biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2021) 65: 351-358 DOI: 10.32615/bp.2021.054

Cloning and functional characterization of a terpene synthase gene *AlTPS1* from *Atractylodes lancea*

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Abstract

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 *AlTPS1* was cloned and functionally characterized. We found that AlTPS1 was a bifunctional enzyme that catalyzed the conversion of farnesyl diphosphate to nerolidol and geranyl diphosphate to linalool *in vitro*. However, it functioned only in the nerolidol production *in vivo* by transient expression of the *AlTPS1* gene in *Nicotiana benthamiana* leaves maybe due to subcellular compartmentalization of the AlTPS1 in the cytosol. Furthermore, *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 *AlTPS1* gene from *A. lancea* were analyzed, and it has provided new insights into terpene biosynthesis in *A. lancea*.

Keywords: *Atractylodes lancea*, linalool, nerolidol, *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

Received 28 January 2021, *last revision* 7 August 2021, *accepted* 1 September 2021.
Abbreviations: DMAPP - dimethylallyl diphosphate; FPP - farnesyl diphosphate; GC-MS - gas chromatography-mass spectrometry; GPP - geranyl diphosphate; IPP - isopentenyl diphosphate; IPTG - β-D-1-thiogalactopyranoside; LB - Luria-Bertani; MEP - methylerythritol phosphate; MVA - mevalonic acid; ORF -open reading frame; RACE - rapid amplification of cDNA ends; TPS - terpenoid synthase.
Acknowledgements: This work was financially supported by the National Natural Science Foundation of China (32000254, 31670341), the Major Program of Technological Innovation of Hubei Province, China (2018ACA124), and the National Key Research and Development Program of China (2017YFC1700704).
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Conflict of interest: The authors declare that they have no conflict of interest.

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 (Koonrungsesomboon *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 *AlTPS1*. 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 randomly selected as the experimental materials. The stem, leaves, rhizomes, and rhizome buds were frozen in liquid nitrogen and stored at -80 °C.

RNA isolation and cDNA synthesis: Total RNA was extracted using the *RNAprep Pure Plant* kit (*Tiangen*, Beijing, China) according to the manufacturer's instructions. The RNA concentration, purity, and quantity were analyzed using a *NanoDrop 2000* (*Thermo Fisher Scientific*, Waltham, MA, USA) and *Agilent 2100* (*Agilent Technologies*, Santa Clara, CA, USA). The first cDNA strand was synthesized using the *PrimeScript™ II* 1st strand cDNA synthesis kit (*TaKaRa*, Dalian, China).

Full-length cloning of *AlTPS1* cDNA: A candidate gene was selected from the *A. lancea* transcriptome data and identified as terpene synthase based on a homology search. A partial *AlTPS1* fragment was amplified by PCR using the primers 5'-CAGTAGAAGATGTGAAGGGATT-3' and 5'-GCCAAACCCAACTCCTTC-3'. Rapid amplification of cDNA ends (RACE) assays was used to obtain the full-length *AlTPS1* cDNA using the *SMARTer® RACE 5'* kit (*TaKaRa*), following the manufacturer's instructions. Gene-specific primers (GSP1, GSP2, and GSP3) were designed based on the sequence fragment of *AlTPS1*. The RACE-ready cDNA was got using the primer GSP1 (5'-TGTATTTTTCACCATTAT-3'); after synthesizing the cDNA, the primers GSP2 (5'-CTTCATCAAGAAACGGCATC-3') and AAP were used to perform the first PCR. GSP3 (5'-GAGCGGCATCCTCAAGTATA-3') and AUAP were used for the second PCR. Finally, the intact open reading frame (ORF) of *AlTPS1* were amplified with primers *AlTPS1*-F (5'-CTTAGGAGCAATTGTCATG-3') and *AlTPS1*-R (5'-CTAGAACATGGAGCTTACA-3'). The PCR amplification conditions were as follows: 94 °C for 2 min; then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and an extension at 72 °C for 10 min.

