Hui-Ling Hsieh, Li-Ting Ma, Sheng-Yang Wang and Fang-Hua Chu*

Cloning and expression of a sesquiterpene synthase gene from *Taiwania cryptomerioides*

Abstract: Taiwania (*Taiwania cryptomerioides* Hayata) is a conifer species native to Taiwan, which is known for several bioactive secondary metabolites extracted from it. In this study, a sesquiterpene synthase (*TPS*) gene isolated from Taiwania was in focus. First, a pair of degenerate primers was designed for reverse transcription-polymerase chain reaction based on the total RNA extracted from the leaves of a mature tree. A DNA fragment with the conserved region of *TPS* gene was obtained. After 5′- and 3′-end amplification, the full-length gene was obtained, which contains an open reading frame of 1791 bp and encodes a predicted molecular mass of 70.2-kDa protein. The gene was highly expressed in young leaves, female flowers, and cones. The expression in leaves was enhanced by salicylic acid. To identify the function of *TPS*, the recombinant protein from *Escherichia coli* (Migula) Castellani & Chalmers was incubated with farnesyl diphosphate. Gas chromatography/mass spectrometry analysis and retention time as well as mass spectrum matching with authentic standards revealed that the major product of TPS is sesquiterpene α-gurjunene. The gene was, therefore, designated as *Tc-Gur*.

Keywords: α-gurjunene, salicylic acid, sesquiterpene synthase, *Taiwania cryptomerioides* Hayata

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Introduction

Taiwania (*Taiwania cryptomerioides* Hayata) is native to East Asia and grows in the mountains of central Taiwan at elevations of between 1800 and 2600 m. Along with *Ginkgo biloba* L., *Sequoiadendron giganteum* (Lindl.) Buch., and *Metasequoia glyptostroboides* Hu & Cheng, Taiwania is a relict plant from the Tertiary Period of the Cenozoic Era. Taiwania is one of the economically most important tree species in Taiwan because of the wide utilization of its quality wood. Over the past 70 years, more than 500 compounds have been isolated from Taiwania, including secondary metabolites such as terpenoids, lignans, isoflavones, and other compounds. Many of the secondary metabolites are bioactive, for example, taiwanin A has antitumor activities and cedrol and α-cadinol show antitermite and antifungal activities, respectively (Kuo et al. 1999; Chang et al. 2003; Chien and Kuo 2009).

Terpenes play diverse roles at the interface between organisms and their environments. Terpenes are partly bioactive, for example, the furano-sesquiterpenes of myrrh (*Commiphora sphaerocarpa* Chiov.) show antitumor, antiparasitic, and healing activities (Dekebo et al. 2002) and artemisinin of *Artemisia annua* L. is revealed to have antimalarial activities (Reale et al. 2008). Terpenes are synthesized from the prenyl diphosphate precursors associated with the mevalonate pathway and the methyl-erythritol 4-phosphate pathway of plants: geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranyl-geranyl diphosphate (GGPP) are the precursors participating on the synthesis of monoterpenes, sesquiterpenes, and diterpenes, respectively. Various terpenes are generated by monoterpene synthases (mono-TPS), sesquiterpene synthases (sesqui-TPS), or diterpene synthases (di-TPS). TPS are the key contributors to the diverse structures of terpenes and are also widely investigated in recent years (Keeling and Bohlmann 2006; Chu et al. 2009; Kuo et al. 2012; Wen et al. 2012; Chien et al. 2013; Ma et al. 2013; Huang et al. 2015).

Sesquiterpenes are abundant in Taiwania and their bioactive effects were also proven in succession (Chang et al. 2003; Chien and Kuo 2009). However, the biosynthesis pathways of sesquiterpenes in this plant are not well documented. In this study, a *sesqui-TPS* gene from Taiwania was cloned and characterized. To understand its biosynthetic mechanism, the biosynthesis of the gene was tested in *Arabidopsis* and its expression was analyzed in different tissues and under different treatment conditions.

***Corresponding author: Fang-Hua Chu,** School of Forestry and Resource Conservation, National Taiwan University, Taipei, Taiwan, Phone: +886-2-33665261, Fax: +886-2-23654520, e-mail: fhchu@ntu.edu.tw; and Experimental Forest, National Taiwan University, Taipei, Taiwan
Hui-Ling Hsieh and Li-Ting Ma: School of Forestry and Resource Conservation, National Taiwan University, Taipei, Taiwan
Sheng-Yang Wang: Department of Forestry, National Chung-Hsing University, Taichung, Taiwan; and Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

Materials and methods

The seeds and tissues of Taiwania (*T. cryptomerioides*) were obtained from the Chi-Tou Tract of the Experimental Forest of National Taiwan University and identified by Dr. Yen-Hsueh Tseng (National Chung-Hsing University). Tissues were harvested immediately, frozen in liquid nitrogen, and then stored at -80°C.

