Plant Physiology and Biochemistry 77 (2014) 99–107

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Biochemical functions of the glutathione transferase supergene family of *Larix kaempferi*

Qi Yang [a,b], Yan-Jing Liu [a], Qing-Yin Zeng [a,*]

[a] State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 7 November 2013
Accepted 5 February 2014
Available online 14 February 2014

Keywords:
Enzyme activity
Kinetic analysis
Gene expression
Glutathione transferase
Gymnosperm
Larix kaempferi

ABSTRACT

Glutathione transferases (GSTs), which are ubiquitous in plants, play a major role in the detoxification of xenobiotics and oxidative stress metabolism. Due to their role in herbicide detoxification, previous studies of plant GSTs have mainly focused on agricultural plants. In contrast, functional information regarding gymnosperm GSTs is scarce. In this study, we cloned 27 full-length GST genes from the deciduous conifer *Larix kaempferi*, which is widely distributed across the cooler regions of the northern hemisphere. As with the angiosperm GST gene family, *Larix* GSTs are divided into eight classes, and tau class GSTs are the most numerous. Compared to the other seven classes of GSTs, *Larix* tau GST genes show substantially more variation in their expression patterns. The purified *Larix* GST proteins showed different substrate specificities, substrate activities, and kinetic characteristics. The pH and temperature profiles of purified *Larix* GST proteins showed broad optimum pH and temperature ranges for enzymatic activity, suggesting that *Larix* GSTs have evolutionary adaptations to various adverse environments. Taken together, this study provides comprehensive insight into the gymnosperm GST gene family.

1. Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) are multifunctional proteins that are ubiquitous in virtually all organisms. GSTs catalyze the conjugation of tripeptide glutathione (γ-glutamyl-cysteinyl-glycine; GSH) to various hydrophobic substrates. Based on analyses of amino acid identity, gene structure, and substrate specificity, plant GSTs have been categorized into eight classes: tau, phi, lambda, theta, zeta, dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase (TCHQD), and γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ) (Lan et al., 2009; Jain et al., 2010; Oakley, 2005). Tau, phi, lambda, and DHAR GSTs are plant-specific, while theta, zeta, TCHQD, and EF1Bγ GSTs exist in both plants and animals. Recently, we identified two new GST classes (hemerythrin and iota) in nonvascular plants (Liu et al., 2013). Plant GST proteins are encoded by a large gene family with more than 55 members in the *Arabidopsis*, poplar, and rice genomes (Lan et al., 2009; Jain et al., 2010; Dixon and Edwards, 2010). In vascular plants, tau and phi GSTs are the most abundant classes, but tau GSTs are absent in moss (Liu et al., 2013).

As a phase II detoxification enzyme, GST proteins display enzymatic activity towards various toxic compounds, including 1-chloro-2,4-dinitrobenzene and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. In plants, tau and phi GSTs can protect cells from a wide range of biotic and abiotic stresses, including pathogen attacks, xenobiotics, heavy metals, toxins, oxidative stress, and UV radiation (Frova, 2003; Jiang et al., 2010). In addition to their role in xenobiotic detoxification, plant GSTs functioning as GSH peroxidases provide protection against oxidative stress, e.g., many plant GSTs can catalyze the reduction of cumene hydroperoxide. Zeta GSTs are believed to play an important role in the isomerization of specific metabolites; for example, an *Arabidopsis* zeta GST catalyzes the GSH-dependent *cis-trans* isomerization of maleylacetoacetate to fumarylacetoacetate, which is a key step in tyrosine degradation (Thom et al., 2001). DHAR is a key enzyme in the ascorbate-glutathione cycle that maintains reduced pools of ascorbic acid and serves as an important antioxidant. It was previously shown that DHARs exhibit activity only towards dehydroascorbic acid. Taken together, plant GST genes have extensive functional divergence.

