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Research article

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

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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

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(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).

2.6. Enzyme assays and kinetics of Larix GST

To assess the specific activities of *Larix* GST enzymes, seven frequently used GST substrates were used. Activities towards 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and 4-nitrobenzyl chloride (NBC) were measured as described by Habig et al. (1974), towards 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) were measured as described by Ricci et al. (1994), and that towards dehydroascorbic acid (DHA), diphenyl ethers (Fluorodifen) and cumene hydroperoxide (Cum-OOH) were measured as described by Edwards and Dixon (2005). Every assay was performed at 25 °C. Protein concentrations were determined by measuring their absorption at 280 nm.

The apparent K_m values for GSH were determined by varying the GSH concentrations from 0.1 to 1.0 mM at a fixed concentration of 1.0 mM CDNB. The apparent K_m values for CDNB were determined by varying the CDNB concentrations from 0.2 to 2.8 mM and a fixed GSH concentration of 1.0 mM. The kinetic parameters were calculated using nonlinear regression analysis by the Hyper32 program available at http://homepage.ntlworld.com/john.easterby/hyper32.html.

The dependence of GST activity on temperature was examined using CDNB as substrate. The assay was carried out in 0.05 M potassium phosphate buffer (pH 6.5) at a range of temperatures from 15 °C to 70 °C at 5 °C intervals. Activity toward CDNB was measured as described by Habig et al. (1974). The dependence of GST activity on pH was investigated as described by Yuen and Ho (2001).

3. Results

3.1. Identification and cloning of GST genes from the L. kaempferi genome

In this study, we cloned 27 full-length genes encoding putative GSTs from the *L. kaempferi* genome (Table 1). Among them, one sequence (*LkGSTU11*) was considered to be a pseudogene, based on the presence of a frame shift disrupting the coding region. After revising the frame shifts by deleting two nucleotides, this sequence was included in phylogenetic and gene expression analyses. The predicted proteins encoded by these 27 genes were initially classified based on a conserved domain analysis from the NCBI, which divided them into eight classes. Tau and phi GSTs were the most numerous, being represented by eleven and seven members, respectively. The lambda and zeta class GSTs contained three and two members, respectively. The theta, TCHQD, DHAR, and EF1Bγ classes contained one member each. Phylogenetic relationships among the 27 *Larix* GSTs and 81 *Populus* GSTs were reconstructed using a maximum-likelihood procedure. *Larix* and *Populus* GSTs belonging to same class were grouped into one clade with high bootstrap support (Supplementary Fig. 1), which further confirms the subfamily designations among the 27 *Larix* GSTs.

3.2. Structural features of Larix tau and phi GSTs

Larix tau and phi GSTs were the most numerous, having eleven and seven members, respectively. Pairwise comparisons of full-length *Larix* tau and phi GST protein sequences revealed some notable features. Eleven tau GST proteins displayed 37.0–96.6% pairwise sequence identity. Seven phi GST proteins displayed 50.4–80.0% pairwise sequence identity. An independent-sample *t*-test revealed that *Larix* phi GSTs were more conserved than tau GSTs (Supplementary Fig. 2, $P = 0.000$). Our previous study identified 26 tau and seven phi GSTs from gymnosperm *Pinus tabuliformis* (Lan et al., 2013). Sequence analysis showed that *Larix* and *Pinus* tau GST proteins shared 29.0–79.4% pairwise sequence identity. *Larix* and *Pinus* phi GST proteins displayed 47.7–90.7% pairwise sequence identity.

Table 1
The GST genes cloned from the *Larix kaempferi* genome. Asterisk denotes the putative pseudogene.

Class	Gene	GenBank accession numbers	Length of gene product	Protein molecular weight (kDa)
Tau	*LKGSTU1*	KF527889	241	26.46
	LKGSTU2	KF527890	234	26.22
	LKGSTU3	KF527891	238	26.53
	LKGSTU4	KF527892	238	26.49
	LKGSTU5	KF527893	239	26.46
	LKGSTU6	KF527894	228	25.95
	LKGSTU7	KF527895	233	26.72
	LKGSTU8	KF527896	236	26.85
	LKGSTU9	KF527897	239	27.02
	LKGSTU10	KF527898	235	26.73
	*LKGSTU11**	KF527899	239	26.42
Phi	*LKGSTF1*	KF527900	216	24.06
	LKGSTF2	KF527901	215	24.13
	LKGSTF3	KF527902	214	24.07
	LKGSTF4	KF527903	212	24.00
	LKGSTF5	KF527904	216	24.20
	LKGSTF6	KF527905	220	24.59
	LKGSTF7	KF527906	215	24.02
Lambda	*LKGSTL1*	KF527907	240	27.08
	LKGSTL2	KF527908	237	26.78
	LKGSTL3	KF527909	237	26.79
Zeta	*LKGSTZ1*	KF527910	226	25.43
	LKGSTZ2	KF527911	229	25.58
Theta	*LkGSTT1*	KF527912	249	28.14
EF1Bγ	*LkEF1Bγ1*	KF438051	422	48.19
DHAR	*LkDHAR1*	KF438049	215	23.93
TCHQD	*LkTCHQD1*	KF438050	264	31.01

