



# Identification of sesquiterpene synthase genes in the genome of *Aquilaria sinensis* and characterization of an $\alpha$ -humulene synthase

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Received: 23 May 2022 / Accepted: 6 July 2022 / Published online: 28 September 2022  
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**Abstract** Sesquiterpenes are the major pharmacodynamic components of agarwood, a precious traditional Chinese medicine obtained from the resinous portions of *Aquilaria sinensis* trees that form in response to environmental stressors. To characterize the sesquiterpene synthases responsible for sesquiterpene production in *A. sinensis*, a bioinformatics analysis of the genome of *A. sinensis* identified six new terpene synthase genes, and 16 sesquiterpene synthase genes were identified as type TPS-a in a phylogenetic analysis. The expression patterns for eight of the sesquiterpene synthase genes after treatment with various hormones or hydrogen

peroxide were analyzed by real-time quantitative PCR. The results suggest that 100  $\mu$ M methyl jasmonate, ethephon, ( $\pm$ )-abscisic acid or hydrogen peroxide could be effective short-term effectors to increase the expression of sesquiterpene synthase genes, while 1 mM methyl salicylate may have long-term effects on increasing the expression of specific sesquiterpene synthase genes (e.g., *As-SesTPS*, *AsVS*, *AsTPS12* and *AsTPS29*). The expression changes in these genes under various conditions reflected their specific roles during abiotic or biotic stresses. Heterologous expression of a novel *A. sinensis* sesquiterpene synthase gene, *AsTPS2*, in *Escherichia coli* produced a major humulene product, so *AsTPS2* is renamed *AsHS1*. *AsHS1* is different from *ASS1*, *AsSesTPS*, and *AsVS*, for mainly producing  $\alpha$ -humulene. Based on the predicted space conformation of the *AsHS1* model, the small ligand molecule may bind to the free amino acid by hydrogen bonding for the catalytic function of the enzyme, while the substrate farnesyl diphosphate (FPP) probably binds to the free amino acid on one side of the RxR motif. Arg450, Asp453, Asp454, Thr457, and Glu461 from the NSE/DTE motif and D307 and D311 from the DDxxD motif were found to form a polar interaction with two  $Mg^{2+}$  clusters by docking. The  $Mg^{2+}$ -bound DDxxD and NSE/DTE motifs and the free RXR motif are jointly directed into the catalytic pocket of *AsHS1*. Comparison of the tertiary structural models of *AsHS1* with *ASS1* showed that they differed in structures in several positions, such as surrounding the secondary catalytic pocket, which may lead to differences in catalytic products. Based on the results, biosynthetic pathways for specific sesquiterpenes such as  $\alpha$ -humulene in *A. sinensis* are proposed. This study provides novel insights into the functions of the sesquiterpene synthases of *A. sinensis* and enriches knowledge on agarwood formation.

Project funding: The work was supported by the National Natural Science Foundation of China (81773844).

The online version is available at <http://www.springerlink.com>

Corresponding editor: Tao Xu.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11676-022-01542-w>.

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**Keywords** Agarwood · *Aquilaria sinensis* · Sesquiterpene synthase · Humulene

## Introduction

Agarwood is a high-grade raw material for the production of essential oils and natural perfumes and has also long been used as a natural digestive, sedative, and anti-emetic medicine. Agarwood is produced from a few species of *Aquilaria* and *Gyrinops* (family Thymelaeaceae) from Southeast Asia in response to an environmental stressor such as disease and physical wounding or damage to the trees from lightning strikes, grazing, and pest infestations (Rasool and Mohamed 2016), which causes the heartwood to become resinous (Kumeta and Ito 2016). In China, these species are mostly distributed in tropical and subtropical regions (Yin et al. 2016).

*Aquilaria sinensis* (Lour.) Spreng is the only certified source of agarwood listed in the China Pharmacopoeia 2020 (China Pharmacopoeia 2020). It is an endangered species and regulated under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Cites 2004). Because agarwood formation can take more than 10 years, in the last decade, various methods such as wounding, burning-chisel-drilling, chemical induction, and biological inoculation have been developed to induce agarwood production (Liu et al. 2013; Wu et al. 2017; Tan et al. 2019; Yan et al. 2019). However, the quality and quantity of these agarwoods are inconsistent and need improvement.