Four-year-old Taiwania saplings grown in a horticulture chamber under natural light were treated by mechanical wounding, for which the twigs with young leaves were cut transversely into segments. For chemical treatments, 3-cm segments were soaked in 100 μM salicylic acid (SA; Sigma, St. Louis, USA) and jasmonic acid (JA; Sigma, St. Louis, USA), whereas controls were soaked in water. Mechanical wounding was performed on 1-cm segments, which were collected at the beginning of the test (0 h) and after 1, 2, 3, 4, 5, and 6 h. The samples were flash frozen in liquid nitrogen and stored at -80°C for RNA analysis. Each treatment was performed from one seedling to minimize the possible intratree variation in terpene metabolism.

For RNA extraction and reverse transcription, scale-like leaves of Taiwania were frozen in liquid nitrogen and powdered by pestle and mortar. Total RNA was extracted and purified from the powder by Plant Total RNA MiniPrep Purification Kit (GeneMark, Taichung, Taiwan) and then dissolved in RNase-free water and stored at -80°C. First-stranded cDNA was synthesized from 1 μg of the total RNA by reverse transcription reaction by SuperScript First-Strand Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, USA).

To find the conserved regions, the following TPS were aligned by the ClustalW program at the European Molecular Biology Laboratory (EMBL; http://www.ebi.ac.uk): design primers of sesqui-TPS, nucleotide and amino acid sequences of conifer sesqui-TPS, δ-selinene-like synthase of *Picea sitchensis* (ABA86249), δ-selinene synthase of *Abies grandis* (AAK83561), longifolene synthase of *Picea abies* (AAS47695), α-farnesene synthase of *Pinus taeda* (AAO61226), (E)-α-bisabolene synthase of *A. grandis* (O81086), and (E)-γ-bisabolene synthase of *Pseudotsuga menziesii* (AAX07266). A pair of degenerate primers 5′-CTGGACGATATGTATGACAC(A/T)TATGGAA-3′ (forward) and 5′-GTA(G/C)ATTCAGGAT(G/T)GTCTTTCAT(A/G)TAACA-3′ (reverse) was used for polymerase chain reaction (PCR). The resulting purified 550-bp fragment was obtained from scale-like leaves of 70-year-old Taiwania. To acquire full-length cDNA, the 5′-end was performed with a rapid amplification of cDNA ends (RACE) kit (Invitrogen) with the reverse primer GSP3 5′-TCAATCCATTGTGCGTCTTGAAGTTGC-3′, and the 3′-end was amplified with the oligo(dT) primer and 5′-GGTTGTCGCTTACTTGATGACTCCAAAG-3′. PCR products were cloned with the pGEM-T Easy Vector System (Promega, Fitchburg, USA) and sequenced with an ABI 377 automatic sequencer (Perkin-Elmer, Foster City, USA).

The sequence analysis and protein structure prediction was performed based on the gymnosperm TPS chosen for the analysis with the cloned *sesqui-TPS* genes listed in Table S1. Multiple alignments were performed with the ClustalW program at EMBL and CLC sequence viewer 7.5 (CLC bio, Aarhus, Denmark). The phylogenetic analysis was conducted by Molecular Evolutionary Genetics Analysis version 4 (Tamura et al. 2007) and the phylogenetic tree was generated by the neighbor-joining algorithm. Distance analyses were completed on 1000 bootstrap replicated data sets. The 3D structure prediction of the sesqui-TPS protein was conducted by ESyPred3D (Swiss-Model Repository (http://swissmodel.expasy.org) and Chimera program (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, USA) Lambert et al. 2002).

Bacterial expression and *in vitro* enzyme assays: The full-length open reading frame (ORF) of the *sesqui-TPS* gene was amplified by PCR with the forward primer ses-pET21a-N 5′-CGGGATCCATGGCTGAAGTGGGTCTCCATGTC-3′ and the reverse primer ses-pET21a-C 5′-CCGCTCGAGTAACATTATAGGTTCAACTAGAATATTC-3′.