The GST proteins were divided into two distinct domains: a conserved N-terminal domain (approximately one-third of the protein) and a more variable C-terminal domain (approximately two-thirds of the protein). In this study, for *Larix* tau GSTs, the N-terminal domain protein sequences displayed 52.8–95.2% pairwise sequence identity, while in the C-terminal domain 58% of the sequences scored <50.0% identity. A pairwise comparison of protein sequences indicated a significant difference between the N- and C-terminal domain protein sequences of *Larix* tau GSTs (paired-sample *t*-test, $P = 0.000$). The N-terminal domain sequences of *Larix* tau GSTs were much more conserved than the C-terminal domain sequences (Fig. 1A). As with the tau GSTs, the N-terminal domain sequences of *Larix* phi GSTs were more conserved than the C-terminal domain sequences ($P = 0.000$) (Fig. 1B).

The three-dimensional structures of ten *Larix* tau GSTs (except for the pseudogene *LkGSTU11*) and seven phi GSTs were built using the MODELER module of InsightII. The ten *Larix* tau GSTs shared the same conformation of structural elements (α-helices and β-sheets), but structural modifications were present in loop regions and in the linker between the N- and C-terminal domains (white arrows, Supplementary Fig. 3A). Structural comparisons of seven phi GSTs indicated that they had similar characteristics to the tau GSTs (Supplementary Fig. 3B).

3.3. Expression patterns of Larix GST genes under normal growth conditions

To examine functional diversification among *Larix* GST genes, the expression patterns of the 27 genes were examined by semi-quantitative PCR under normal conditions. The expression of all 27 genes in four different tissues (root, bud, needle, and phloem in the stem) was analyzed (Fig. 2). Some *Larix* GST genes (e.g., *LkGSTZ1* and *LkDHAR1*) could be detected by PCR using 24, 26, 28, or 30

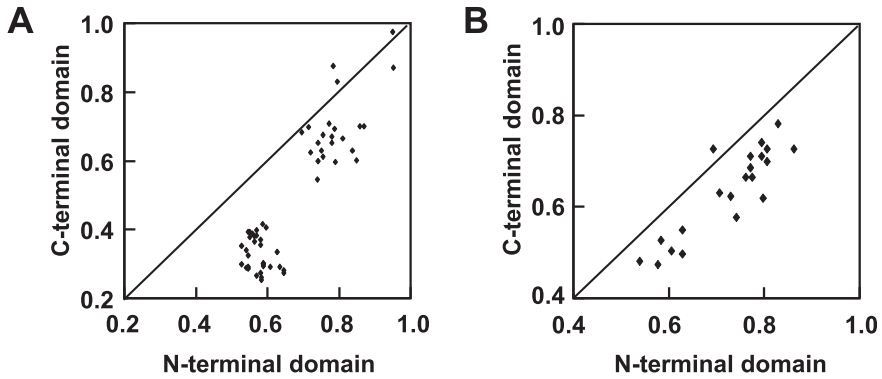

Fig. 1. Pairwise protein sequence identity plots for the C-terminal domain versus the N-terminal domain of *Larix* tau (A) and phi (B) GSTs.

amplification cycles, while some genes (e.g., *LkGSTU2* and *LkGSTU10*) were only detected by PCR using 30 amplification cycles. Thus, we only focused on the expression patterns of *Larix* GST genes using 30 PCR amplification cycles. All PCR products were confirmed by sequencing. Greater variation in expression pattern was found among the *Larix* tau GSTs than among members of the other seven classes of GSTs. Among the eleven *Larix* tau GSTs, three (*LkGSTU5*, *7*, and *8*) were expressed in all tissues tested, while the other eight were selectively expressed in some of the tissues. *LkGSTU2* was weakly expressed in needles and buds, while *LkGSTU10* was weakly expressed only in bud tissues. *LkGSTU6* and *11* were expressed in root, needle, and phloem tissues. We found that *LkGSTU1*, *3*, and *9* were expressed only in root tissues, and the expression of *LkGSTU4* and *7* in root tissues was much higher than that in the other three tissues tested. Thus, these five genes (*LkGSTU1*, *3*, *4*, *7*, and *9*) might play important roles in *Larix* roots.

L. kaempferi contains seven phi GST genes. Among these, *LkGSTF2* was expressed only in root tissues, while the other six (*LkGSTF1*, *3*, *4*, *5*, *6*, and *7*) were expressed in all tissues tested. Except for *LkGSTZ2*, which was weakly expressed only in needle tissues, the other six classes of *Larix* GST genes (*LkGSTL1*, *2*, and *3*; *LKGSTZ1*; *LkGSTT1*; *LkEF1Bγ1*; *LkDHAR1*; and *LkTCHQD1*) were expressed in all four tissues tested.