Bioinformatics analysis of the *AlTPS1* gene: The ORF of the full-length *AlTPS1* sequence was discovered and the deduced amino-acid sequences of orthologs from other species were aligned and analyzed using *DNAMAN*. The physicochemical properties of the predicted protein were analyzed using *ExPASy* (https://web.expasy.org/protparam/) and *SOPMA* (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The protein signal peptides were analyzed using the *SignalP* method. Its subcellular localization was evaluated using *ProtComp 9.0* (web.expasy.org/protscale/) and the phosphorylation sites were determined on the *Net NGlyc 1.0* server. The secondary and tertiary structures of the protein were predicted using *SOPMA* (http://www.cbs.dtu.dk/services/SignalP/) and *SWISS-MODEL* (https://www.swissmodel.expasy.org/), respectively. Its phylogeny was analyzed using the neighbor-joining (NJ) method in *MEGA 6*.

Prokaryotic expression of *AlTPS1* in *Escherichia coli*: First, we optimized the codons based on the *AlTPS1* amino acid sequence and constructed the prokaryotic expression vector pET28a-*AlTPS1*. Three molecular chaperones (pGr07, pTf16, and pG-KJE8) were transformed into strain BL21(DE3) to improve the soluble expression level. To examine function, a single recombinant *Escherichia coli* colony was inoculated in Luria-Bertani (LB) medium containing kanamycin (50 mg dm^{-3}) and chloramphenicol (20 mg dm^{-3}) or tetracycline (10 mg dm^{-3}), and grown at temperature of 37 °C and shaking at 200 rpm to absorbance A_{600} = 0.5 - 0.6 before induction with β-D-1-thiogalactopyranoside (IPTG; 0.1 mM) and l-arabinose (0.5 - 4 g dm^{-3}) and changing of the temperature to 16 °C. The cells were harvested by centrifugation and homogenized by sonication using a *Misonix Sonicator*

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 AlTPS1 *in vitro*: In the enzyme assay, 0.2 cm^3 reaction mixtures containing 0.1 cm^3 purified protein, 10 mM substrate (FPP or GPP), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 25 mM HEPES buffer (pH 7.5) were overlaid with 0.3 cm^3 hexane and incubated at 30 °C for 1 h. The samples were mixed and centrifuged at 1000 *g* for 10 min. Boiling-inactivated protein was used as a control. The product was detected by gas chromatography-mass spectrometry using an injection port temperature of 250 °C and an initial temperature of 80 °C, which was increased to 180 °C at 5 °C min^{-1}, and then to 250 °C at 20 °C min^{-1}. The helium flow rate was 1.0 cm^3 min^{-1}.

The characterization of AlTPS1 *in vivo*: The full-length ORF of AlTPS1 was cloned into the pCambia1301 vector using BamHI and SalI restriction sites in the forward (CGCGGATCCGCGATGTTCACGACCAGCAACC) and reverse primers (ACAGCTCCTCGCCCTTGCTCACCA TGTCGACGAACATGCTACTAACGAAATCCTTC 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 *AlTPS1* were cloned into 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 $2^{-\Delta\Delta Ct}$ 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 1min 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

