· Research Papers ·

Cloning and Expression Analysis on 3-Hydroxy-3-methyl-glutaryl-coenzyme A Reductase from *Aquilaria sinensis*

XU Yan-hong[1], YANG Xin[1], WEI Jian-he[1, 2*], ZHANG Xing-li[1], ZHANG Zheng[1, 2], GAO Zhi-hui[1], SUI Chun[1]

1. Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China
2. Hainan Provincial Key Laboratory of Resources Conservation and Development of Southern Medicine, Hainan Branch Institute of Medicinal Plant, Chinese Academy of Medical Sciences & Peking Union Medical College, Wanning 571533, China

Abstract: **Objective** To clone the full-length cDNA of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) from *Aquilaria sinensis* (AsHMGR1) and to analyze its expression profile in different tissues and in response to different treatments. HMGR is the first rate-limiting enzyme for sesquiterpene synthesis in the mevalonate pathway. **Methods** RT-PCR and RACE were used to clone the full-length cDNA of HMGR from *A. sinensis* based on the conserved HMGR gene fragments. The bioinformatic analysis was performed on its nucleic acid and protein sequence. The expression profile of AsHMGR1 in different tissues and in response to different treatments was analyzed by quantitative RT-PCR. **Results** The full-length AsHMGR1 cDNA was 2026 bp, containing a 1719 bp open reading frame which encoded a protein of 572 amino acids. Amino acid sequence homology alignment and phylogenetic analysis demonstrated that AsHMGR1 belonged to the HMGR gene family. The detection of tissue expression patterns showed that AsHMGR1 was mainly expressed in the stem, followed by roots and branches. AsHMGR1 could be stimulated by methyl jasmonate and H_2O_2 to varying degrees in a time-dependent manner. **Conclusion** These data will provide a foundation for further investigation on AsHMGR1 functions and regulatory mechanisms in sesquiterpene synthesis in *A. sinensis*.

Key words: agarwood; *Aquilaria sinensis*; expression; 3-hydroxy-3-methylglutaryl-coenzyme A reductase; molecular cloning
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Introduction

The plants of *Aquilaria* spp. belonging to the family Thymelaeaceae as typical evergreen trees mainly distribute throughout Southeast Asia. The resinous portion of their branches and trunks, known as agarwood, is widely used in traditional medicine as a digestive, sedative, and anti-emetic, and also popularly in incense and perfume. In the international market, high quality agarwood is more expersive than gold. It is noteworthy that only wounded trees could produce agarwood. Under natural conditions, wild mature *Aquilaria* trees randomly affected by wind breakage, thunder strikes, ant or insect attack, or microorganism invasion can produce agarwood from their heartwood. However, these biological processes develop very slowly over several years. To date, most agarwood comes from wild *Aquilaria* resources, and due to huge demand for agarwood, all species of this genus are regarded as endangered species in Appendix II of the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES, 2004). Despite its economic and pharmacological values, little is known about the molecular mechanisms of agarwood formation.

* Corresponding author: Wei JH Address: Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, No. 151, Malianwa North Road, Haidian District, Beijing 100193, China
E-mail: wjianh@263.net Fax: +86-10-5783 3016
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Studies have revealed that the major bioactive components of agarwood are sesquiterpenes and phenylethyl chromones (Hashimoto *et al*, 1985; Chen *et al*, 2011; 2012). By the end of June 2010, 132 components were isolated from agarwood. Among these, sesquiterpenes and 2-(2-phenylethyl) chromone derivatives account for 52% and 41%, respectively (Chen *et al*, 2012). Thus, elucidating the biosynthesis of sesquiterpenes is important for understanding the mechanisms of agarwood formation.

In higher plants, sesquiterpenes could be biosynthesized from the mevalonate (MVA) pathway (Gardner and Hampton, 1999) and/or 2-*C*-methyl-*D*-erythritol-4-phosphate (MEP/DXP) pathway (Rohmer, 1999). 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC: 1.1.1.34), which catalyses the conversion of HMG-CoA to MVA, is considered to be the first rate-limiting enzyme in the plant MVA pathway, and by increasing the activity or content of HMGR, the contents of terpenoids or their precursors in plant secondary metabolism could be improved. There have been many successful reports on using transgenic technology to transfer HMGR genes into target plants to improve the content of active ingredients (Aquil *et al*, 2009; Ram *et al*, 2010; Alam and Abdin, 2011).