3.4. Substrate specificities and activities of Larix GST proteins

To investigate the catalytic characteristics of *Larix* GST proteins, which may be related to their biological functions, we selected ten tau, seven phi, and one DHAR GSTs for protein expression and purification. Because *LkGSTU11* is a pseudogene, we did not consider its biochemical function. Among the 18 GSTs selected for protein expression and purification, six (LkGSTF1, 4, 5, and 6; and LkGSTU7 and 10) were expressed as inclusion bodies in *E. coli* BL21 (DE3), while the other twelve (LkGSTU1, 2, 3, 4, 5, 6, 8, and 9; LkGSTF2, 3, and 7; and LkDHAR1) were expressed as soluble proteins in *E. coli* BL21 (DE3). Thus, in this study, only the twelve soluble GST proteins were purified using a Nickel-Sepharose High Performance column. The purified *Larix* GST proteins were assayed to determine their enzymatic activities toward seven GST substrates (Table 2): 1-chloro-2,4-dinitrobenzene (CDNB), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), 1,2-dichloro-4-nitrobenzene (DCNB), 4-nitrobenzyl chloride (NBC), fluorodifen, cumene hydroperoxide (Cum-OOH), and dehydroascorbic acid (DHA).

For the eight *Larix* tau GSTs, all purified proteins displayed enzymatic activity toward three substrates: CDNB, NBD-Cl, and fluorodifen. In contrast, not all of the proteins displayed activity toward DHA (Table 2). With the exception of LkGSTU6, the other tau GSTs displayed activity toward NBC. With the exception of LkGSTU4 and 6, the other tau GSTs displayed activity toward Cum-OOH. Only LkGSTU2, 5, and 6 displayed activity toward DCNB. Interestingly, among the eight purified *Larix* tau GSTs, LkGSTU5 displayed the greatest enzymatic activity toward CDNB, NBD-Cl, and fluorodifen. Compared to the other seven tau GSTs, LkGSTU5 had 3.4–251.3-fold greater activity toward CDNB, 2.1–137.6-fold greater activity toward NBD-Cl, and 2.3–731.0-fold greater activity toward fluorodifen.

Three *Larix* phi GSTs (LkGSTF2, 3, and 7) did not display enzymatic activity toward DCNB, fluorodifen, and DHA. Although three phi GSTs displayed activity toward NBC and Cum-OOH, LkGSTF2 displayed much greater enzymatic activity toward these two substrates than LkGSTF3 and 7. Among three phi GSTs, LkGSTF2 had weak activity toward NBD-Cl (0.06 μmol min^{-1} mg^{-1}). LkGSTF2 and 7 displayed enzymatic activity toward CDNB, whereas LkGSTF3 did not. As with other angiosperm and moss DHARs, LkDHAR1 displayed activity only toward substrate DHA.

3.5. Kinetic properties of Larix GSTs

GSTs can catalyze the conjugation of GSH to a variety of electrophilic substrates. In this study, the apparent kinetic constants of seven tau GSTs and two phi GSTs were determined using GSH and CDNB as substrates (Table 3). Except for LkGSTU8, the apparent K_m^{GSH} values of the six *Larix* tau GSTs fell within the range of 0.07–0.81 mM, indicating that they have similar, strong affinities for GSH. This is in accordance with the highly conserved nature of the N-terminal domain of the GSTs (Fig. 1), where the enzyme conjugates GSH. Obvious variations in substrate affinity (K_m^{CDNB}) and catalytic efficiency ($[k_{cat}/K_m]^{CDNB}$) to CDNB were observed among the seven *Larix* tau GSTs (K_m^{CDNB} 1.33–30.62 mM and $[k_{cat}/K_m]^{CDNB}$ 0.12–35.35 mM^{-1} s^{-1}). Of the seven *Larix* tau GSTs, LkGSTU5 had the highest k_{cat}^{CDNB} values, resulting in the protein displaying the greatest catalytic efficiency (highest $[k_{cat}/K_m]^{CDNB}$ values). For the *Larix* phi GSTs, because LkGSTF3 did not display any enzymatic activity towards CDNB, we only determined the apparent kinetic constants for LkGSTF2 and 7 (Table 3). LkGSTF7 displayed much greater affinity and catalytic efficiency toward GSH than LkGSTF2, but these two proteins had a similar affinity and catalytic efficiency toward CDNB.

3.6. pH and temperature profiles of Larix GSTs

The intracellular pH can change during plant defense responses against various stresses (Kader and Lindberg, 2010). Thus, for natural enzymes, a tolerance to changes in pH is fundamental to plant survival under stressful conditions. To investigate the evolutionary adaptation of *Larix* GSTs to a variety of pH levels, we examined the

