Cloning and functional characterization of a terpene synthase gene \textit{AlTPS1} from \textit{Atractylodes lancea}

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Abstract

\textit{Atractylodes lancea} (Thunb.) DC has been used widely as a medicinal herb for centuries and is now being used to treat COVID-19 pneumonia. Terpenoids are thought to be its main pharmacologically active constituents. However, their biosynthesis remains uncharacterized in this species. In this study, the terpene synthase gene \textit{AlTPS1} was cloned and functionally characterized. We found that \textit{AlTPS1} was a bifunctional enzyme that catalyzed the conversion of farnesyl diphosphate to nerolidol and geranyl diphosphate to linalool \textit{in vitro}. However, it functioned only in the nerolidol production \textit{in vivo} by transient expression of the \textit{AlTPS1} gene in \textit{Nicotiana benthamiana} leaves maybe due to subcellular compartmentalization of the \textit{AlTPS1} in the cytosol. Furthermore, \textit{AlTPS1} was highly expressed in leaves, considered to be the sites of nerolidol synthesis. This study is the first in which the cloning and expression of the \textit{AlTPS1} gene from \textit{A. lancea} were analyzed, and it has provided new insights into terpene biosynthesis in \textit{A. lancea}.

\textbf{Keywords}: \textit{Atractylodes lancea}, linalool, nerolidol, \textit{Nicotiana benthamiana}, subcellular compartmentalization, terpene synthase.

Introduction

Terpenoids form the largest class of plant secondary metabolites are very structurally diverse, with more than 50 000 natural products identified (Vattekkatte et al. 2018). They have essential functions in various basic plant processes (e.g., signaling molecules and phytohormones) and myriad roles in plant secondary metabolism, such as the repelling of herbivores, attraction of beneficial organisms, communication between plants, and mediation of complex interactions with the environment (Pichersky and Raguso 2018). Their extensive use in cosmetics, as flavorings, in pharmaceuticals, in the chemical industry, and as biofuel substitutes has made terpenoids indispensable (Pyne et al. 2019).

Although terpenoids have different chemical structures, they are biosynthesized from two interconvertible five-carbon compounds: isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). These compounds are generated separately by the methylerythritol phosphate (MEP) and mevalonic acid (MVA) pathways in plastids and the cytoplasm, respectively (Vattekkatte et al. 2018). IPP and DMAPP are then condensed head-to-tail by prenyltransferases to produce the terpene precursors
geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate, which are then converted into monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20), respectively, by a large family of enzymes known as terpene synthases (TPSs) (Chen et al. 2011). The TPS gene family is a medium-sized family, based on extensive studies in many plant species, including Arabidopsis thaliana (Aubourg et al. 2002), Oryza sativa (Chen et al. 2020), Vitis vinifera (Martin et al. 2010), and Solanum lycopersicum (Falara et al. 2011). According to their sequences and functional characteristics, TPS genes are classified into eight subfamilies (TPS-a-h): three angiosperm-specific subfamilies (TPS-a, TPS-b, and TPS-g), a gymnosperm-specific subfamily (TPS-d), a conserved land plant subfamily (TPS-c), and two conserved vascular plant subfamilies (TPS-e and TPS-f) (Chen et al. 2011).

Atractylodes lancea has long been used as an herbal medicine in many countries. The rhizomes of *A. lancea* are prescribed and used widely, mainly to treat digestive disorders, rheumatic diseases, body fluid imbalance, and night blindness (Koonningsomsomboon et al. 2014). According to the ancient Chinese medicine literature, the rhizomes of *A. lancea* were frequently prescribed to control plagues, currently, they are being used to treat COVID-19 pneumonia (National Health Commission of the People’s Republic of China 2020; http://www.nhc.gov.cn). The essential oil in the *A. lancea* rhizome, which consists primarily of sesquiterpenes such as β-eudesmol, hinesol, and atractylone, is the main pharmacologically active component (Jun et al. 2018).

Despite the isolation and identification of these chemical components, their biosynthesis remains elusive. No TPS gene in *A. lancea* has been functionally characterized. The aim of this study was functionally identified and characterized a TPS gene from *A. lancea* and it was named *AITPS1*. We also characterized this gene’s subcellular localization and expression.