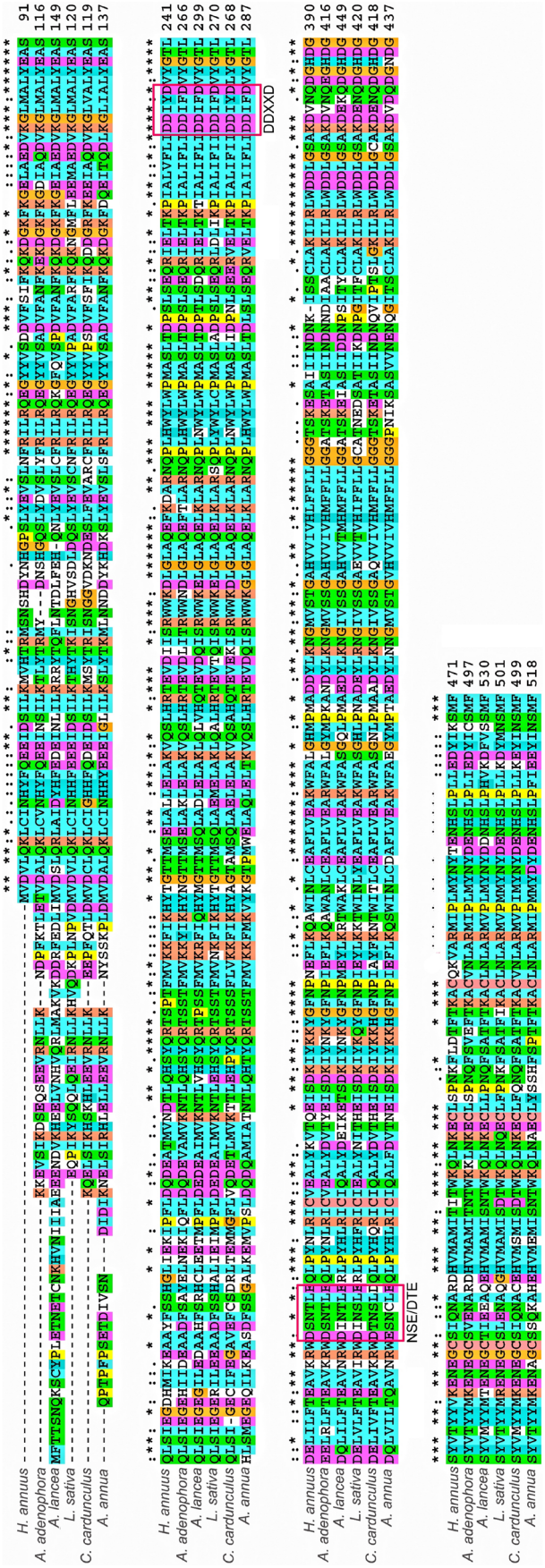
A full-length cDNA of putative terpene synthases was cloned by RACE assay based on previous transcriptome data and contained a 1 593-bp (Fig 1. Suppl.). The gene was named *AlTPS1* and submitted to GenBank (accession No. MT847607). The theoretical molecular mass of the predicted AlTPS1 protein was 61.62 kDa, and its isoelectric point was 5.06. The predicted protein was hydrophilic.

A blastp search of the deduced AlTPS1 amino acids revealed 71.26, 71.19, 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_024994581.1), 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 AlTPS1 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 AlTPS proteins, a phylogenetic tree of AlTPS and proteins from other plant species was constructed using the NJ method. In the tree, TPS proteins from various plants clustered into seven subfamilies. AlTPS1 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 AlTPS1 protein. The predicted tertiary structure of AlTPS1 was shown in Fig. 3. The prediction of subcellular localization indicated that AlTPS1 was located on the cytoplasm for the most likely case, with a probability of 45 %. The analysis of phosphorylation site confirmed that AlTPS1 has 41 phosphorylation sites: 18 serine, 18 threonine, and 5 tyrosine sites.

To identify the function of AlTPS1, 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. 2*A* 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. 2*B*,*C* Suppl.). The purified protein was obtained by Ni-chelating affinity chromatography (Fig. 4*A*). The functional activity of AlTPS1 was assayed using GPP and FPP as substrates. This analysis revealed that AlTPS1 converted FPP to nerolidol (Fig. 4*B*) and produced the monoterpene linalool when incubated with GPP (Fig. 4*C*).

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 monoterpene products was found (Fig. 4*D*).

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

Fig. 1. The multiple alignments of AlTPS1 and other plant TPS proteins. DDXXD motifs and NSE/DTE motif are shown in red boxes.