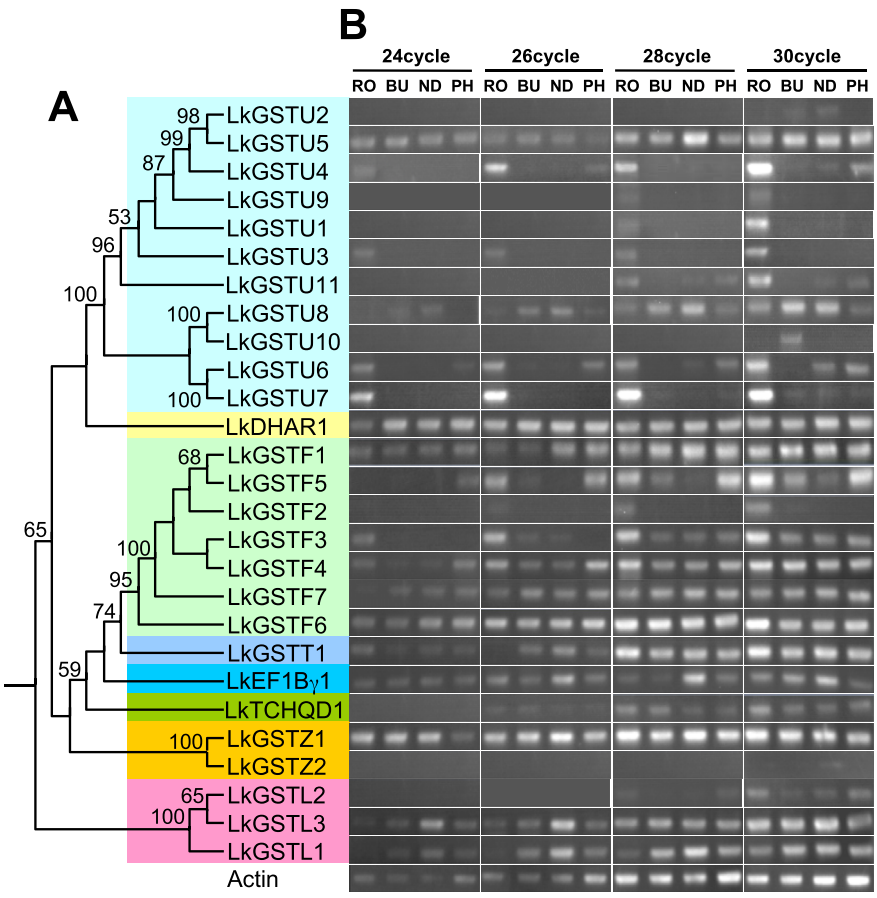

Fig. 2. Phylogenetic relationships among *Larix* GSTs (A) and their expression patterns (B). The numbers at each node in the phylogenetic tree are bootstrap values, with only values >50% shown. RO, BU, ND, and PH represent root, bud, needle, and phloem tissues, respectively.

effect of pH on *Larix* GST activity (Fig. 3A). All of the tested ten *Larix* GST proteins displayed maximal activity at alkaline pH values: LkGSTU1 and LkDHAR1 displayed maximal activity at pH 8.0, six GSTs (LKGSTU2, 5, 6, and 8; and LKGSTF2 and 7) at 8.5, and LKGSTU4 and 9 at a pH of 9.0. The enzymatic activity of the ten *Larix* GSTs almost ceased at pH 5.5. LkGSTU1, 2, 4, and 9 retained >57% of their maximum activity over a pH range from 7.0 to 9.5, while LkGSTU5, 6, and 8 retained >53% of their maximum activity over a pH range from 7.5 to 9.5. Compared to the other *Larix* GSTs, LkGSTF7 exhibited broad-spectrum adaptation to pH; it retained >60% of its maximum activity at pH values ranging from 6.5 to 10.0.

The activity of the *Larix* GSTs was temperature-dependent. A temperature profile analysis of the ten purified *Larix* GSTs indicated a broad optimal temperature range for enzymatic activity (Fig. 3B). However, the *Larix* tau GSTs displayed variations in their optimum temperature. Specifically, LkGSTU2 and 4 displayed >50% of their

Table 2
Specific activities of the *Larix* GSTs. Values shown are means ± SD, calculated from three replicates. n.d., no activity detected.