**Materials and methods**

**Plants:** *Atractylodes lancea* (Thunb.) DC was collected from the Medicinal Botanical Garden of Hubei University of Chinese Medicine in September 2019; the original plants were from Yingshan County, Hubei Province, China (31°00′ N, 115°36′ E). Three-year-old plants were from Yingshan County, Hubei Province, of Chinese Medicine in September 2019; the original plants were from the Medicinal Botanical Garden of Hubei University.

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3000 (Branson W185 D, Farmingdale, NY, USA) in chilled extraction buffer (50 mM Tris, pH 8.0, with 20 mM imidazole, and 150 mM sodium chloride). Following centrifugation at 15 000 g for 30 min, the supernatant was collected and affinity-purified using Ni-nitrilotriacetic acid chromatography.

**Enzymatic reaction of AITPS1 in vitro:** In the enzyme assay, 0.2 cm$^3$ reaction mixtures containing 0.1 cm$^3$ in vitro Enzymatic reaction of AlTPS1:

The characterization of AITPS1 in vivo: The full-length ORF of AITPS1 was cloned into the pCambia1301 vector using BamHI and SalI restriction sites in the forward primer (CGCGGATCCGCGATGTTCACGACCAGCAACC) and reverse primers (CAACAGCTCTCGCCCTTGCTACCA TGTGCACGAACATGCTAATCGAAATCTCTC ACTAA), respectively. After sequence verification, it was transformed into Agrobacterium competent cells (EHA105) by electroporation. The Agrobacterium was grown in LB media containing 50 mg dm$^{-3}$ kanamycin and 25 mg dm$^{-3}$ rifampicin at 28 °C. When the culture had grown to $A_{600} = 0.3$, the cells were collected and suspended in MES buffer (10 mM MES, 200 μM acetosyringone, and 10 mM MgCl$_2$). Then, the suspension was injected into 4-week-old Nicotiana benthamiana leaves, cultured for 4 - 6 d. The leaves were harvested and analysed by headspace-gas chromatography.

**Subcellular localization:** The full-length coding sequences of AITPS1 were cloned into the pCambia1301-GFP vector to create C-terminal GFP-fused constructs. The constructs were transformed into the Agrobacterium EHA105 by electroporation as mentioned above. The fluorescence of leaves was observed 5 d after agroinfiltration with laser scanning confocal microscopy (Leica Microsystems CMS GmbH, Mannheim, Germany). Excitation wavelength of 488 nm, emission wavelength of 495-510 nm was used to detect GFP, and 650-750 nm was used for chloroplast autofluorescence.

**Expression analysis:** Total RNA was extracted from the stem, leaf, rhizome bud, and rhizome tissues of A. lancea and reverse transcribed using PrimeScript RT Master Mix (TaKaRa, Dalian). Real-time qPCR analyses were performed using the Quant One Step RT-PCR kit (SYBR Green, TianGen) with the iCycler IQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR amplification reactions were performed at 50 °C for 30 min and 95 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 20 s. The relative gene expression was quantified using the $2^{-△△Ct}$ method and normalized to the housekeeping GAPDH gene. Three biological replicates of each sample were performed.

**The terpenes detected of A. lancea leaves:** Leaves were collected and ground to a power with liquid nitrogen. About 5 g of powder was dissolved in 50 cm$^3$ hexane and shaken for 2 h at room temperature 26 °C, then ultrasonic treated for 30 min. The extract was concentrated in a rotary evaporator and detected by GC/MS. The gas chromatography-mass spectrometry (GC/MS) conditions were as follows: initial temperature of 50 °C held for 1 min and increased to 210 °C at 3 °C min$^{-1}$, then increased to 280 °C at 5 °C min$^{-1}$. The helium flow rate was 1.0 cm$^3$ min$^{-1}$.