The amplified product containing the *Bam*HI/*Sac*I restriction sites was cloned into a pET-21a(+) vector (Novagen, Merck Bioscience, Darmstadt, Germany). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) for expression. Functional expression and enzyme assays have been previously described by Fäldt et al. (2003) and Martin et al. (2004). Recombinant *E. coli* cells were grown in 5 ml Luria-Bertani medium containing ampicillin (100 g ml^{-1}). Proteins were then induced with 1 mM isopropyl-β-D-thiogalactoside and grown for another 24 h at 20°C. Cells were collected by centrifugation and disrupted in 1 ml chilled extraction buffer [25 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 10% (v/v) glycerol, 10 mM dithiothreitol] by sonication, extracts were cleared by centrifugation, and the supernatant was collected and assayed for TPS activity with 50 μM FPP (Sigma, St. Louis, USA), 1 mM ascorbic acid, and protease inhibitor (Roche, Mannheim, Germany) and then overlaid with 1 ml pentane to trap the volatile products. After incubation at 30°C for 1 h, the reaction mixture was extracted with pentane (3×1 ml) and the combined extracts were purified over a silica column (70 Å, Phenomenex, Torrance, USA) to a sample vial and stored at 4°C. Negative controls for enzyme assays were performed from the extracts of *E. coli* BL21 transformed with pET-21a(+) without the insert.

The products produced from TPS were analyzed by gas chromatography/mass spectrometry (GC/MS). The extracts were absorbed by solid-phase microextraction (SPME) fibers. SPME holders and carboxen-polydimethylsiloxane (75 μm) fibers were purchased from Supelco (Bellefonte, USA). SPME fibers were conditioned by heating in the hot injection port of a gas chromatograph at 200°C for 15 min. For analysis, the sample vials were sealed and placed in a water bath at 40°C for 30 min, and the SPME fiber was introduced into the vial headspace and held for 15 min to collect the volatiles. The fiber was then removed from the headspace and desorbed at the GC injection port (230°C) of a PolarisQ Ion Trap GC/MS system (Thermo Fisher Scientific, Waltham, USA) equipped with a 30 m×0.25 mm×0.25 μm DB-5ms capillary column (Agilent J&W Scientific, Folsom, USA). The program was immediately started, and the SPME fiber was removed after a period of 1 h. Temperature program: initial 50°C→200°C (4°C min^{-1})→ 300°C (15°C min^{-1}) and hold for 5 min. Other parameters: injection at 250°C; ion source at 230°C; EI 70 eV; He as carrier gas (1.0 ml min^{-1}); split ratio 1:20; mass range 40–550 *m/z*. For substance identification, the Wiley/NBS Registry of Mass Spectral Databases and NIST and authentic reference compounds were available. The Kováts indices (KI) were calculated based on a homologous series of n-alkanes C9-C24 on a DB-5ms capillary column.

For tissue-specific gene expression, the total RNA of scale-like leaves of a 70-year-old tree (old leaves), awl-like leaves of a 2-year-old tree (young leaves), male flowers, female flowers, phloem, xylem, cones, bark, root, and 22-day-old seedling tissues of Taiwania was isolated. For the gene expression in wounded and chemically treated samples, total RNA was isolated 0 (untreated), 1, 2, 3, 4, 5, and 6 h after treatment. Each experiment was performed with three biological repeats, and each biological experiment was repeated three times. The transcription of the novel gene was monitored by reverse transcription-PCR (RT-PCR) analysis using specific primers: forward 5′-GTTTATTTAGTGCTATTGATATGTATGT-3′ and reverse 5′-ACAATACAAATTATTACATGATTTCCA-3′. The two specific primers used for amplification of *Actin* were

forward 5′-TGCTTATGTTGTTTTAGTTATTTGC-3′ and reverse 5′-CACAGATATACTCTCTAAAACCTTAC-3′. For real-time PCR analysis, the specific primers were the same as in the case of RT-PCR. The cDNA (100 ng) from each sample was analyzed by means of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, USA) on a StepOnePlus cycler with *Actin* as the endogenous control. For treatment, the 0 h samples of treatment and the less expression samples served as calibrators to quantify the relative expression ratios of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results and discussion