GSTs	Specific activities (μmol min^{-1} mg^{-1})						
	CDNB	NBD-Cl	DCNB	NBC	Fluorodifen	Cum-OOH	DHA
LkGSTU1	2.10 ± 0.02	3.02 ± 0.10	n.d.	1.11 ± 0.03	2.06 ± 0.02	0.56 ± 0.01	n.d.
LkGSTU2	5.26 ± 0.08	3.87 ± 0.32	0.05 ± 0.01	1.06 ± 0.03	2.25 ± 0.11	0.17 ± 0.02	n.d.
LkGSTU3	0.84 ± 0.01	0.43 ± 0.01	n.d.	0.60 ± 0.03	0.63 ± 0.02	0.05 ± 0.01	n.d.
LkGSTU4	4.45 ± 0.11	6.49 ± 0.37	n.d.	1.11 ± 0.03	19.44 ± 0.34	n.d.	n.d.
LkGSTU5	40.20 ± 0.50	13.76 ± 0.33	0.04 ± 0.01	1.67 ± 0.07	43.86 ± 0.55	0.56 ± 0.02	n.d.
LkGSTU5(V177W)	2.25 ± 0.07*	6.36 ± 0.50*	n.d.	1.48 ± 0.08*	0.69 ± 0.08*	0.09 ± 0.01*	n.d.
LkGSTU5(V177F)	10.78 ± 0.31*	7.51 ± 0.77*	0.04 ± 0.01	0.72 ± 0.05*	7.84 ± 0.18*	0.38 ± 0.03*	n.d.
LkGSTU6	11.87 ± 0.23	4.08 ± 0.15	0.36 ± 0.01	n.d.	0.56 ± 0.02	n.d.	n.d.
LkGSTU8	0.27 ± 0.01	0.10 ± 0.01	n.d.	0.69 ± 0.03	3.05 ± 0.10	0.03 ± 0.01	n.d.
LkGSTU8(W175V)	0.36 ± 0.01*	0.09 ± 0.01	n.d.	0.86 ± 0.04*	6.03 ± 0.13*	n.d.	n.d.
LkGSTU9	0.16 ± 0.01	2.99 ± 0.04	n.d.	1.36 ± 0.06	0.06 ± 0.05	0.06 ± 0.01	n.d.
LkGSTU9(F177V)	0.25 ± 0.01*	6.06 ± 0.40*	n.d.	0.70 ± 0.06*	0.02 ± 0.01	0.05 ± 0.01	n.d.
LkGSTF2	0.11 ± 0.01	0.06 ± 0.01	n.d.	1.82 ± 0.04	n.d.	0.46 ± 0.02	n.d.
LkGSTF3	n.d.	n.d.	n.d.	0.35 ± 0.03	n.d.	0.04 ± 0.01	n.d.
LkGSTF7	0.08 ± 0.01	n.d.	n.d.	0.63 ± 0.03	n.d.	0.06 ± 0.01	n.d.
LkDHAR1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.94 ± 0.01

*Significant difference ($P < 0.05$) in enzymatic activities between the wild-type and its mutants.

Table 3
Kinetic constants of *Larix* tau GSTs towards GSH and CDNB. Values shown are means ± SD, calculated from three replicates.

GSTs	K_m^{GSH} (mM)	k_{cat}^{GSH} (S^{-1})	$(k_{cat}/K_m)^{GSH}$ ($mM^{-1}\ S^{-1}$)	K_m^{CDNB} (mM)	k_{cat}^{CDNB} (S^{-1})	$(k_{cat}/K_m)^{CDNB}$ ($mM^{-1}\ S^{-1}$)
LkGSTU1	0.33 ± 0.02	5.11	15.48	1.82 ± 0.12	11.50	6.32
LkGSTU2	0.78 ± 0.04	15.08	19.33	1.33 ± 0.09	19.71	14.82
LkGSTU4	0.27 ± 0.01	5.85	21.67	6.94 ± 0.34	42.30	6.10
LkGSTU5	0.81 ± 0.06	64.11	79.15	4.72 ± 1.33	166.86	35.35
LkGSTU6	0.51 ± 0.03	31.02	60.82	1.68 ± 0.01	53.10	31.61
LkGSTU8	3.28 ± 0.13	1.07	0.33	5.70 ± 0.61	1.60	0.28
LkGSTU9	0.07 ± 0.02	0.12	1.71	30.62 ± 6.44	3.61	0.12
LkGSTF2	0.18 ± 0.02	0.11	0.61	1.32 ± 0.06	0.18	0.14
LkGSTF7	0.03 ± 0.01	0.06	2.00	1.11 ± 0.17	0.14	0.13

maximum activity over a temperature range of 20–60 °C, while LkGSTU8 displayed >50% of its maximum activity over a temperature range of 15–30 °C (this protein lost its enzymatic activity toward CDNB at 45 °C). LkGSTF2 and 7 retained >57% of their maximum activity over a temperature range of 40–70 °C, indicating that these two proteins might possess an evolutionary adaptation for tolerance to high temperatures.

3.7. Site-directed mutagenesis of the LkGSTU5, 8 and 9 genes

Among the eight *Larix* tau GSTs, LkGSTU5 showed the greatest enzymatic activity and the highest catalytic efficiency toward substrate CDNB (Tables 2 and 3). Based on sequence alignment, we found a conserved Trp/Phe residue in *Larix* tau GSTs (alignment position 189 in Fig. 4). The X-ray structure of TaGSTU4 (1GWC) indicated that the residue was an important component of the hydrophobic substrate-binding pocket of the protein (Fig. 5). But this conserved Trp/Phe residue is substituted by Val in LkGSTU5 (Fig. 4). This substitution might be responsible for that LkGSTU5 protein had the high level of activity and catalytic efficiency towards substrate CDNB. To test this hypothesis, we constructed two sets of mutants for biochemical assays. First, the Val177 residue of LkGSTU5 was replaced by Trp and Phe residues, respectively. Compared with wild-type LkGSTU5 protein, the mutants V177W and V177F showed much lower enzymatic activities towards substrate CDNB (Table 2). Secondly, we mutated Trp175 of LkGSTU8 and Phe177 of LkGSTU9 to Val residue. Compared with wild-type proteins, the mutants W175V and F177V showed much higher enzymatic activities towards substrate CDNB (Table 2).