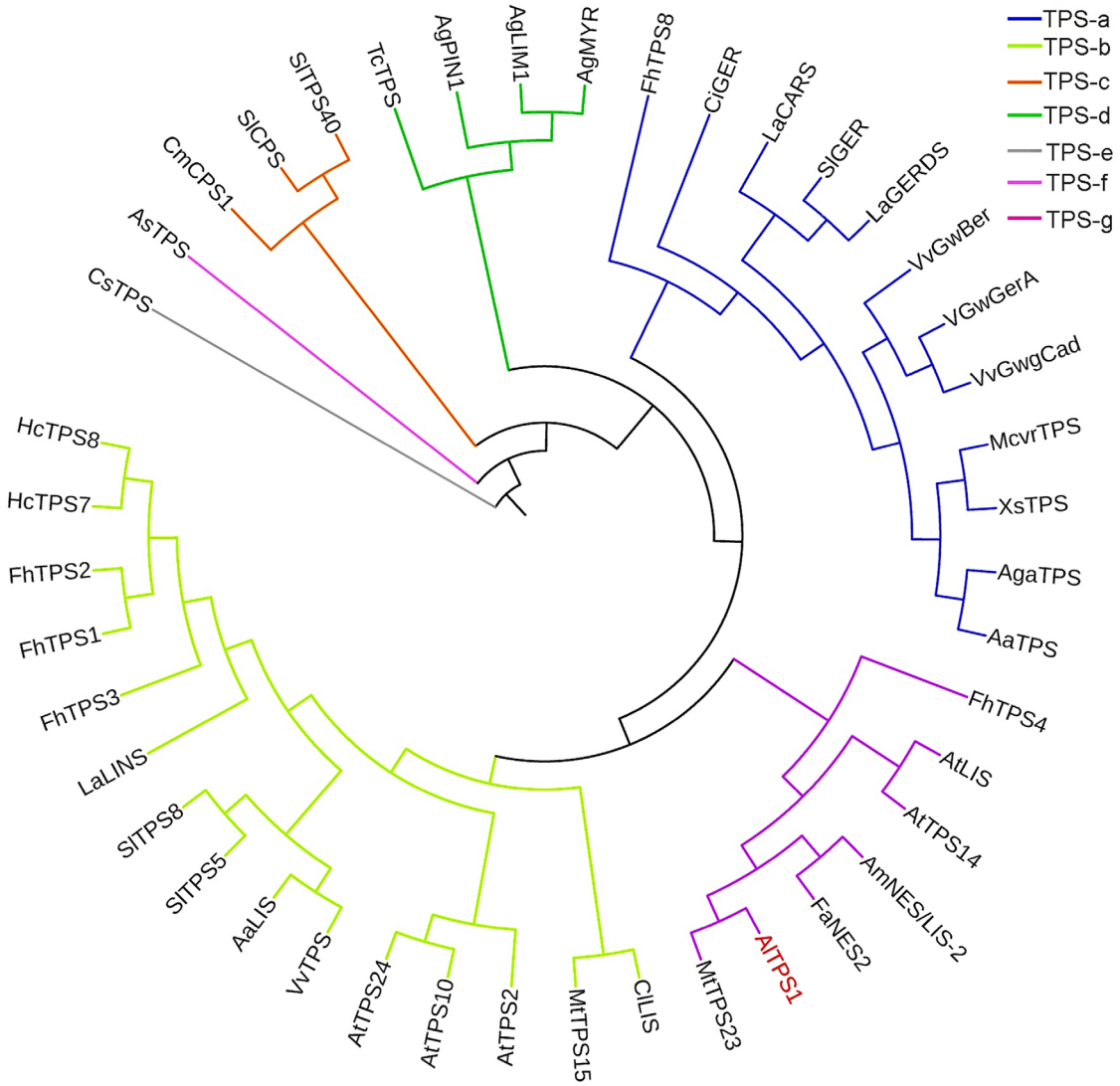


Fig. 2. The phylogenetic analysis of AlTPS and other plant TPS proteins. AlTPS proteins in this study are highlighted in red. The TPS-a, TPS-b, TPS-c, TPS-d, TPS-e, TPS-f, and TPS-g clades are highlighted with differently coloured lines. The names and accession numbers of TPS are provided in Table 2 Suppl.

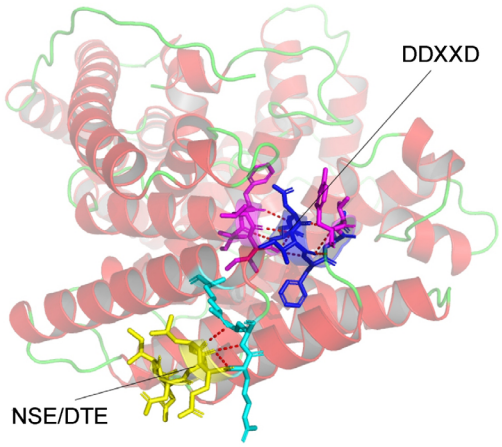


Fig. 3. The predicted tertiary structure of the AlTPS1 protein. DDXXD motifs are shown in *blue* and NSE/DTE motif is shown in *yellow*.

cations and stabilization of the active site (Marrero 1992).