Cloning and sequence analysis of *TPS* gene

A full-length cDNA clone was obtained from scale-like leaves of Taiwania using degenerate primers for PCR and iterative RACE methods. This putative TPS consists of a 1791-bp ORF, a 57-bp 5′ noncoding region, and a 168-bp 3′ noncoding region. The ORF encodes a protein of 596 amino acids with a calculated molecular mass of 70.2 kDa and a predicted pI of 5.13. The protein contains the characteristic conserved sequences of TPS, including the highly conserved $D^{350}D^{351}XXD^{354}$ motif, a crucial site for the divalent action and substrate binding, and the $R^{21}R^{22}X_8W^{31}$ motif, the common sequence of cyclizing mono-TPS for targeting the protein into plastids (Figure 1; Davis and Croteau 2000; Keeling and Bohlmann 2006; Nieuwenhuizen et al. 2009). The multiple sequence alignment of this putative TPS showed 53%–58% amino acid similarity and 31%–36% identity with the mono-TPS, 39%–62% amino acid similarity and 24%–39% identity with the sesqui-TPS, and lower amino acid similarity with di-TPS (38–39% amino

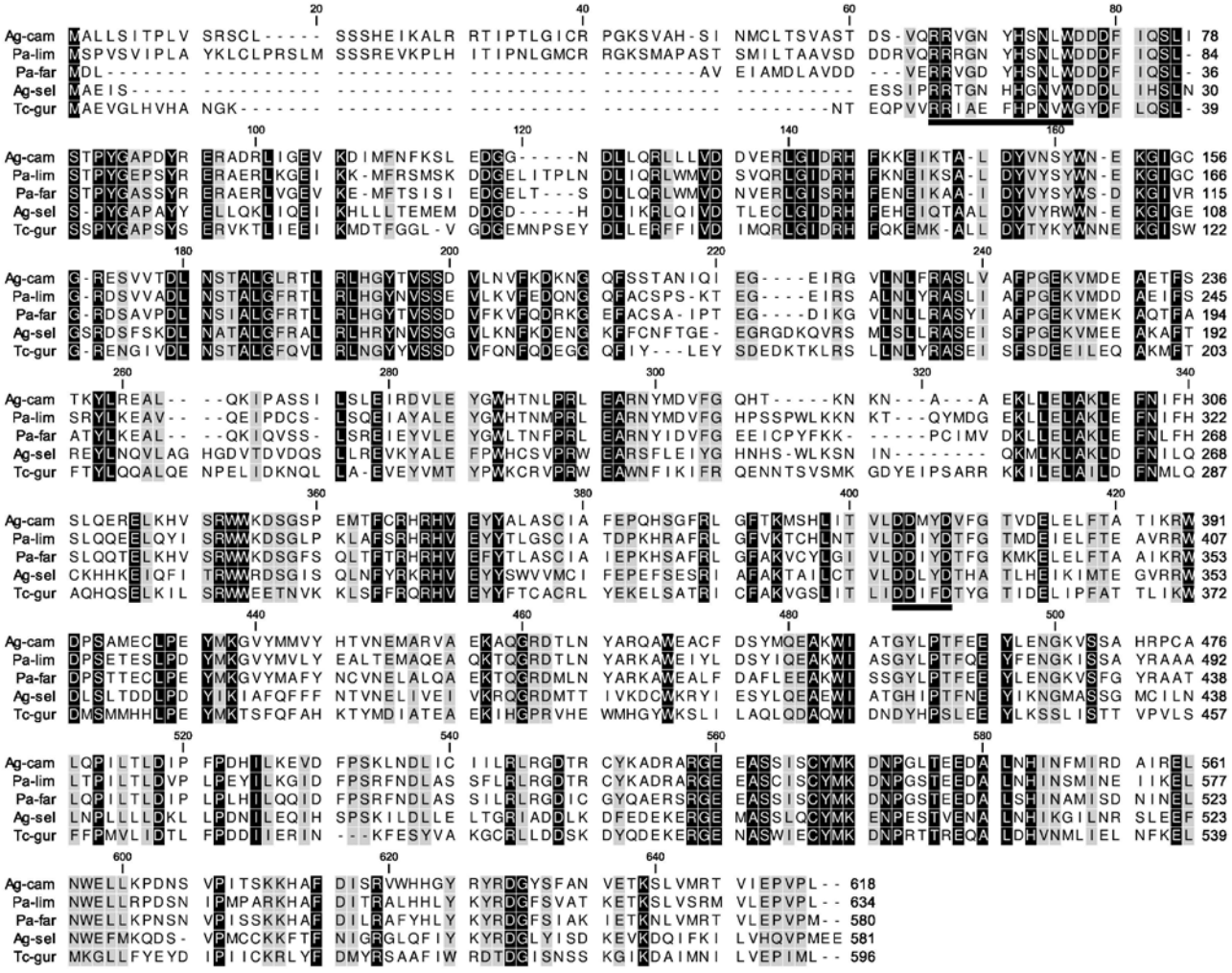

Figure 1 Amino acid sequence alignment of Tc-Gur (*Tc-gur*) and other gymnosperm TPS.
(-)-α-Camphene synthase from *A. grandis* (Ag-cam; AAB70707), (-)-α-limonene synthase from *P. abies* (Pa-lim; AAS47694), δ-selinene synthase from *A. grandis* (Ag-sel; AAC05727), and *E*,*E*-α-farnesene synthase from *P. abies* (Pa-far; AAS47697). Horizontal lines mark the highly conserved RRX_8W and DDXXD motifs.

