hydrophobic compounds (H-site) (Edwards and Dixon 2000). Moreover, $\beta\alpha\beta\alpha\beta\beta\alpha$ topological arrangements of the G-site secondary structure elements were detected on the *PbGSTZ1* isozyme (figures 1 and 3) (Frova 2003; Soranzo *et al*. 2004). The SSCXWRVRIAL motif and conserved serine residues were also present in N-terminus of the protein. The genomic clone *PbGSTZ2* also had 226 amino acids with the conserved

Nucleotide sequence of *PbGSTZ2*

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ATGGCGTCCGTGAGTCAGGAAAACCCCGCAACCAGTGTTACCTCTTCTAGACTGAAGCTG
TACTCTTACTGGCGCAGTTCATGTTCTTGGCGAGTCAGGATTGCTCTCAATCTGAAAGGG
TTACCATATGAGTACAAAGCAGTGAATATTGTGCAAGGAGAGCAATTTAGTGAAGAGTTT
ACCAAATTGAACCCCCTTCAATTTGTTCCAACACTGGTGGATGGAGATACAATTGTTTCA
GATTCGCTTGCCATATCGTTGTACTTGGAGTACAAATTTCCTGAACACCCACTACTCCCA
GATGATCATCATTTGAAAGCAATTAGCTTACAGGCCGCCTCTATTATTGGATCAAACATA
CAACCTCTTCAGAACTTGGTGGTCTTGAATTTGATCGAGGAGAAACTGGGAGTAGAGGAG
CGTTTGGCATGGCCTAAACCATTCATAGAGAGAGGTTTTACTGCTTTGGAGAAGTTGTTG
AAAGATGTTGCAGGGAAATATTCTGTAGGTGATCAGCTTACATTGGCAGATATCTTTCTT
GTCCCACAAGTTTTTGGTGCGAGGCGTTTCAACGTTGACATGTCAAAATTCCCAACTTTG
AATAGAATTGACAAAGAATTAGCAGAACTTCCAGAATTCCAGGCTGCTCTGCCTGCAAGA
CAACCTGATGCAAAAGCATGA
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Aminoacid sequence of *PbGSTZ2*

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MASVSQENPATSVTSSRLKLYSYWRSSCSWRVRIALNLKGLPYEYKAVNIVQGEQFSEEFT
KLNPLQFVPTLVDGDTIVSDSLAISLYLEYKFPEHPLLPDDHHLKAISLQAASIIGSNIQP
LQNLVVLNLIEEKLGVEERLAWPKPFIERGFTALEKLLKDVAGKYSVGDQLTLADIFLVPQ
VFGARRFNVDMSKFPTLNRIDKELAELPEFQAALPARQPDAKA*
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Figure 2. Nucleotide and deduced amino acid sequence of PbGSTZ2.

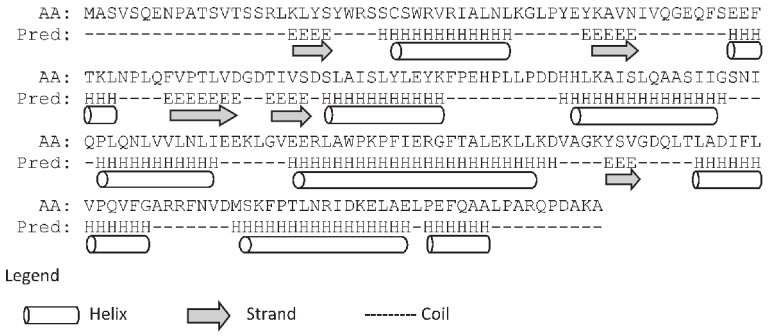

Figure 3. Topological arrangement of the PbGSTZ1 isozyme.

features of zeta class GST genes. The sequence comparison of the putative genomic clone (*PbGSTZ2*) with the corresponding cDNA clone (*PbGSTZ1*) showed 87% identity at the nucleotide level and 81% identity at amino acid level (figure 4a). According to the extensive bioinformatic analyses, the amino acid sequence of the genomic clone contains several amino acid substitutions distributed throughout ORF without stop codon. Thus, it was concluded that *PbGSTZ2* clone could be an allelic or a paralogue version of *PbGSTZ1* gene. To confirm the exon/intron structure of the *PbGSTZ,* ORF was partially amplified with primers more closely located to the start site (figures 1 and 4). The same size band (443 bp) was observed as expected (figure 4b). This indicated that this copy of the GST zeta gene (*PbGSTZ2*) did not contain any intron. It has been known that multiple copies of GST zeta genes have been presented in plants. This genomic