**Results**

A full-length cDNA of putative terpene synthases was cloned by RACE assay based on previous transcriptome data and contained a 1 593-bp (Fig 1. Suppl.). The gene was named AITPS1 and submitted to GenBank (accession No. MT847607). The theoretical molecular mass of the predicted AITPS1 protein was 61.62 kDa, and its isoelectric point was 5.06. The predicted protein was hydrophilic. A blastp search of the deduced AITPS1 amino acids revealed 71.26, 69.92, 68.19, and 63.81 % similarity with genes of Lactuca sativa (XP_023748879.1), Helianthus annuus (XP_022017131.1), Ageratina adenophora (AYV65218.1), Cynara cardunculus var. Scolymus (XP_024944581.1), and Artemisia annua (PWA37709.1), respectively. A search of the NCBI conserved domain database showed that the protein had a substrate-Mg$^{2+}$ binding site and aspartate region that belonged to terpene cyclase plant C1 and the isoprenoid biosyn-C1 superfamily. Multiple sequence alignment confirmed that AITPS1 contained the highly conserved aspartate-rich motif (DDXXD) and NSE/DTE motif in the C-terminal domain, which are required for the binding of divalent cations, typically Mg$^{2+}$, to catalyze terpene biosynthesis (Fig. 1).

To understand the potential roles of AITPS proteins, a phylogenetic tree of AITPS and proteins from other plant species was constructed using the NJ method. In the tree, TPS proteins from various plants clustered into seven subfamilies. AITPS1 belonged to clade TPS-g, which is related most closely to (3S,6E)-nerolidol synthase 1 from Medicago truncatula (Fig. 2).

Secondary structural analysis showed that the alpha helix, extend strand, beta turn, and random coil accounted for 73.58, 3.40, 4.34%, and 18.68 %, respectively, of the predicted AITPS1 protein. The predicted tertiary structure of AITPS1 was shown in Fig. 3. The prediction of subcellular localization indicated that AITPS1 was located on the cytoplasm for the most likely case, with a probability of 45 %. The analysis of phosphorylation site confirmed that AITPS1 has 41 phosphorylation sites: 18 serine, 18 threonine, and 5 tyrosine sites.

To identify the function of AITPS1, the recombinant
AlTPS1 protein was expressed in E. coli for determining the enzymatic activity in vitro, however, inclusion bodies were the major expression pattern (Fig. 2A Suppl.). Three molecular chaperones that aid proper protein folding were co-expressed with AlTPS1 in E. coli (Kwon et al. 2019). Compared with that of the other two molecular chaperones, pGr07 co-expression markedly increased the solubility of the recombinant AlTPS1 protein (Fig. 2B,C Suppl.). The purified protein was obtained by Ni-chelating affinity chromatography (Fig. 4A). The functional activity of AlTPS1 was assayed using GPP and FPP as substrates. This analysis revealed that AlTPS1 converted FPP to nerolidol (Fig. 4B) and produced the monoterpane linalool when incubated with GPP (Fig. 4C).

Furthermore, we investigated whether AlTPS1 serve the same function in vivo using Agrobacterium-mediated transient gene expression in tobacco leaves. The N. benthamiana expressed AlTPS1 clearly produced nerolidol, but none of the monoterpe products was found (Fig. 4D).

Subcellular localization prediction indicated that AlTPS1 is most likely located on cytoplasm. To reveal the actual subcellular localization, green fluorescent protein (GFP) was used as a reporter gene, and transient GFP expression was observed by confocal laser scanning microscopy. As expected, the analysis confirmed AlTPS1 protein were distributed throughout the cytosol (Fig. 5).

Expression analysis of AlTPS1 in different plant organs (stem, leaf, rhizome bud, and rhizome) was performed to investigate its spatial regulation in A. lancea. AlTPS1 showed tissue-specific expression, with the highest expression observed in the leaf and lowest in the rhizome, an important officinal part of A. lancea (Fig. 6). Furthermore, we detected the terpenes of A. lancea leaves in order to confirm whether nerolidol could be present in the leaf. A total of 6 terpenes were discovered in the leaves, including a nerolidol (Table 1 Suppl.).

**Discussion**

Terpenoids in the essential oil of A. lancea are thought to be the major active components and quality determinants of this medicinal material. To understand terpene biosynthesis in A. lancea, terpene synthase, which is considered to be pivotal in the formation of the different terpene structural classes, was cloned and characterized functionally.