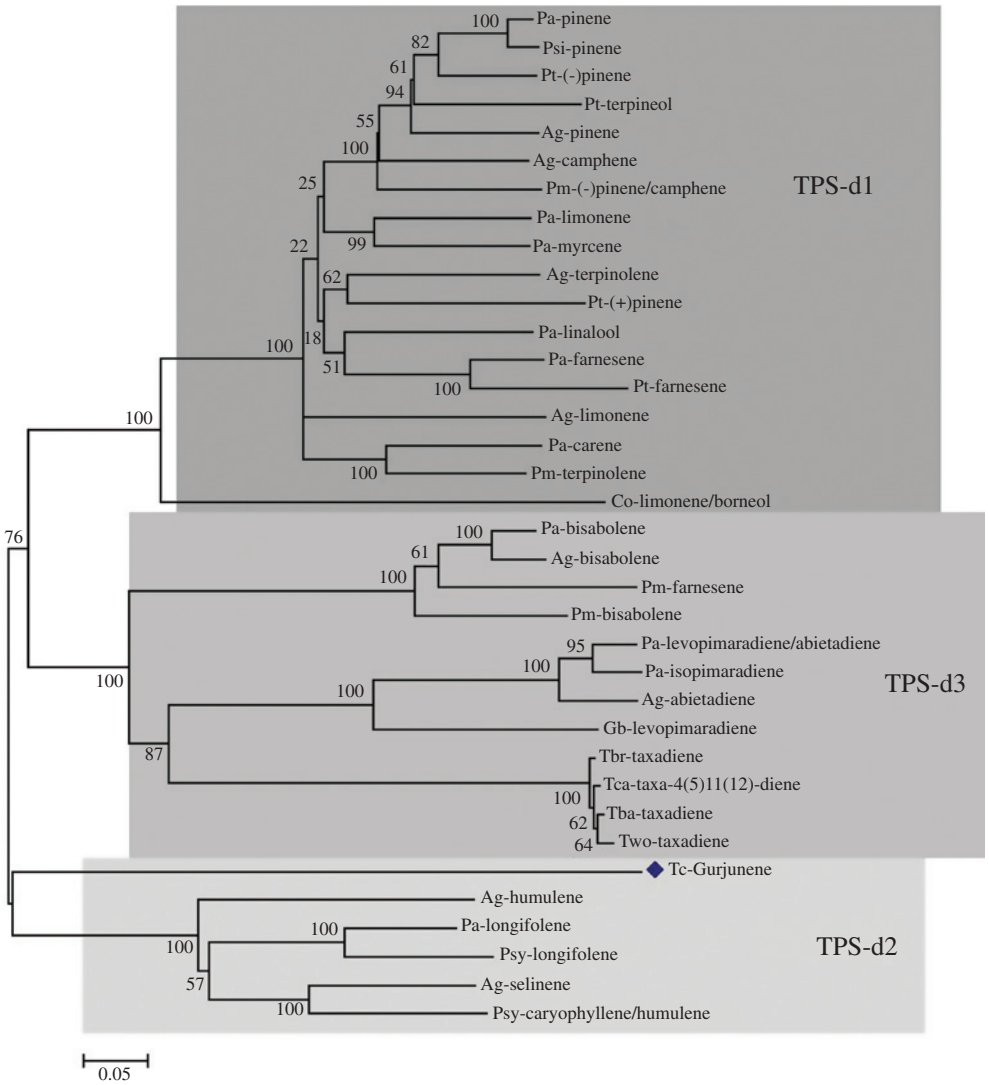

Figure 2 Phylogenic tree showing *Tc-Gur* (marked with a diamond) among gymnosperm TPS amino acid sequences.
Three groups, TPSd1, TPSd2, and TPSd3, are differentiated. (Pa, *Picea abies* L.) H. Karst.; Psi, *Picea sitchensis* (Bong.) Carr.; Pt, *Pinus taeda* L.; Ag, *Abies grandis* (Douglas ex D. Don) Lindley; Pm, *Pseudotsuga menziesii* (Mirb.) Franco; Co, *Chamaecyparis obtusa* (Siebold & Zucc.) Endl.; Gb, *Ginkgo biloba* L.; Tbr, *Taxus brevifolia* Nutt.; Tca, *Taxus canadensis* Marshall; Tba, *Taxus baccata* L.; Twc, *Taxus wallichiana* var. *chinensis* (Pilger) Florin; Tc, *Taiwania cryptomerioides*; Psy, *Pinus sylvestris*. L.) Scale bar represents 0.05 amino acid substitutions per site. The numbers show bootstrap values from 1000 replicates.

acid similarity and 22–24% identity; Table S1). However, this putative TPS showed higher similarity/identity to sesqui-TPS (58–62% amino acid similarity and 35–39% identity) by excluding a motif of 210 amino acids common to all di-TPS (Bohlmann et al. 1998b): *E*-α-bisabolene synthase of *P. abies* (Martin et al. 2004) and *A. grandis* (Bohlmann et al. 1998a) and (*E*)-γ-bisabolene/(*E*)-β-farnesene synthase of *P. menziesii* (Huber et al. 2005). The phylogenetic comparison (Figure 2) showed that this putative TPS belongs to the TPS-d2 subfamily of gymnosperm sesqui-TPS (Bohlmann et al. 1998b; Martin et al. 2004). The protein structure of this putative TPS was modeled by 5-*epi*-aristolochene (5EAU) synthase (a sesqui-TPS of *Nicotiana tabacum* L.; Starks et al. 1997) by means of the Swiss-Model, and a 34.22% identity was found. Overlaying the two proteins showed a possible interaction site of the substrate (FPP), divalent cations (Mg^{2+}), and the DDXXD motif in this putative TPS (Figure S1).

Heterologous expression and functional identification

This putative TPS coding region was cloned into the pET21a vector and transformed into *E. coli* BL21 (DE3) cells for expression. The molecular mass of this recombinant