(a)

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PbGSTZ2 MASVSQENPATSVTSSRLKLYSYWRSSCSWRVRIALNLKGLPYEYKAVNIVQGEQFSEEF 60
        MASVSQE P  S TS+RLKLYS+WRSSC+WRVRIALNLKGLPYEYKAVN+ QGEQFSEEF
PbGSTZ1 MASVSQETPGISSTSARLKLYSFWRSSCAWRVRIALNLKGLPYEYKAVNLRQGEQFSEEF 60

PbGSTZ2 TKLNPLQFVPTLVDGDTIVSDSLAISLYLEYKFPEHPLLPDDHHLKAISLQAASIIGSNI 120
        TKLNP  FVPTLVDGD IV+DS AI  YLE KFP HPLLPDD   KAISLQAA +IGSNI
PbGSTZ1 TKLNPTHFVPTLVDGDIIVADSFAILSYLEDKFPGHPLLPDDLQSKAISLQAAVLIGSNI 120

PbGSTZ2 QPLQNLVVLNLIEEKLGVEERLAWPKPFIERGFTALEKLLKDVAGKYSVGDQLTLADIFL 180
        QPLQN+ VLNLI+EKLG +E  AWPK FIE+GFTALEKLLKDVAGKYS+GD+LTLADIFL
PbGSTZ1 QPLQNISVLNLIQEKLGPKEHQAWPKHFIEKGFTALEKLLKDVAGKYSIGDKLTLADIFL 180

PbGSTZ2 VPQVFGARRFNVDMSKFPTLNRIDKELAELPEFQAALPARQPDAKA  226
        VPQV+ AR +NVDMSKFPTLNRID+ LAELPEFQAA+P RQPDAKA
PbGSTZ1 VPQVYNARSYNVDMSKFPTLNRIDQALAELPEFQAAVPERQPDAKA  226
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(b)

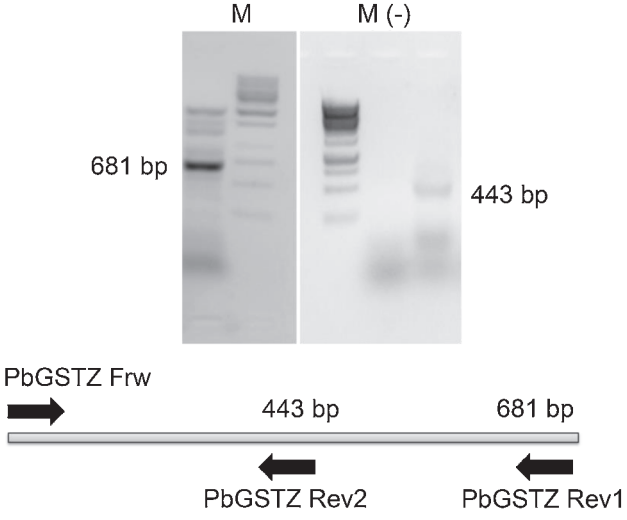

Figure 4. (a) The aminoacid comparison of the genomic PbGSTZ2 and PbGSTZ1. (b) Analyis of exon/intron structure by partial genomic PCR. Fragments, 681 and 443 bp were amplified by a set of PbGSTZ Frw/PbGSTZ Rev1 and PbGSTZ Frw/PbGSTZ Rev2 primers, respectively.