HMGR-homologous genes have been cloned from many plants, such as *Arabidopsis thaliana* (L.) Heynh (Caelles, 1989), *Catharanthus roseus* (L.) G. Don (Maldonado-Mendoza, Vincent, and Nessler, 1992), *Camptotheca acuminate* Decne. (Maldonado-Mendoza, Vincent, and Nessler, 1997), *Morus alba* L. (Jain, Vincent, and Nessler, 2000), *Eucommia ulmoides* Oliv. (Jiang *et al*, 2006), and *Euphorbia pekinensis* Rupr. (Cao *et al*, 2010). To date, it is hard to find any information about genes encoding HMGR in *Aquilaria* Lam. species, although many sesquiterpenes have been isolated from the plants of *Aquilaria* spp. (Chen *et al*, 2012). Thus, it is necessary to identify HMGR genes from this economically and medically important genus. Here, we report the molecular cloning and characterization of the full-length cDNA of an HMGR gene from *A. sinensis*, and analyze its expression profile in different tissues and in response to treatments of methyl jasmonate (MJ) and H_2O_2. Our results lay the foundation for the further study on AsHMGR1 function and metabolic regulation of sesquiterpene synthesis.

Materials and methods

Plant materials

A. sinensis plants were grown in a greenhouse at Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College. The roots, young branches, stems, and leaves were collected from 3-year-old trees.

A. sinensis calluses originating from stems were used to investigate the effect of MJ and H_2O_2 on AsHMGR1 expression. The calluses were treated with 100 μmol/L MJ or H_2O_2 for 2, 4, 6, 8, 12, and 24 h, respectively before sampled for analysis.

All materials were frozen in liquid nitrogen and stored at −70 ℃ until use.

RNA extraction and cDNA synthesis

The total RNA was isolated from the stems of 3-year-old *A. sinensis* and calluses with the Total RNA Purification Kit (Aidlab, China) supplemented with on-column DNA digestion according to the manufacturer's instructions. RNA integrity was verified on 1% agarose gel. RNA quantity was determined using a NanoDrop 2000C Spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized according to SMART™ cDNA Library Construction Kit (Clontech, USA) protocols.

Cloning of a conserved AsHMGR1 gene fragment

To amplify the conserved fragment, we used the first-strand cDNA as a template with two degenerate primers designed from highly conserved HMGR regions. The nucleotide sequences of these primers were as follows: fHMGR (5′-ATI (T/C) TIGGICA (G/A) TG (T/C) TG (T/C) GAG-3′) and rHMGR (5′- IC (G/T) (G/A) TTGTA (C/T) TTCATGT G-3′). The PCR conditions were 30 cycles at 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 1 min, and final extension at 72 ℃ for 5 min. The PCR product was purified, cloned to the pMD18-T vector (Takara, Dalian), and sequenced.

RACE PCR and full-length cDNA cloning

5′-RACE PCR was performed using nested PCR with gene-specific primers derived from the cloned conserved sequence 5′ end (reverse primer 1, 5′-CGCT AGTGGCGCCTCCAGACAAG-3′, and reverse primer 2, 5′-TTGGAACTGAATACTCCCTCCCGTC-3′) and the SMART III oligonucleotide as forward primer. 3′-RACE PCR was conducted with gene-specific

primers derived from the cloned conserved sequence 3′ end (forward primer 1, 5′-GACTCAACTAGCATCTC-AATCGGCG-3′, and forward primer 2, 5′-GGCTGCT-TGCAACGATTGTGGC-3′) and CDSIII/3′ as reverse primer. A first-round PCR was carried out for 20 cycles at 94 ℃ for 30 s, 68 ℃ for 6 min, and 68 ℃ for 7 min. The PCR product was diluted by 100 times and used as the template for a second PCR. The second PCR was carried out for 30 cycles at 94 ℃ for 1 min, 60 ℃ for 30 s, 72 ℃ for 1 min, and final extension at 72 ℃ for 5 min. 5′ and 3′ RACE products were cloned into the pMD18-T vector and sequenced. Splicing the three fragments, and thereby designing a pair of specific primers (forward primer 5′-GTTCCCCGTGTGTCAC-GCAG-3′ and reverse primer 5′-TGGTATAAACAATT-CCGCACAAGG-3′), a full-length cDNA encoding the putative HMGR was obtained by PCR amplification. All clones were sequenced to ensure that the gene sequence was correct.