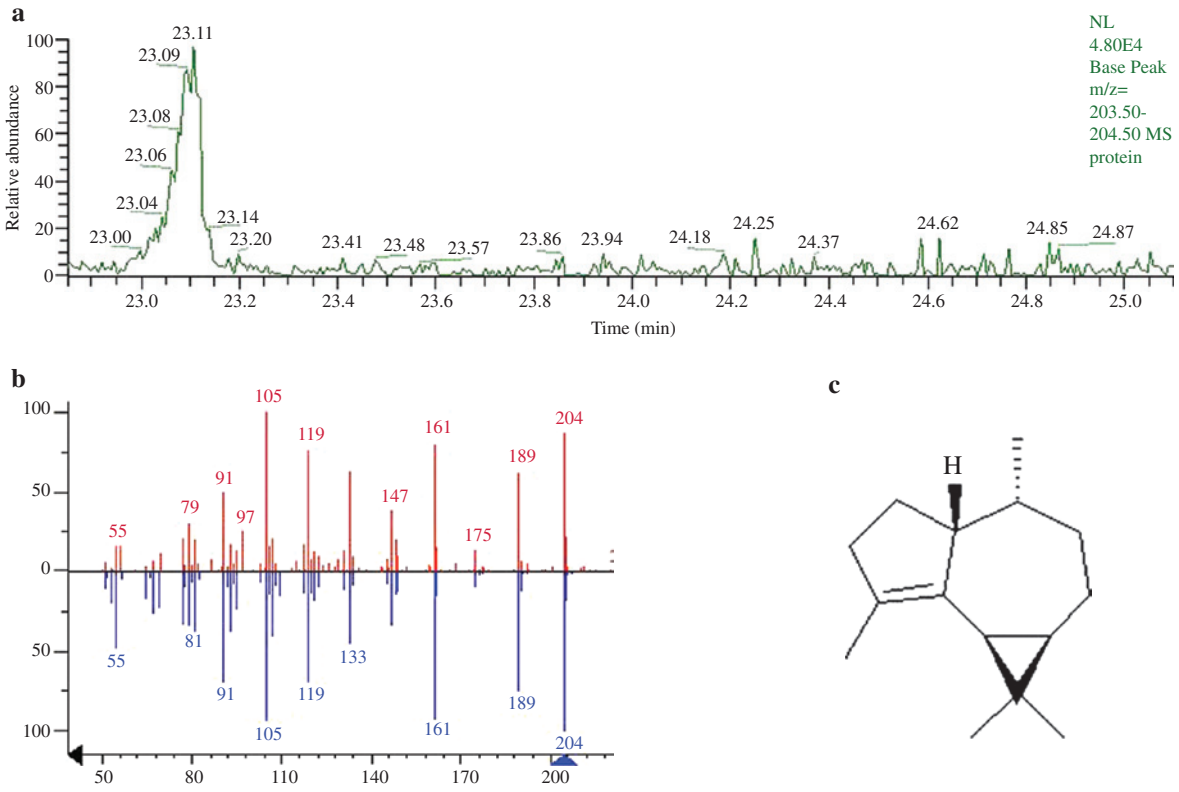

Figure 3 GC-MS chromatograph of the sesquiterpene product of Tc-Gur.
(a) GC/MS analysis of sesquiterpene products generated by recombinant *Tc-Gur* enzyme *in vitro*. (b) Mass spectrum of compound RT: 23.11 produced by *Tc-Gur* (top) and the authentic α-gurjunene (Sigma, St. Louis, USA). (c) α-Gurjunene.

putative TPS protein was approximately 70.2 kDa, and it was only active with FPP, not with GPP and GGPP. The compounds produced in the reaction incubated with FPP were analyzed by GC/MS. Based on this and Kováts retention indices (KI), the major product was α-gurjunene (RT: 23.11 min, KI: 1396; Figure 3) and was designated as *Tc-Gur*. α-Gurjunene is an aromadendrane-type sesquiterpenoid. Although this type is not the major structure of compounds in Taiwania, it may be modified in the living plant (Figure S2). In other species, the biosynthetic studies of (-)-α-gurjunene from *Solidago canadensis* L. (Schmidt et al. 1999) demonstrated that approximately 38% of the compounds of the essential oil from plant extracts are cyclocolorenone components; however, when the substrate FPP was added to crude extracts, no cyclocolorenones were detected but only the compound (-)-α-gurjunene. This can be rationalized by the oxidation of (-)-α-gurjunene to cyclocolorenone in the living plant by *P*450 enzymes (Schmidt et al. 1999). Cyclocolorenone is associated with allelopathic, antimicrobial (Jacyno et al. 1991; Schmidt et al. 1999) and antiparasitic (Ribeiro et al. 2008) bioactivities; α-gurjunene is the major constituent of spikenard