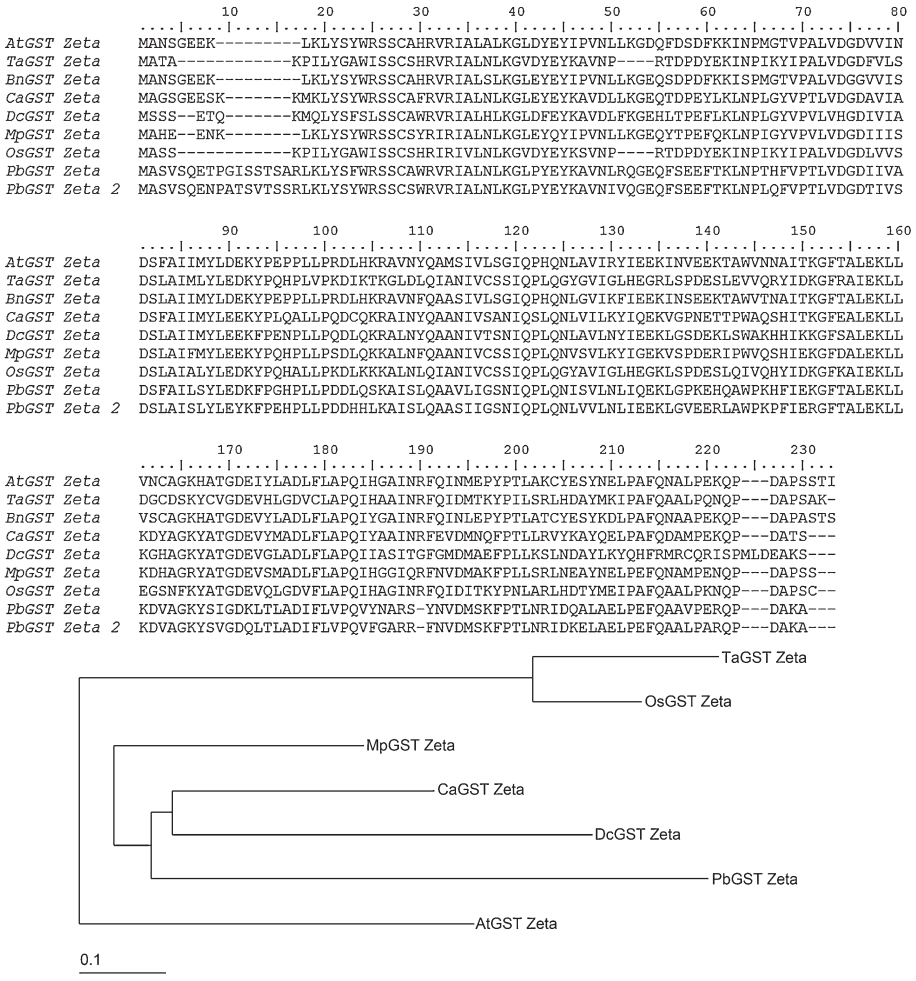

Figure 5. Sequence alignment and genetic distances calculation (UPGMA method) of plant zeta class GSTs. A sequence alignment was created using the following sequences: *Triticum aestivum*, GenBank® accession number 004437 (TaGSTZ); *Oryza sativa*, GenBank® accession number AAK98533 (OsGSTZ); *Malva pusilla*, GenBank® accession number AAO61856 (MpGSTZ); *Dianthus caryophyllus*, GenBank® accession number CAA41279 (DpGSTZ); *Capsicum annuum*, GenBank® accession number ABQ88335 (CaGSTZ); *Brassica napus*, GenBank® accession number AAO60042 (BnGSTZ) and *Arabidopsis thaliana*, GenBank® accession number AAO60039 (AtGSTZ).

clone might be one of the GST zeta copies present in the genome.

A multiple sequence alignment of the *PbGSTZ1* protein with other plant GSTs is shown in figure 5. *PbGSTZ1* shared maximum identity with CaGST zeta with 58%. Although, the overall amino acid sequence identities of *PbGSTZ1* to other *GST zeta* genes were less than 58%, there was a high degree of identity in the conserved domains that were characteristic of plant GSTs. Phylogenetic analysis of the PbGSTZ1 was performed from the deduced amino acid sequences with a wide range of plants belonging to different families of either monocots or eudicots. The neighbour-joining method was used in the tree reconstruction of PbGSTZ1 and the zeta class plant GSTs from other plant species with Bioedit (ClustalW) software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Figure 5 shows the three branches of zeta class GST proteins. TaGSTZ and OsGSTZ are classified into same group. AtGSTZ is found in another group. CaGSTZ and DcGSTZ classified into the same group while PbGSTZ1 classified into another related group.

Northern blot analysis

Glutathione is the most abundant and important low molecular mass thiol, widely distributed in living cells and involved in many biological reactions such as maintaining the homeostasis of heavy metal ions inside the plant cell (Ridnour *et al*. 1999; Diopan *et al*. 2010). Tree-specific accumulation of transcripts in the same forest area from *PbGSTZ* genes, in fresh leaves, was examined by Northern blot analysis (figure 6). Needles were chosen since transcripts for PbGSTZs were accumulated generally in leaf, whereas these were not observed in roots (Tsuchiya *et al.* 2004). From the blot analysis, it was concluded that there was not much variation in the zeta class GST steady state mRNA levels

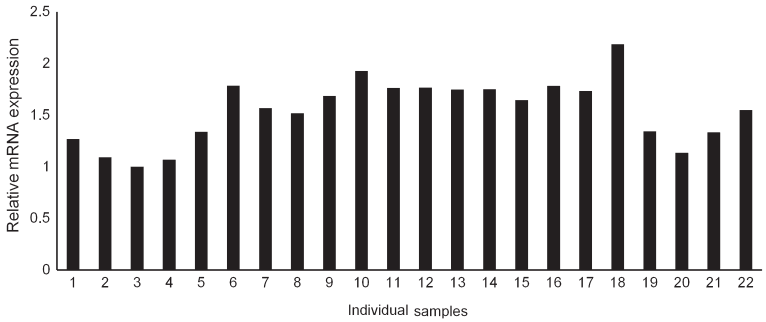

Figure 6. Northern blot, densitometric analysis of 20 different RNA samples from *Pinus brutia* Ten.