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 AlTPS1 protein with FPP generated acyclic nerolidol, whereas AlTPS1 produced linalool from GPP (Fig. 4*B*,*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 AlTPS1-infiltrated tobacco leaves in comparison to control leaves. This could be attributed to the cytosol subcellular localization of AlTPS1 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 plastic localization was clearly determined to VvRILinNer functions in the linalool production *in vivo* (Zhu *et al*. 2014). Therefore, we inferred that the AlTPS1 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 *AlTPS1* 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

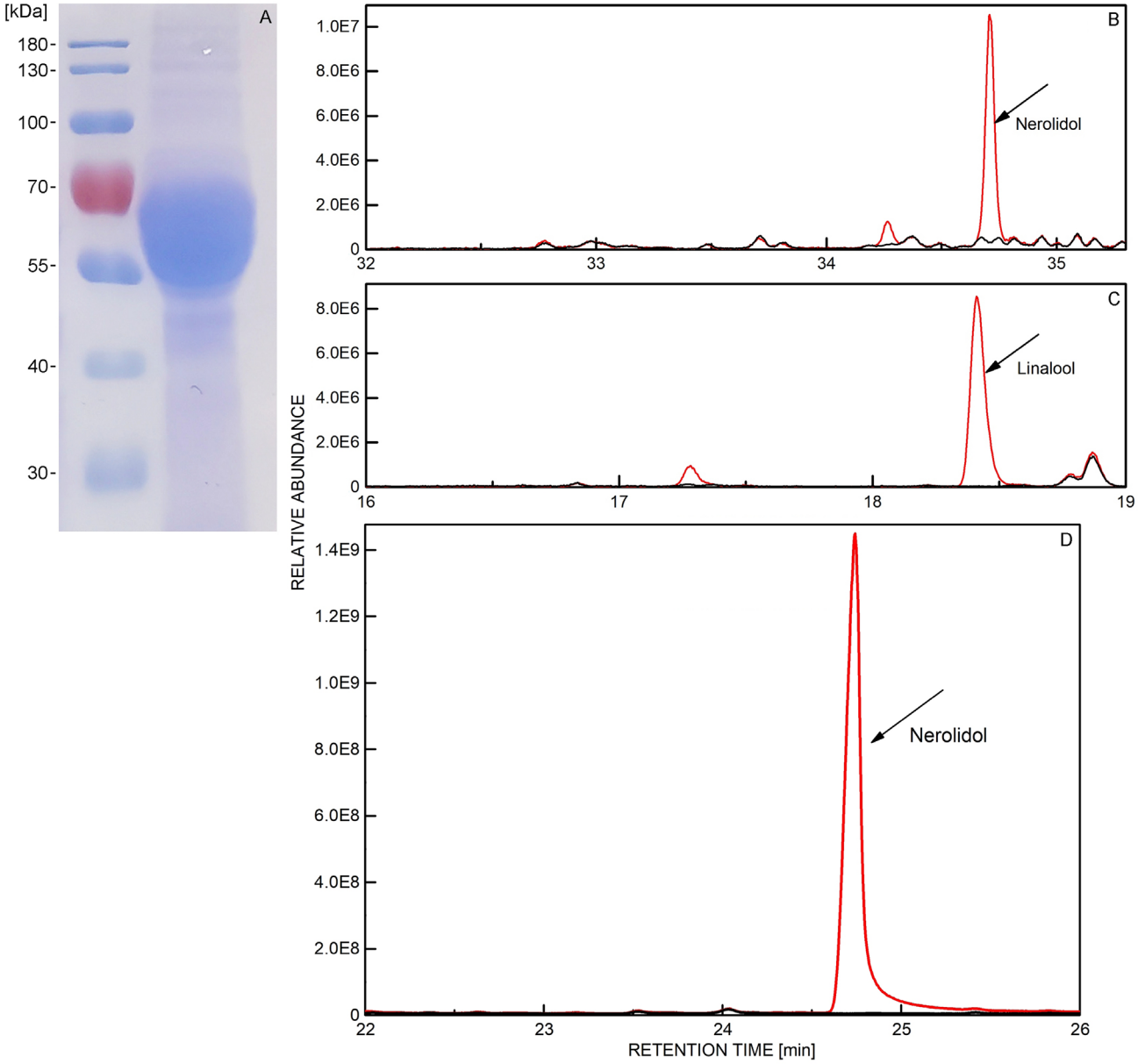


Fig. 4. The SDS-PAGE analysis of purified AlTPS1 protein and function analysis of AlTPS1. The purified AlTPS1 protein (*A*). *In vitro* enzyme assays of recombinant AlTPS1 using farnesyl diphosphate FPP (*B*) or geranyl diphosphate GPP (*C*) as the substrate. GC-MS analysis of *in vivo* products in *N. benthamiana* overexpressed *AlTPS1* (*D*).