extract and the essential oil of agarwood, which showed sedative effects on rats (Takemoto et al. 2008); and α-gurjunene from the essential oils of *Calea clematidea* has been reported to possess antimicrobial bioactivity (Flach et al. 2002; Wang et al. 2005). The bioactivity of α-gurjunene, the product of *Tc-Gur*, reveals that *Tc-Gur* may have a role in defense in Taiwania.

Tissue-specific gene expression

To determine the tissue-specific expression of *Tc-Gur*, RNA was isolated from the scale-like leaves of a 70-year-old tree (old leaves), awl-like leaves of a 2-year-old tree (young leaves), male flowers, female flowers, phloem, xylem, cones, bark, root, and 22-day-old seedlings, and RT-PCR analysis was performed with actin as a reference. The expression of *Tc-Gur* was present in old leaves, young leaves, female flowers, and cones but absent in male flowers, phloem, xylem, bark, root, and 22-day-old seedlings (Figure 4). High levels of expression appeared in cones and young leaves. To investigate the quantitative expression level in each tissue, real-time PCR was performed. As

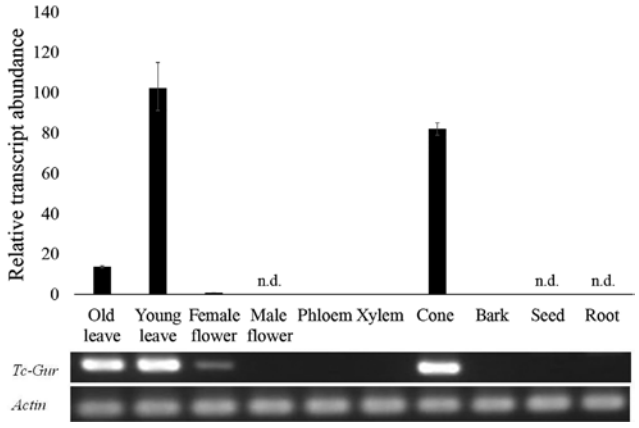

Figure 4 Bar chart of gene expression of *Tc-Gur* in specific tissues corresponding to real-time PCR analysis.

shown in Table S2, female flower tissue served as a calibrator for comparative analysis; the expression of *Tc-Gur* was approximately 102 times greater in young leaves, 82 times greater in cones, and 14 times greater in old leaves than the expression in female flowers (the expression in other tissues was too weak to be calculated).