Given the important role of plant GSTs in herbicide detoxification, most studies of the GST gene family have focused on agricultural plants (e.g., rice, wheat, and maize) and the model plant *Arabidopsis* (Hatton et al., 1996; Wagner et al., 2002; Wisser et al., 2011). Gymnosperms represent a large group of plants with a long evolutionary history. Extant gymnosperms consist of about 1026 species

* Corresponding author. Tel.: +86 10 62836440; fax: +86 10 62590843.
E-mail address: qingyin.zeng@ibcas.ac.cn (Q.-Y. Zeng).

http://dx.doi.org/10.1016/j.plaphy.2014.02.003
0981-9428/

(Christenhusz et al., 2011). Most gymnosperms such as conifers are important forest-forming species. Although some studies have reported the characteristics of gymnosperm GSTs (Zeng et al., 2005; Lan et al., 2013; Schroder and Wolf, 1996), compared to those of angiosperms and especially agricultural plants, evolutionary and functional information regarding gymnosperm GSTs is scarce. To better understand the evolutionary and functional characteristics of GST family on the whole-plant scale, more information on gymnosperm GSTs at a genome-wide level is required. Thus, in this study, we focused on structural and functional characterizations of the GST supergene family in Japanese larch (*Larix kaempferi*). The *Larix* genus (a major genus of the Pinaceae) contains at least ten species. *Larix* is the only deciduous conifer genus, and is an important wood product for sustainable harvest from intensively managed plantations (San Jose-Maldia et al., 2009). *L. kaempferi* displays good cold tolerance and extensive adaptability (Fujimoto and Koga, 2010), resulting in a wide distribution across the cooler regions of the northern hemisphere. Thus, *L. kaempferi* is an ecologically and economically important forest species. In this study, we cloned 27 full-length GST genes from the *L. kaempferi* genome, which were divided into eight classes. By integrating the gene expression patterns, structural features, and enzymatic characteristics of *Larix* GSTs, this study provides comprehensive insight into the gymnosperm GST gene family.

2. Methods

2.1. Molecular cloning and nomenclature

To identify the *L. kaempferi* GSTs, a collection of 57575 *Larix* nucleotide sequences from the National Center for Biotechnology Information (NCBI) were searched using the full-length *Pinus tabuliformis* GST protein sequences (Lan et al., 2013) with the TBLASTN algorithm. NCBI Conserved Domain search was applied for the identification of typical GST N- and C-terminal domains in protein structures. Based on the predicted *Larix* GST gene sequences, primers were designed to amplify the coding regions (Supplemental Table 1). Total RNA was isolated from a mixture of *Larix* needles and buds using an RNAprep Pure Plant Kit (Polysaccharides & Polyphenolics-rich) (Tiangen Biotech Co., Ltd., Beijing, China) and the first-strand cDNA was synthesized using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Dalian, China). Amplifications of the *Larix* GST genes were performed using conditions of 94 °C for 3 min (1 cycle), 94 °C for 30 s, 55 °C for 40 s, 72 °C for 1 min (35 cycles), and 72 °C for 3 min (1 cycle) in a 25 μl reaction mixture containing 1 μl of template cDNA, 2.5 μl of 10 × TaKaRa Ex Taq buffer, 0.5U of Ex Taq DNA polymerase (TaKaRa), 0.1 mmol of each dNTP (TaKaRa), and 10 pmol of each primer. PCR products were separated on a 1% agarose gel and recovered using a DNA Fragment Quick Purification/Recover Kit (DingGuo, Beijing, China). The recovered fragments were then cloned into the pEasyT3 Vector (Transgen, Beijing, China) and sequenced in both directions to verify the gene sequences.

Followed the system suggested by Dixon et al. (2002a) for plant GSTs, a univocal name was assigned to each *Larix* GST gene, consisting of two italic letters denoting the source organism, the subfamily name (e.g., *GSTU*, *GSTF*, *GSTT*, *GSTZ*, *GSTL*, *DHAR*, *EF1Bγ*, and *TCHQD* corresponding to tau, phi, theta, zeta, lambda, DHAR, EF1Bγ, and TCHQD classes, respectively), and a progressive number for each gene (e.g., *LkGSTU1*).