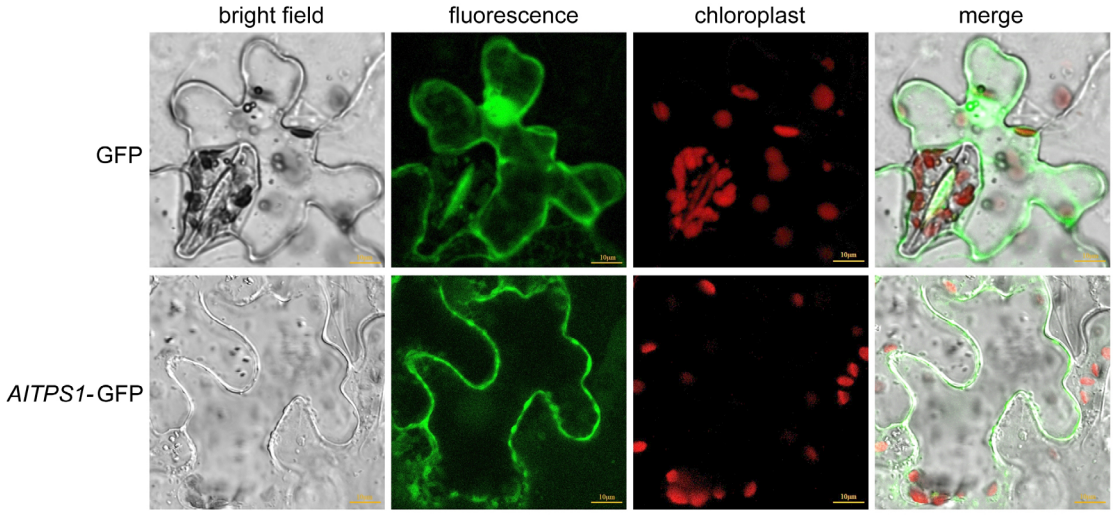


Fig. 5. The subcellular localization of AlTPS1 in *Nicotiana benthamiana*. Images are of bright-field fluorescence, GFP fluorescence (*green*), chlorophyll autofluorescence (*red*), and merged (*green* and *red*) fluorescence.

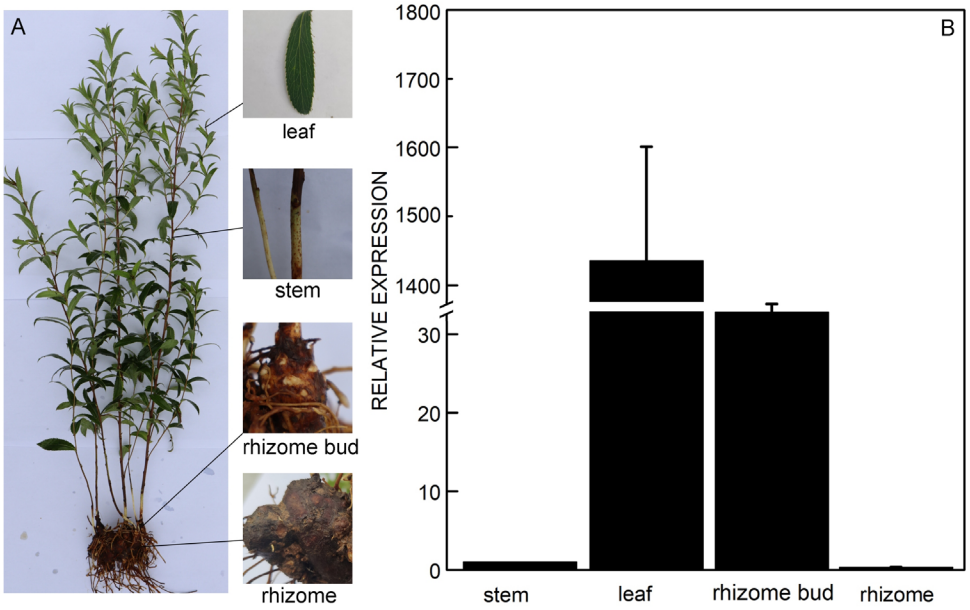


Fig. 6. The different tissues of *A. lancea* (*A*), and the relative *AlTPS1* expression in different *A. lancea* tissues (*B*).

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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