Bioinformatic analysis

Expasy Proteomics Server (http://www.expasy.org) was used to calculate physical and chemical parameters for the deduced amino acid sequence. Transmembrane domains were predicted by TMHMM 2.0 software. Coding sequence comparison was performed online (http://www.ncbi.nlm.nih.gov) and Clustal W was used for multiple alignment analysis of the full-length HMGR amino acid sequences. An HMGR phylogenetic tree was constructed with MEGA4.

Quantitative real-time PCR analysis

Total RNA was isolated, and a 2 μg aliquot was subjected to first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega, USA) and an oligo (dT18) primer. The suitability of the oligonucleotide sequences in terms of annealing efficiency was evaluated in advance using the Primer 5.0 program. Forward 5′-GGCCAGGACCTCCACATCTC-3′ and reverse 5′-TCCTTGTTCGACCTATTGTACTTCAT-3′ primers were used for AsHMGR1 amplification. A fragment of the AcHistone gene (Kumeta and Ito, 2010) was also amplified as an internal control, using primers forward 5′-GTACCGCTACCGGAGGGAAGTTGAA GA-3′ and reverse 5′-CTTCTTGGGCGACTTGGTA GCCTTGGT-3′. The qRT-PCR analysis was performed using a BioRad Real-Time System CFX96TM C1000 Thermal Cycler (Singapore). All experiments were repeated for three times along with three independent repetitions of the biological experiments.

Results

Molecular cloning of full-length AsHMGR1 cDNA

We obtained the cDNA sequence from *A. sinensis* by taking the advantage of the conserved domains of previously cloned HMGRs. Using newly-designed degenerate primers corresponding to two conversed domains found in most HMGRs (-ILGQCCE-, and -SHMKYNR-), a 1047 bp conserved fragment was obtained. We carried out 5′ and 3′ RACE and were able to deduce a full-length cDNA (named AsHMGR1) putatively encoding an HMGR, which was subsequently confirmed by sequencing. The full-length cDNA was 2026 bp, with a 135 bp 5′-UTR, a 352 bp 3'-UTR, a poly (A) tail of 29 bp, and a 1719 bp open reading frame (ORF) encoding a protein of 572 amino acids. The cDNA sequence has been deposited in GenBank under the accession number JQ990217.

Bioinformatic analysis of AsHMGR1

The predicted AsHMGR1 protein had a calculated molecular weight of 6.17×10^4 and a pI value of 6.24 (http://www.cn.expasy.org/tools/protparam.html).

TMHMM2.0 Analysis predicted that AsHMGR1 contains two transmembrane domains, one located between F_{39} and A_{61} and the other between M_{81} and I_{103} along the polypeptide chain (Fig. 1). Multiple sequence alignment demonstrated that AsHMGR1 had high amino acid sequence similarity to *Vitis vinifera* (78.34%), *Tilia miqueliana* (78.27%), *Corylus avellana* (76.70%), and *M. alba* (75.92%). This result suggests that AsHMGR1 belongs to the HMGR superfamily (Fig. 2). The *N*-terminal end of AsHMGR1 was significantly diverse in both length and composition, while the *C*-terminal catalytic domain was highly similar to HMGRs from other plant species. The putative amino acid sequence of AsHMGR1 contains two HMG-CoA-binding motifs ("EMPIGY-VQIP" and "TTEGCLIA") and two NADP(H)-binding motifs ("DAMGMNM" and "GTVGGGT"). These motifs are functionally important and highly conserved in all HMGR proteins. To elucidate the evolutionary relationship between *A. sinensis* and other plant species, a phylogenetic tree was constructed using the Clustal W program based on

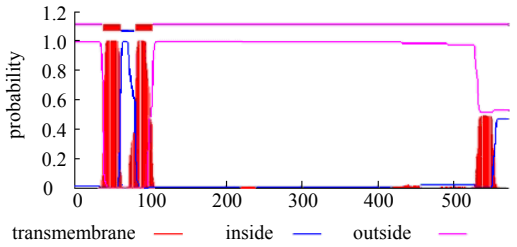

Fig. 1 Results of transmembrane helix prediction by TMHMM

Transmembrane helices are colored red; helices outside the membrane are colored pink; helices inside the membrane are colored blue

HMGR sequences obtained from GenBank. This revealed that AsHMGR1 had the highest homology with the HMGR (ABK88909) cloned from *Atractylodes lancea* (Fig. 3).