The main fragrant compounds in agarwood are sesquiterpenes and phenylethyl chromone derivatives, but the sesquiterpenes are more abundant and provide aromatic qualities suitable for perfumes (Ogita et al. 2015; Kristanti et al. 2018). Agarwood contains at least 210 sesquiterpenes, which vary in the type of skeletons, including eudesmanes, eremophilanes, guaianes, agarospiranes, acoranes, cadinanes, prezizaanes, zizaanes and humulanes (Chen et al. 2012; Gao et al. 2019; Li et al. 2021b). Terpene synthases (TPSs) are the key enzymes for the biosynthesis of terpenes, including sesquiterpenes. The TPS family is a mid-size family that is generally divided into seven clades, with the angiosperm sesquiterpene synthases (STSs) mainly included in the TPS-a clade (Chen et al. 2011). To date, TPS genes have been identified in many economically significant plant species such as *Magnolia grandiflora* (Lee and Chappell 2008), *Gossypium hirsutum* (Yang et al. 2013), *Camellia sinensis* (Zhou et al. 2020), and *Chrysanthemum indicum* (Zhou et al. 2021). Recently, Nong et al. (2020) revealed the *A. sinensis* TPS gene loci based on genomic sequencing. Li et al. (2021a) also identified the TPS gene family in *A. sinensis* based on the genome reported by Ding et al. (2020). In addition, Das et al. (2021) analyzed the genome

of *A. agallochum* and classified the TPS genes. Some STSs from *Aquilaria* species have also been identified and characterized using transgenic expression and chemical analyses. Different classes of guaiane synthases are also present in *Aquilaria* species, producing  $\delta$ -guaiane as the major product and  $\alpha$ -guaiane as a minor product with other minor products such as germacrene A (e.g., GS-1 in *A. microcarpa*, Lee et al. 2014),  $\alpha$ -humulene (e.g., AcC2- AcC4 in *A. crassna*, Kumeta and Ito 2010),  $\beta$ -elemene (e.g., ASS1–ASS3 in *A. sinensis*, Xu et al. 2013; GS-2 in *A. macrocarpa*, Kurosaki et al. 2015) or both  $\beta$ -elemene and  $\alpha$ -humulene (e.g., GS-3, GS-4 from *A. microcarpa*, Kurosaki et al. 2015). Other STSs in *Aquilaria* trees include  $\alpha$ -humulene synthases AcHS1-3 from *A. crassna* (Kumeta and Ito 2016), a nerolidol synthase As-SesTPS from *A. sinensis* (Ye et al. 2018), a vetispiradiene synthase AsVS from *A. sinensis* (Ding et al. 2019), and a sesquiterpene synthase AsSS4 from *A. sinensis*, which yields  $\beta$ -elemene,  $\alpha$ -guaiane,  $\alpha$ -caryophyllene,  $\delta$ -guaiane and cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl) (Liang et al. 2014). Thus, the STSs in *Aquilaria* trees are very complex and diverse. Here, based on a genome-wide analysis of *AsTPS* genes in *A. sinensis*, 16 sesquiterpene synthase genes belonging to type TPS-a were identified. The expression patterns of sesquiterpene synthase genes from *A. sinensis* were analyzed by real-time quantitative PCR. Sesquiterpene synthase gene *AsHS1* was amplified from the transition part of the white wood and brown agarwood of a tree of *A. sinensis*, and heterologous expression of this gene in *E. coli* produced a humulene synthase that catalyzes the production of  $\alpha$ -humulene from FPP. The three-dimensional structures of this enzyme were predicted and compared with other STSs. Based on the results, biosynthetic pathways for several sesquiterpenes in *A. sinensis*-cultured cells are postulated. The research provides new insights into the catalytic mechanism of sesquiterpene synthase in *A. sinensis* and will help to improve agarwood induction techniques.

## Materials and methods

### Plant materials and hormone treatments

*A. sinensis* suspension cells and branches from a 5-year-old *A. sinensis* tree were used. Calli were generated from fresh, young stem tips of a shoot as described by Liu et al. (2015). Viable, loose calli were used to prepare suspension cells by shaking in liquid Murashige and Skoog (MS) medium containing  $\alpha$ -naphthalene acetic acid (NAA) ( $2.0 \text{ mg L}^{-1}$ ), 6-benzylaminopurine (6-BA) ( $1.0 \text{ mg L}^{-1}$ ) and casein hydrolysate ( $0.5 \text{ g L}^{-1}$ ) at 120 rpm and  $22 \text{ }^\circ\text{C}$ . After viable cell suspensions were produced, methyl salicylate (MeSA), methyl jasmonate (MeJA), ethephon (ETH), ( $\pm$ )-abscisic