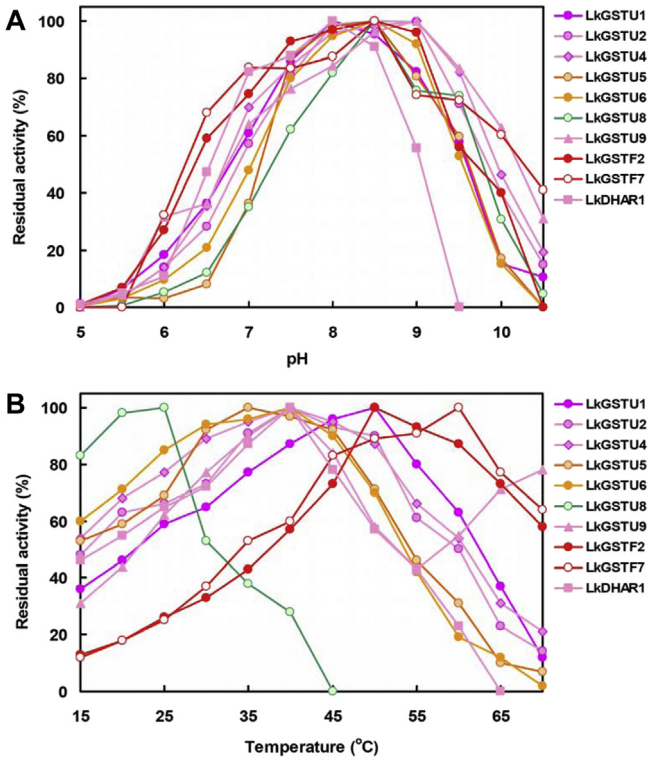

Fig. 3. Effects of pH (A) and temperature (B) on *Larix* GST activity.

4. Discussion

In this study, we cloned 27 full-length GST genes from the *L. kaempferi* genome, which were divided into 8 classes. As with the angiosperm GST gene family, the tau and phi GST classes were the most numerous in the *L. kaempferi* genome, being represented by eleven and seven members, respectively. Many studies have shown that tau and phi GSTs play important roles in stress tolerance, development, and secondary metabolism (Wisser et al., 2011; Dixon et al., 2011; Chen et al., 2012). The X-ray structures of several angiosperm tau and phi GSTs have been determined (Thom et al., 2002; Axarli et al., 2009), which have allowed us to understand the structural and functional characteristics of gymnosperm tau and phi GSTs. These X-ray structures indicate that GSTs contain two distinct domains: a conserved N-terminal domain and a more variable C-terminal domain. The GSH-binding residues are located in the conserved N-terminal domain. The X-ray structure of TaGSTU4 (1GWC) indicated that the Ser15, Lys42, Ile56, Glu68 and Ser69 (Fig. 5) are GSH-binding site (G-site) residues and form hydrogen bonds with GSH (Thom et al., 2002). Based on sequence and structural comparisons, five G-site residues, corresponding to Ser20, Lys47, Ile61, Glu73, and Ser74 of LKGSTU5 (alignment positions 21, 51, 65, 78, and 79 in Fig. 4), were predicted for *Larix* tau and phi GSTs (blue arrows, Fig. 4).

The Ser20 residue of LKGSTU5 (alignment position 21 in Fig. 4) is highly conserved in *Larix* tau and phi GSTs. This highly conserved Ser residue is thought to be responsible for stabilization of the thiolate anion of enzyme-bound GSH (Dirr et al., 1994). This conserved Ser residue is replaced by Cys in plant DHARs and lambda GSTs, and by Tyr residues in animal alpha, mu, and pi GSTs (Dixon et al., 2002b; Sheehan et al., 2001). Ile61 of LKGSTU5 (alignment position 65 in Fig. 4) is located in the loop connecting the α-helix 2 to β-strand 3 in the N-terminal domain (Fig. 5). In four tau and six phi *Larix* GSTs, this critical Ile residue was replaced by Val (Fig. 4). Compared to wild type, replacing this Ile by Ala in *Pinus* PtGSTU1 resulted in lower enzymatic activity and affinity (Zeng and Wang, 2005). In *Cathaya* CaGSTF, removing the side residues of this Ile resulted in the loss of all enzymatic activity (Wei et al., 2012). Thus, this residue is considered to be critical for the GSH-binding and catalytic functions of plant tau and phi GSTs.

For the *Larix* tau GSTs except LkGSTU5, a conserved Trp/Phe residue was observed in alignment position 189 in Fig. 4. In GST proteins, this amino acid is considered to be an important residue in hydrophobic substrate-binding sites (H-sites) (Thom et al., 2002; Rossjohn et al., 1998). This Trp residue contains a large indole (benzopyrrole) side chain, which is an important component of the hydrophobic substrate-binding pocket of the protein. When this amino acid is replaced by a residue with a smaller side chain (e.g., Ala or Val), the hydrophobic substrate-binding pocket might become larger, resulting in a conformation change within the protein that affects its catalytic activity and efficiency. For example, in *Proteus mirabilis* PmGSTB1-1, the substitution of this Trp residue with Ala resulted in 17-fold enhancement of its enzymatic activity and a 22-fold increase in its catalytic efficiency toward CDNB (Allocati et al., 2005). Of the eight *Larix* tau GSTs, LkGSTU5 displayed the greatest enzymatic activity and had the highest catalytic