Expression profile of AsHMGR1 in different tissues of *A. sinensis*

Agarwood is a non-timber dark resinous wood that forms in the stems, branches, or roots of *Aquilaria* trees after wounding. Since HMGR is the first rate-limiting enzyme in the plant MVA pathway, it is of interest to

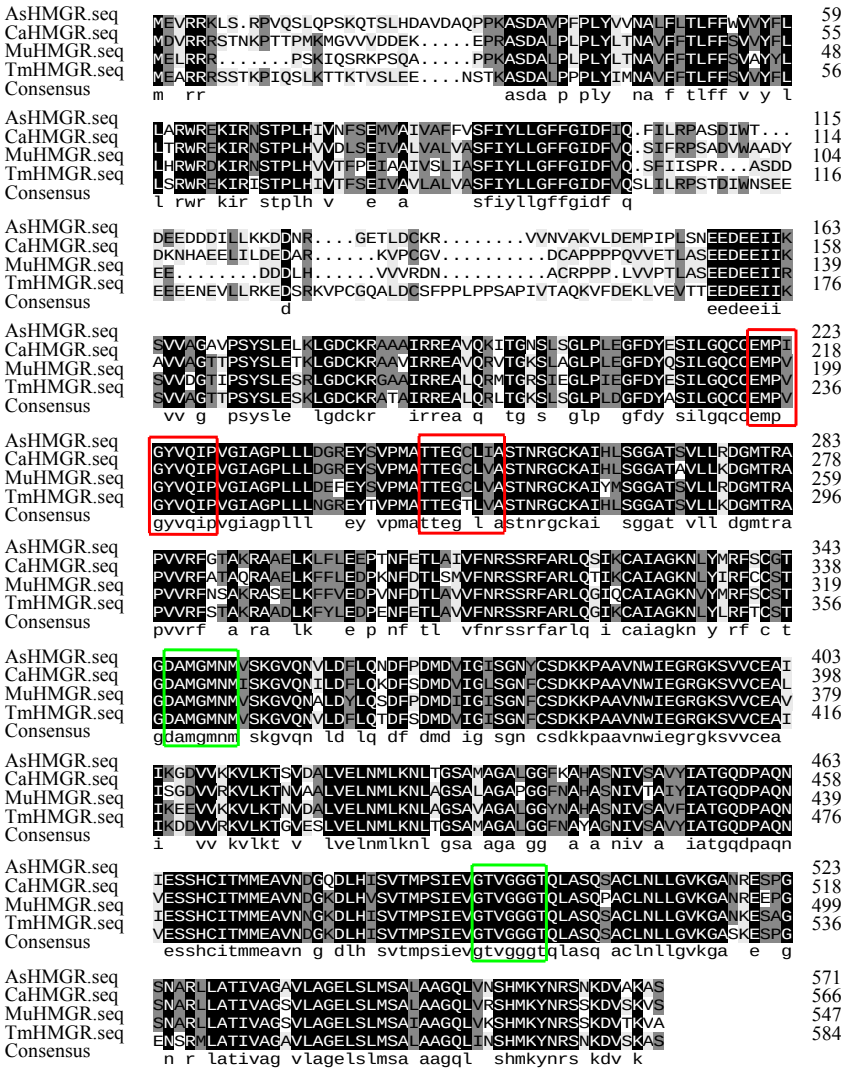

Fig. 2 Alignment of deduced amino acid sequences for AsHMGR1 and HMGRs from *Corylus avellana* (CaHMGR), *Morus alba* (MuHMGR), and *Tilia miqueliana* (TmHMGR)

Residues shaded in black are conserved among the four sequences. Two putative HMG-CoA-binding motifs ("DAMGMNM" and "GTVGGGT") are indicated with red boxes, and two NADP(H)-binding motifs ("EMPIGYVQIP" and "TTEGCLIA") are indicated with green boxes

determine whether AsHMGR1 expression is positively correlated with the active principle content in different tissues of *A. sinensis*. Total RNA is isolated from the roots, stems, young branches and leaves. RT-PCR analysis shows that AsHMGR1 expression level is the highest in the stem, followed by roots and branches, and the lowest in the leaves, which is the lowest in the leaves (Fig. 4).