acid (ABA), and hydrogen peroxide ( $H_2O_2$ ) were added to a final concentration of 100  $\mu$ M. Then, control and short-term treatment samples were cultured in darkness for 1 and 2 h, respectively. To analyze the effects of longer treatments of hormones and oxidative stress, MeJA, ETh, ABA, and  $H_2O_2$  were added to a cell suspension of *A. sinensis* to a final concentration of 1 mM, and control and treatment samples were cultured for 24 or 48 h. The calli samples were stored and used for quantitative real-time PCR to quantify sesquiterpene synthase gene expression under various hormone and  $H_2O_2$  treatments. For isolating the sesquiterpene synthase gene, transition areas between the white wood and brown wood (agarwood) of branches were cut from a 5-year-old tree in Hainan, which was identified as *Aquilaria sinensis* (Lour.) Spreng by Prof. Zheng Zhang and used as material for gene amplification.

### Prediction and structural analysis of *A. sinensis* TPS genes

The *A. sinensis* genome and annotation data released by Ding et al. (2020) were downloaded from GigaDB (<http://gigadb.org/dataset/100702>). The Hidden Markov Model (HMM) files of the C-terminal domain of TPSs (Pfam ID: PF03936) and the N-terminal domain of TPSs (Pfam ID: PF01397) were downloaded from the Pfam database (<http://pfam.xfam.org/>) (El-Gebali et al. 2019). Potential *A. sinensis* terpene synthase genes were found using HMMER3.1b to search for the predicted proteins from the *A. sinensis* genome released by Ding et al. (2020) using the PF01397 and PF03936 model data as queries (Bateman et al. 2004). Significant hits (E-values <  $10^{10}$ ) were identified as possible *AsTPS* genes and used for further analysis. The gene annotation files were analyzed to reveal the structural organization of these genes, and the results were visualized using TB-tools (Chen et al. 2020). The sequences of *ASSI-3*, *As-SesTPS*, and *AsVS* genes could not be located in the *A. sinensis* genome provided by Ding et al. (2020), but were located in the draft map of the genome released by Nong et al. (2020). The genomic structures of the three genes were identified using the Splign online tool (Kapustin et al. 2008).

### Sequence alignment and phylogenetic tree construction

MUSCLE was used to align the amino acid sequences of the plant TPSs. The alignment results, with marked conserved regions and sites, were illustrated in BioEdit software. A phylogenetic tree was constructed using the maximum likelihood method and the Jones-Taylor-Thornton (JTT) model with 1000 bootstrapped replications in the IQ tree software (Minh et al. 2020a, b).

### Sequence character analysis of *AsTPS* genes

To identify the sequence characters, we acquired the theoretical pI and predicted molecular weight (MW) using the tool at the ExPASy portal ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)). The locations of signal peptides of the *AsTPS*s were predicted by the online servers TargetP 2.0 and ChloroP 1.1 (Emanuelsson et al. 2007, 1999).

### Quantitative reverse transcription PCR

Total RNAs were extracted from the precipitates of *A. sinensis* calli using EASYspin Plus Plant RNA Kits (Aidlab, China). RNA integration was analyzed using a 1% agarose gel, and RNA was quantified using a NanoDrop2000c spectrophotometer (Thermo Scientific, USA). Reverse transcription was carried out using M-MLV Reverse Transcriptase following the manufacturer's instructions (Promega, USA). Real-time PCR (qPCR) was performed using TransStart Top Green qPCR Supermix (Transgene, China) and a Roche LightCycler96 Real-Time System. The relative expression for each candidate gene was calculated using the  $2^{-\Delta\Delta CT}$  method. Three independent technical replicates were used for each experiment, and the *AsTUA* gene was selected as the internal reference (Gao et al. 2012b). The qPCR primers for analyzing the expression of terpene synthase genes in *A. sinensis* calli were designed using the online primer design tool Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3manager.cgi>; Table S1).