(figure 6). Densitometric quantification revealed that the mRNA levels varied up to 1.12 fold.

Discussion

This study was undertaken to identify zeta class genes from *P. brutia* Ten. The full length cDNA of GST zeta gene, *PbGSTZ1* was isolated by RT-PCR based strategy using EST-based primers derived from *P. taeda.* The full-length genomic clone, *PbGSTZ2* was also obtained by genomic PCR. Genomic clone revealed some discrepancies compared to cDNA clone with 87% identity at the nucleotide level and 81% identity at amino acid level. The zeta class of GST genes mostly has high copy number in plants. Multiple GSTZ copies were indicated for *O. sativa* L., *A. thaliana*, *Zea mays* and *Triticum aestivum* GSTZs (Subramaniam *et al*. 1999). Our findings indicate that genomic version of *PbGSTZ2* could be another copy of GST *zeta* genes in *P. brutia.* This suggests that *PbGSTZ2* gene could be a paralogue or an allelic version of *PbGSTZ1.* PbGSTZ1 and PbGSTZ2 were composed of 226 amino acids and had a number of common features with other zeta class plant GST enzymes. There was particularly a high percentage identity in the N-terminal region (SSCXWRVRIAL motif) of PbGSTZ1 and PbGSTZ2 (figure 1) (Board *et al.* 1997). Active site serine residue was also conserved in *PbGSTZ1* and *PbGSTZ2* genes. The overall appearance of the alignment indicated that the structure of *PbGSTZ1* and *PbGSTZ2* strongly resembles that of other zeta class plant GSTs. PbGSTZ1 shared maximum identity with CaGST zeta with 58% (figure 5). In contrast with the other classes, the zeta class has been extremely well-conserved over a considerable evolutionary period, such that 38% identity remains between the carnation and human sequences (Itzhaki and Woodson 1993). It was striking to identify closely related GSTs from such divergent species. As can be seen in the phylogenetic tree from figure 5, the category of the AtGSTZ, OsGSTZ and TaGSTZ was not close may be they evolved from the same ancestor along different pathways. GST zeta genes of PbGSTZ, CaGSTZ and DcGSTZ have high homology with each other (Chelvanayagam *et al.* 1997). Gene organizations of plant GSTs have been very well-established. Gene structures of GST zeta genes were more complex than those of GST genes of other classes owing to the fact that the coding sequences were split into 10 exons (Dixon *et al.* 2000). However, on an attempt of isolating genomic clone of *PbGSTZ1*, we isolated the different copy of PbGST zeta from genomic PCR using the same primers used in cDNA amplifications. To elucidate exon/intron structure of *PbGSTZ2*, we further performed PCR using the genomic DNA with different primers amplifying the coding sequence partially (443 bp) and the full length of *PbGSTZ2* (681 bp). When genomic DNA was used as PCR template, 443 and 681 bp of PCR products were generated for *PbGSTZ2* as expected. These results indicated that *PbGSTZ2* gene is a processed gene without introns interrupting their ORF.

Glutathione is an important storage and transport form for reduced sulphur in many plant species (Rennenberg 1982). In addition, glutathione has been implicated in cellular defense mechanisms against herbicides, oxidants and other environmental stresses (Smith *et al.* 1990). Various environmental factors like, sulphur content of the soil and atmosphere, growth at high altitudes, temperature is known to affect the concentration of glutathione in plant cells. The expression levels of GST zeta among individual trees of *P. brutia* were also compared to assess the presence of genetic variation. Therefore, the cDNA was utilized as the probe for the application of Northern blot analysis. As stated before, the densitometric quantification revealed *PbGST zeta* mRNA levels within 20 individuals varied up to 1.12-fold and there was no correlation between the zeta GST expression and GSH concentration in the needles of 20 individuals of *P. brutia* Ten.

To date, only tau class GST enzymes are characterized from some pinus species (Zeng and Wang 2006). This study is the first to report isolation of the two zeta class GST genes from *P. brutia* Ten.

Acknowledgements

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