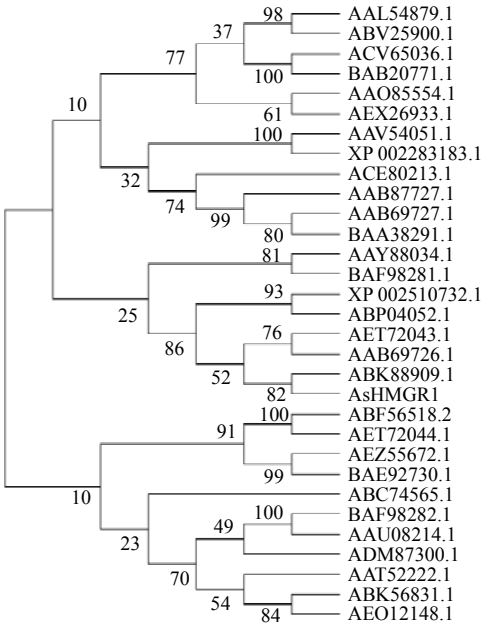

Fig. 3 Dendrogram of translated full-length AsHMGR1 sequence from *A. sinensis* and some characterized HMGR sequences from other plant species

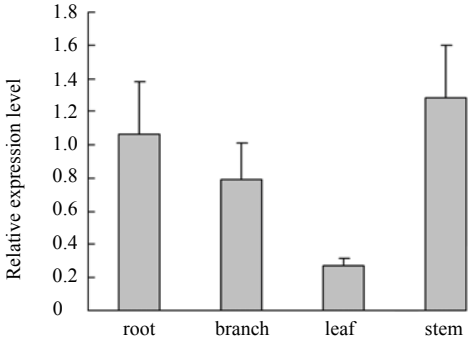

Fig. 4 Expression patterns of AsHMGR1 in different tissues of *A. sinensis* analyzed by RT-PCR

Induction of AsHMGR1 by MJ and H_2O_2

MJ and H_2O_2 are considered to be signal molecules in plant secondary metabolism (Reymond and Farmer, 1998; Leon, Rojo, and Sanchez-Serrano, 2001; Zhao, Davis, and Verpoorte, 2005), and MJ could induce the sesquiterpenoid production in *Aquilaria* Lam. (Ito *et al*, 2005; Okudera and Ito, 2009; Kumeta and Ito, 2010). Thus, we investigated the effects of MJ and H_2O_2 on AsHMGR1 gene expression at the mRNA level. We treated *A. sinensis* calluses with MJ and H_2O_2 and sampled them at pointed time after treated for 2, 4, 6, 8, 12, and 24 h. Total RNA was extracted and AsHMGR1 expression was quantified by RT-PCR. The results showed that AsHMGR1 could be stimulated by MJ and H_2O_2 to varying degrees with time-dependent effects (Fig. 5). Expression of AsHMGR1 was gradually induced and reached the maximum at 12 h when treated with MJ; while treated with H_2O_2, expression of AsHMGR1 was induced from 2 to 8 h, and reached the maximum at 6 h, then decreased immediately.

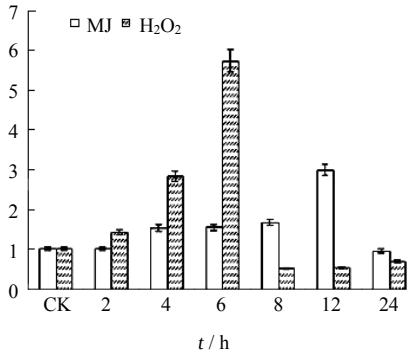

Fig. 5 Expression profiles of AsHMGR1 after treatment with 100 μmol/L MJ and H_2O_2

Discussion

Agarwood is a valuable resinous heartwood of *Aquilaria* plants widely used in traditional medicine, incense, and perfume. Sesquiterpene is one of its major bioactive components (Hashimoto *et al*, 1985; Chen *et al*, 2011; 2012). However, to date, the molecular basis of sesquiterpene biosynthesis and agarwood formation has rarely been studied, and most of the genes in the sesquiterpene biosynthesis pathway have not yet been cloned, except for five δ-guaiene synthase genes (Kumeta and Ito, 2010). HMGR is considered as the first key rate-limiting enzyme in the MVA pathway, and an important regulation point in the metabolism of sesquiterpene. It has been reported that the content of artemisinin in transgenic *Artemisia annua*, which was co-transcformed with HMGR and FPS, was eveated 17.4% (Lin, 2011). Over-expression SmHMGR and SmGGPP in *Salvia miltiorrhiza* could improve the synthesis of tanshinones synergistically. The contents of tanshinones in transgenic lines were all improved, and the highest one was elevated by 4.7 times relative to the non-transgenic lines (Xu, 2011). This achievement is a major breakthrough in *S. miltiorrhiza* metabolic engineering research in the international.