2.2. Phylogenetic analysis

The full-length GST protein sequences were aligned using MUSCLE3.8.31 software and further adjusted manually using BioEdit v7.0.0 software. The maximum likelihood trees was constructed using PhyML v2.4.4 software with the optimal amino acid substitution model selected by modelgenerator version 0.85 program and one hundred bootstrap replicates. Previously study has considered the GRX2 protein as a putative ancestor of cytosolic GSTs (Oakley, 2005). Thus, GRX2 protein was used as an outgroup for phylogenetic analysis.

2.3. Homology modeling

To predict the tertiary structures of *Larix* tau and phi GSTs, the X-ray structures of a tau GST (Protein Data Bank accession number 1GWC) and a phi GST (Protein Data Bank accession number 1AW9) were used as templates. Alignment of each target with template sequence was achieved through the Align 2D program of the InsightII package. The structure of each target was automatically built using the Modeler Module. The resulting models were evaluated by the profile-3D program.

2.4. Tissue-specific expression of Larix GST genes

To investigate the tissue-specific expression patterns of the *Larix* GST genes, three mature *Larix* trees were sampled. Total RNA was isolated from bud, needle, root, and phloem in the stem using RNAprep Pure Plant Kit (Polysaccharides & Polyphenolics-rich) (TIANGEN), treated with RNase-free DNase I (Promega) and reverse transcribed into cDNA using the RNA PCR Kit (AMV) version 3.0 (TaKaRa). The *Larix* actin gene was used as an internal control. Twenty-seven specific primer pairs were designed basing on multiple sequence alignment of all *Larix* GST sequences (Supplemental Table 2). PCR was performed in a volume of 25 μl containing 3 μl of first-strand cDNA, 2.5 μl of 10 × TaKaRa Ex Taq buffer, 0.5U of Ex Taq DNA polymerase, 0.1 mmol of each dNTP, and 10 pmol of each primer. PCR conditions consisted of 3 min initial denaturation at 94 °C, followed by cycles of 30 s at 94 °C, 40 s at 55 °C and 40 s at 72 °C, with a 3 min final extension step at 72 °C. To be in the linear range, the numbers of cycles used for amplification with each primer pair were 24, 26, 28, and 30, respectively.

2.5. Expression and purification of recombinant Larix GST proteins

To investigate the enzymatic functions of *Larix* GSTs, the *Larix* GST genes were subcloned into pET30a expression vectors (Novagen) using primers detailed in Supplemental Table 3. The methods of Site-directed mutagenesis were based on our previous description (Zeng and Wang, 2005). All the mutagenesis primers are shown in Supplemental Table 3. Colonies containing the appropriate insert were identified by sequencing. The resultant recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) and verified by sequencing. *E. coli* BL21 (DE3) strains were grown using Luria–Bertani liquid medium. *E. coli* BL21 (DE3) strains harboring the appropriate colonies were cultured at 37 °C overnight, and then subcultured (1:100) into fresh media until the optical density (A_{600}) reached 0.5. The expression of recombinant GST proteins was initiated by adding a final concentration of 0.1 mM isopropyl-β-D-thiogalactopyranoside. After 10 h induction, cells were harvested by centrifugation (8000 × g, 3 min, 4 °C), resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 7.4). The resuspended cells were sonicated on ice and centrifuged (10,000 × g, 10 min, 4 °C). The resulting particulate material and a small portion of the lysate were analyzed by SDS-PAGE. The rest of the lysate was loaded onto a Nickel-Sepharose High Performance column (GE Healthcare Bio-Sciences), washed with binding buffer and the GST proteins were eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 500 mM imidazole, pH 7.4).