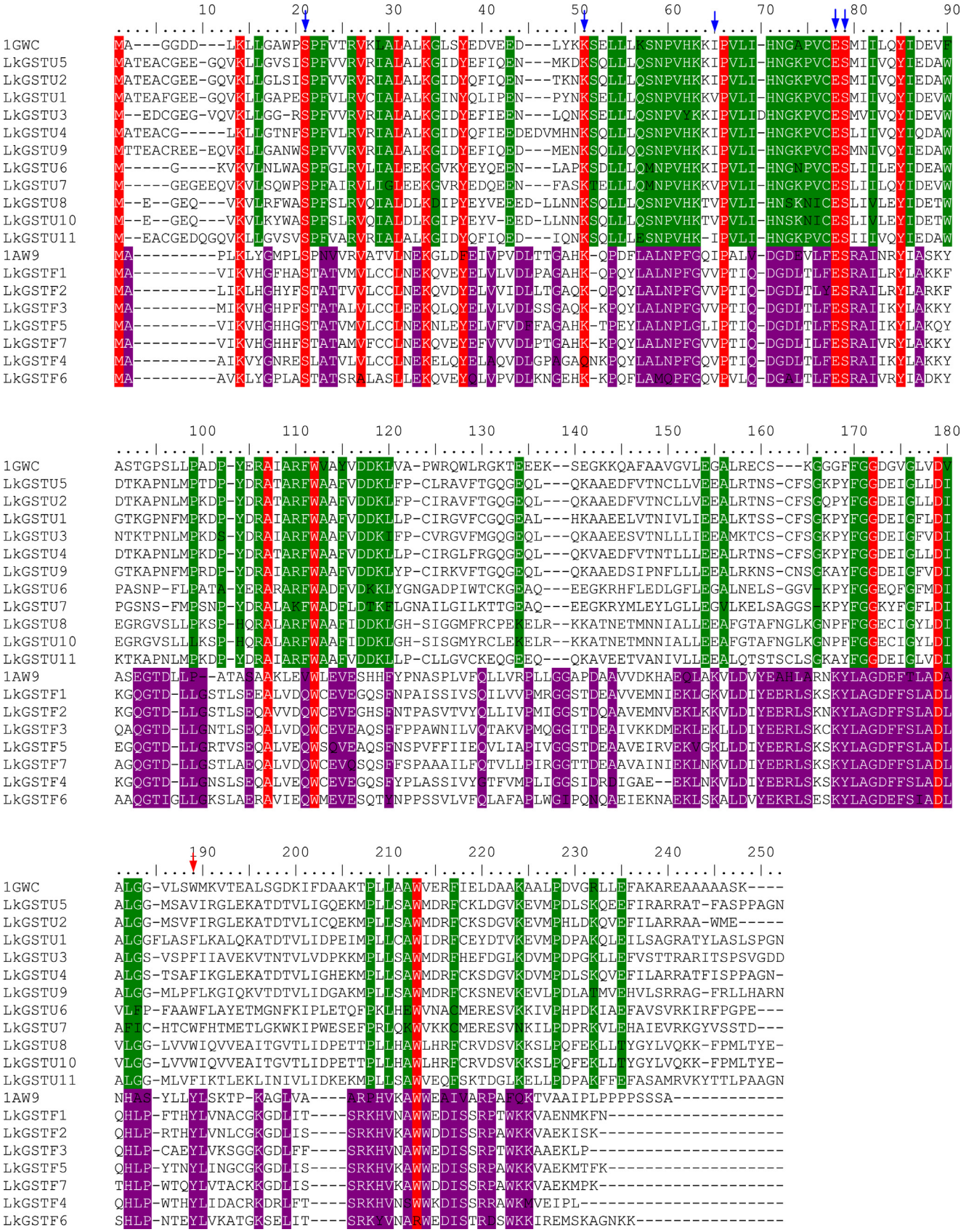

Fig. 4. Sequence alignment of *Larix* tau and phi GSTs. Conserved residues in all tau and phi GSTs are marked in red. Residues marked in green and purple indicate conserved residues in tau and phi GSTs, respectively. Predicted G- and H-sites are marked with blue and red arrows, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

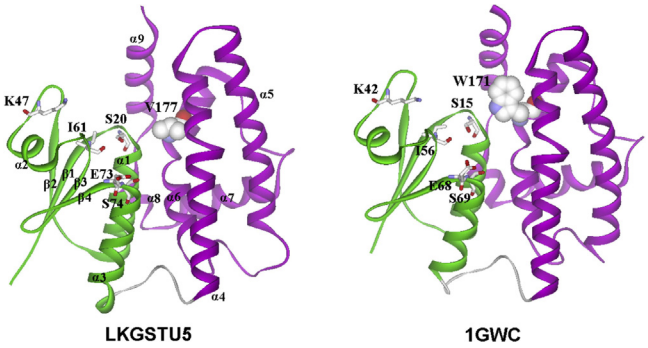

Fig. 5. Structural comparison between LkGSTU5 and wheat tau GST (1GWC). The N- and C-terminal domains are shown in green and purple, respectively, while the linker between the two domains is shown in white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