AlTPS1 gene isolated in this study from A. lancea encoded 530 amino acids which is in accordance with other studies reporting that TPSs generally contain 550-850 amino acids and have relative molecular masses of predicted protein 61,62 kDa (Herde et al. 2008). AlTPS1 shared considerable homology with (3S,6E)-nerolidol synthase 1-like from L. sativa and contained the conserved aspartate-rich DDxxD and NSE/DTE motifs in the C-terminus (Fig. 1), involved in the chelation of divalent...
The TPS family is divided into seven subfamilies designated TPS-a-g (Chen et al. 2011). The TPS-g subfamily has a highly conserved RRX8W motif in the N-terminus, which is thought to aid initiation of the isomerization cyclization reaction (Williams et al. 1998). Thus, this group is responsible for acyclic terpenoid production. Snapdragon (E)-ocimene synthase in clade TPS-g can use GPP to generate acyclic (E)-ocimene as its main product (Dudareva et al. 2003). FhTPS4 isolated from Freesia cultivars is also a member of the TPS-g family, it catalyzes the formation of acyclic monoterpenes and sesquiterpenes using FPP and GPP, respectively, as substrates (Gao et al. 2018). In the phylogenetic analysis, AlTPS1 belonged to the TPS-g subfamily (Fig. 2). To clarify the potential roles of the AlTPS1 protein, its soluble expression was improved by co-expression of the pGr07 molecular chaperone, which binds to and refolds...
misfolded proteins (Farajnia et al. 2020). As expected, incubation of the purified recombinant AITPS1 protein with FPP generated acyclic nerolidol, whereas AITPS1 produced linalool from GPP (Fig. 4B,C), indicating that it could be a bifunctional enzyme that plays roles in acyclic monoterpene and sesquiterpene biosynthesis in A. lancea.

However, linalool was not detected in AITPS1-infiltrated tobacco leaves in comparison to control leaves. This could be attributed to the cytosol subcellular localization of AITPS1 because the subcellular compartmentalization of biosynthesis C10 and C15 compounds from GPP and FPP differs (Fig. 5) (Hemmerlin et al. 2011, Nagegowda 2010). Similarly, AmNES/LIS-1 was localized in the cytosol and was responsible for nerolidol biosynthesis, whereas AmNES/LIS-2 was localized in the plastids, and accounts for linalool formation in snapdragon (Nagegowda et al. 2008). In addition, in young grape, VvRiLinNer converted FPP and GPP into nerolidol and linalool in vitro, but its plastid localization was clearly determined to VvRiLinNer functions in the linalool production in vivo (Zhu et al. 2014). Therefore, we inferred that the AITPS1 acquired in this study mainly participated in the synthesis of sesquiterpene nerolidol of A. lancea.

Monoterpenes and sesquiterpenes are active constituents in rhizomes (Gu et al. 2007). Here, we examined the expression of AITPS1 in different organs, and found that its expression was obviously higher in the leaf than in the other three tissues (Fig. 5) and the nerolidol

Fig. 4. The SDS-PAGE analysis of purified AITPS1 protein and function analysis of AITPS1. The purified AITPS1 protein (A). In vitro enzyme assays of recombinant AITPS1 using farnesyl diphosphate FPP (B) or geranyl diphosphate GPP (C) as the substrate. GC-MS analysis of in vivo products in N. benthamiana overexpressed AITPS1 (D).
could be emitted in the leaf (Table 1 Suppl.), implying that leaves are important for acyclic nerolidol synthesis. The transportation and regulatory networks controlling the biosynthesis of terpenoids deserve further study.

In summary, we successfully cloned the AlTPS1 gene from A. lancea. In vitro assays showed that AlTPS1 was found to be a bifunctional enzyme that could catalyse the synthesis both of nerolidol and linalool. The function was further confirmed that AlTPS1 was mainly responsible for nerolidol production in vivo. The subcellular localization experiment discovered that AlTPS1 was localized in the cytosol and was specifically expressed in leaves by expression analysis. These findings contribute to our understanding of the complex molecular mechanisms of terpene biosynthesis and provide the foundation for improvement of the quality of medicinal herbs through the use of genetic engineering in breeding.

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