In this study, a full-length HMGR cDNA, containing a 1719 bp ORF, was successfully cloned from *A. sinensis* stems for the first time. Bioinformatic analysis showed that AsHMGR1 contained two HMG-CoA-binding motifs and two NADP(H)-binding

motifs, and shared high identity with HMGRs identified from other plants, demonstrating that AsHMGR1 belongs to the HMGR superfamily, and thus may share the function of other HMGRs. Unlike animals which have single-copy HMGR gene, plant HMGRs are encoded by multigene families of two or more members, depending on the species. Different members play the different biological roles in the regulation of plant metabolism (Venkatachalam *et al*, 2009). Here, only one AsHMGR1 was cloned from *A. sinensis*. Whether other HMGR members exist in this species needs to be determined.

Expression analysis shows that AsHMGR1 is expressed in all the tissues we examined including roots, stems, branches, and leaves, but the expression levels were significantly lower in leaves (five times lower than in stems), which is consistent with the location of sesquiterpene production in *A. sinensis*. Many studies have shown that HMGR expression is tissue-specific. For example, rubber HMGR1 is mainly expressed in latex (Venkatachalam *et al*, 2009); In *E. pekinensis*, EpHMGR is mainly expressed in roots (Cao *et al*, 2010); In *S. miltiorrhiza*, SmHMGR is mainly expressed in leaves (Dai *et al*, 2011). These different expression patterns suggest that this enzyme might have a specialized function in directing the sesquiterpene biosynthetic pathway in *A. sinensis*. However, the exact roles of AsHMGR1 in these tissues and its regulatory mechanisms need further investigation. Our exogenous MJ and H_2O_2 treatment experiment demonstrated that AsHMGR1 could be stimulated by 100 μmol/L MJ and H_2O_2, and the induction of H_2O_2 could be faster and stronger than MJ.

Although *Aquilaria* Lam. is the most important plant source of agarwood, and all *Aquilaria* Lam. species were listed in Appendix II of *CITES* in 2004, little progress has been made in understanding the mechanisms of agarwood formation. Identifying genes encoding the enzymes involved in sesquiterpene biosynthesis is important for understanding the mechanisms of agarwood formation, as sesquiterpene is one of its major bioactive components (Hashimoto *et al*, 1985; Chen *et al*, 2011; 2012). HMGR, the first enzyme in the MVP pathway of sesquiterpene metabolism, controls carbon flux into the sterol biosynthesis pathway that is closely associated with resistance, growth, and development. Our results will facilitate the further investigation of AsHMGR1 physiological functions and regulatory roles in sesquiterpene biosynthesis, and thus in agarwood formation.

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Introduction of Cover Photo

Salvia yunnanensis C. H. Wright is a perennial plant that is native to Yunnan, Guizhou, and Sichuan provinces in China, found growing on grassy hillsides, forest margins, and dry forests at 1800 to 2900 m (5900 to 9500 ft) elevation. *S. yunnanensis* has tuberous roots and grows on erect stems to 30 cm (12 in) tall, with simple oblong-elliptic leaves that are 2 to 8 cm (0.79 to 3.1 in) long and 1.5 to 3.5 cm (0.59 to 1.4 in) wide.

Inflorescences are widely spaced 4—6 flowered verticillasters in terminal racemes or panicles, with a 2.5 to 3.0 cm (0.98 to 1.2 in) blue-purple corolla.

S. yunnanensis is a habitually used medicinal plant in Yunnan. It distributed widely over the southwest China, and its contents of tanshinone II_A and protocatechuic aldehyde are higher than those of *S. miltiorrhiza*. Therefore, *S. yunnanensis* is one of the best resources of the medicinal plant.

Provided by LI Min-hui