efficiency toward CDNB. A sequence alignment analysis revealed that in LkGSTU5, this important H-site residue is substituted by Val (alignment position 189 Fig. 4). When this Val residue of LkGSTU5 were replaced by Trp and Phe, the mutants V177W and V177F had much lower enzymatic activities towards substrate CDNB than that of wild-type protein. By contraries, when Trp175 of LkGSTU8 and Phe177 of LkGSTU9 were replaced by Val residue, compared with wild-type proteins, the mutants W175V and F177V had much higher enzymatic activities towards substrates CDNB. Thus, the Val177 residue was responsible for that LkGSTU5 protein had the high level of activity and catalytic efficiency towards substrate CDNB.

Compared to the other seven classes of GSTs, the *Larix* tau GST genes displayed much more variation in their expression patterns. *LkGSTU1*, *3*, and *9* were expressed only in root tissues, while the expression of *LkGSTU4* and *7* in root tissues was much higher than in the other three tissues tested (bud, needle, and phloem in the stem). Plant tau GST genes are believed to play important roles in defence responses against biotic stressors. For example, a maize tau GST was found to function in multiple disease resistance responses (Wisser et al., 2011), while the rice GST gene family is among 4 of 145 families found to be significantly associated with quantitative trait loci for resistance to various diseases (Wisser et al., 2005). Due to their sessile nature, plants are constantly exposed to a multitude of biotic stresses. Because soils contain a multitude of microbial species, including plant pathogenic bacteria and fungi, plant roots are exposed to these pathogenic microorganisms (Casadevall and Pirofski, 2007; Nazir et al., 2010). Thus, those *Larix* tau GST genes that are specifically expressed in root tissues might be related to multiple disease resistance responses to pathogenic microorganisms.

Sequence and structural comparisons of the *Larix* tau GSTs indicated that the N-terminal domain is much more conserved than the C-terminal domain. The GSH-binding site residues are located in the conserved N-terminal domain, while the hydrophobic substrate-binding sites are mainly located in the C-terminal domain. For *Larix* tau GSTs, the apparent K_m values toward GSH fell within the range of 0.07–3.28 mM, while the K_m values toward the hydrophobic substrate CDNB ranged from 1.33 to 30.62 mM. The affinity of *Larix* tau GSTs for GSH was far more conserved than for the hydrophobic substrate CDNB ($P = 0.006$, Mann–Whitney U test). This is in accordance with the highly conserved nature of the N-terminal domain of the GSTs. In addition, this study found that *Larix* GST proteins had maximal activity at alkaline pH values. Our previous study showed *Pinus* PtGSTU1 had the highest activity in the pH range 8.5–9.0 (Zeng et al., 2005). The optimum activity of the rice OsGSTU5 was observed at pH 8.5 (Cho et al., 2006). For plant GSTs, the possible mechanism for increased activity at alkaline pH might be the promotion of formation of the thiolate anion of GSH.

Based on the phylogenetic tree of *Larix* GSTs, we identified six recent duplicate gene pairs (*LkGSTU2/5*, *6/7*, and *8/10*; *LkGSTF1/5*; *LkGSTZ1/2*; and *LkGSTL2/3*). For the two duplicate gene pairs (*LkGSTF1/5* and *LkGSTL2/3*), both of the duplicated genes displayed similar expression patterns: they were expressed in all tissues tested. However, for the remaining four duplicate gene pairs, two duplicated genes displayed different expression patterns. For example, the duplicate gene pair *LkGSTZ1/2*, *LkGSTZ1* was expressed in all test tissues, while *LkGSTZ2* was expressed only in needle tissues. In addition, we found that some duplicate gene pairs displayed different biochemical characteristics. E.g., for the duplicate gene pairs *LkGSTU6/7* and *8/10*, LkGSTU7 and 10 were expressed as inclusion bodies in *E. coli*, while LkGSTU6 and 8 were expressed as soluble proteins. For the duplicate gene pair *LkGSTU2/5*, although LkGSTU2 and 5 were expressed as soluble proteins in *E. coli*, the two proteins displayed different enzymatic activities toward the five substrates (CDNB, NBD-Cl, NBC, fluorodifen, and Cum-OOH), and different catalytic efficiencies towards GSH and CDNB.

5. Conclusion

Glutathione transferases play important roles in stress tolerance and detoxification in plants. In this study, 27 GST genes were cloned from the deciduous conifer *L. kaempferi*. As with the angiosperm GST gene family, *Larix* GSTs are divided into eight classes, and tau class GSTs are the most numerous. *Larix* tau GST genes showed substantially variations in their expression patterns. The purified *Larix* GST proteins showed different substrate specificities, substrate activities, and kinetic characteristics. This study provided comprehensive insight into the gymnosperm GST gene family.

Contributions

Conceived and designed the experiments: Qing-Yin Zeng. Performed the experiments: Qi Yang. Analyzed the data: Yan-Jing Liu and Qing-Yin Zeng. Wrote the paper: Qi Yang and Qing-Yin Zeng.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.02.003

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