The antifungal and allelopathic bioactivity of α-gurjunene was interpreted as that *Tc-Gur* may have a defense function in Taiwania. The tissue-specific gene expression of *Tc-Gur* supports this hypothesis. Young trees (young leaves) have less physical protection, in comparison to thick trunks, resin, and seed coats, which may account for the higher expression of *Tc-Gur* to supply chemical defense. The expression of *Tc-Gur* is increasing during the developmental period from female flowers to cones as seen on the weaker expression of female flowers in comparison with the expression in cones.

Effect of JA and SA treatment and physical wounding

To test whether *Tc-Gur* gene expression contributes to plant resistance against microbes or insects, herbivore attack was simulated by JA treatment and physical wounding, but these did not show any effect (data not presented). However, after SA treatment for 2 h, the expression of *Tc-Gur* was increased significantly, whereas no similar trend was observed in the control group (Figure 5). For quantitative investigation, real-time PCR performed with the sample without soaking served as a calibrator for comparative analysis. The expression of *Tc-Gur* soaked in SA for 2 h was 61 times greater than the expression of the untreated sample (Table S3). SA treatment affected the gene expression of *Tc-Gur*. This result differs from the majority of TPS of gymnosperms, where gene expression is increased by wounding and JA treatment simulated by herbivores and induced spawning. The sensitivity of SA of *Tc-Gur* suggests that it is likely

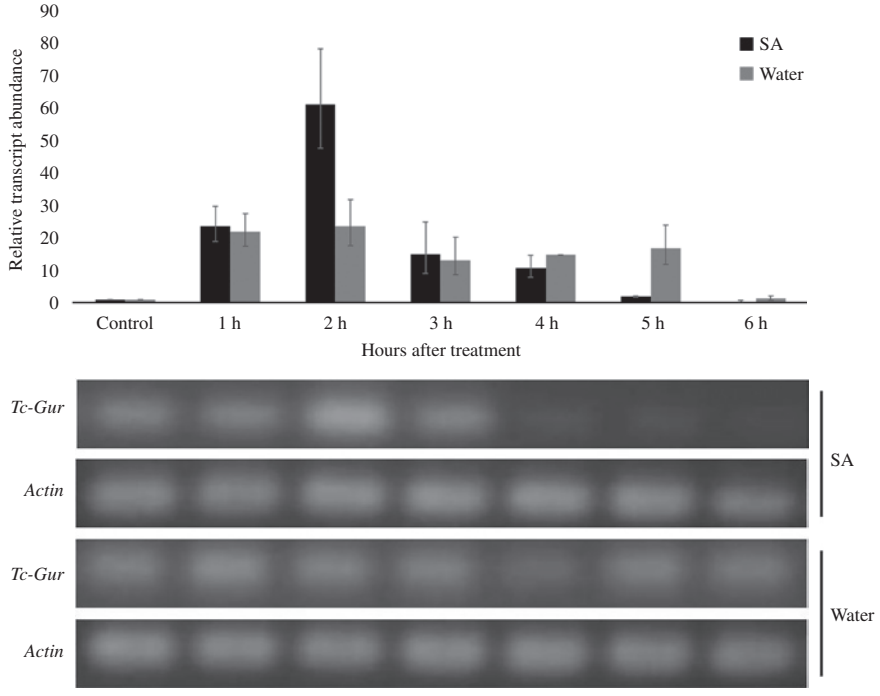

Figure 5 Effect of water and SA treatment on *Tc-Gur* expression by real-time PCR analysis corresponding to the RT-PCR analysis.

to be associated with the systemic acquired resistance system of plants.

Conclusions

The cloning and characterization of *sesqui-TPS* from Taiwania demonstrated its role as α-gurjunene synthase. The tissue-specific gene expression and treatment revealed that the protein might play a pivotal role in defense of this tree. The roles of *Tc-Gur* in the life cycle of Taiwania and in female flower development need a further in-depth investigation.

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