### Heterologous expression of an *AsSTS* gene in *Escherichia coli*

A prokaryotic expression plasmid was constructed using the EasyGeno Assembly Cloning kit (Tiangen, China). The coding sequence of *AsHS1* was amplified by reverse transcription PCR with primers of *AsHS1*-F: 5'-ATGTCAGCTGCTCAGGTCTCAC-3' and *AsHS1*-R: 5'-TCATATAGTAATTGGATGGACCAGCAATGAAG-3'. The pET-28a (+) plasmid (Novagen, Madison, WI, USA) was linearized using restriction enzymes BamHI and HindIII, and recombined with the coding sequence of *AsHS1*. The constructed plasmid was transformed into *E. coli* BL21 (DE3) pLys S cells and His-tag fused protein was produced after the induction of 0.2 mM isopropyl thio-galactoside (IPTG) for 4 h at 18 °C and 180 rpm. Fusion protein was purified using His-Tagged Protein Purification Kits (CoWin Biosciences, Beijing, China). The presence of the expected protein in the bacterial lysates and purification product were examined by SDS-PAGE.

## Determination of enzyme activity

The reaction took place in a 2 mL vial with a solid-top polypropylene cap. The reaction buffer contained 25 mM Tris–HCl buffer (pH 7.0), 10% glycerol, 5 mM dithiothreitol (DTT), 100 mM MgSO<sub>4</sub>, and 46 μM farnesyl pyrophosphate (FPP). To initiate the reaction, 100 μL of crude proteins (0.2 mg mL<sup>-1</sup>) was pipetted into 100 μL of reaction buffer. After incubation of the mixture at 30 °C for 1 h, SPME fibers were inserted into the headspace of the vial and pre-saturated for 30 min. Then, the volatiles were collected after 30 min and transferred to the injection port of the Varian 450 GC Gas Chromatograph–Varian 300 mass spectrometer (GC–MS) (Varian, Palo Alto, CA, USA) equipped with a VF-5 MS quartz capillary column (30 m×0.25 mm i.d.; film thickness 0.25 μm) and. The carrier gas was helium, and the column flow rate was 1 mL min<sup>-1</sup>. The inlet temperature was 250 °C in splitless mode. The ionization mode was EI with an ionization voltage of 70 eV. The ion source temperature was 250 °C, and the scanning mass value range was 30–500 amu. The starting temperature was 50 °C and was increased by 10 °C min<sup>-1</sup> to 280 °C, then held for 10 min. The types of sesquiterpenes were identified based on retention time, and the results of mass spectra versus those of authentic standards.

## Molecular modeling

Three-dimensional protein structural models were predicted using the SWISS-MODEL server (<https://swissmodel.expasy.org>) (Waterhouse et al. 2018). The crystal structure of the 5-*epi*-aristolochene synthase M4 mutant from *Nicotiana tabacum* (SMTL ID: 5ikh.1) (Koo et al. 2016) was used as a template to model AsHS1, AcHS1 and ASS1, which had 97% coverage, 0.79 Global Model Quality Estimate (GMQE) and 44.32% identity to AsHS1, and even higher homology with AcHS1 and ASS1. The amino acid residues in the protein structure were classified according to their secondary structure using the standardized algorithm Define Secondary Structure of Proteins (DSSP). The possible presence of active catalytic pockets of proteins was predicted by POCASA 1.1 (<https://g6altair.sci.hokudai.ac.jp/g6/service/pocasa/>). The possibility of docking between the substrate ligand small molecule FPP and the protein in the pocket structure was predicted by AutoDock 4.2.6 software (Rizvi et al. 2013). Structural figures were drawn using PyMol (<http://www.pymol.org/>).

## Results

### Identification of sesquiterpene genes and structural analysis

A total of 46 gene models encoding TPSs of *A. sinensis* were acquired through search against the protein data set released by Ding et al. (2020) using HMMER3.1b software and with the HMM files of terpene synthase domains (PF01397 and PF03936) as queries (Bateman et al. 2004; Ding et al. 2020). Based on previous reports, TPSs mostly comprise 350–860 amino acids (Bohlmann et al. 1998; Durairaj et al. 2019). So, we chose 22 AsTPS members that comprise 350–860 amino acids as candidates for further analysis. The structural organization of the 22 AsTPS genes was analyzed, of which 17 AsTPS genes (*AsTPS1n–AsTPS17n*) are shown in Fig. S1. The five sequences that were not located included *AsVS* (MH378283), *As-SesTPS* (KF135950) and *ASS1–ASS3* (JQ712682/3/4). *ASS1–ASS3* had highly similar sequences and were putatively located on chromosome 5 (26,125,981–26,122,559) but interrupted by a GT-rich sequence (Fig. S2). The difficulties in locating the coding regions of these genes may be due to imperfections in the genome sequence. The five AsTPS genes were used in a search against another *A. sinensis* genome released by Nong et al. using the Blastn program and were finally located in the corresponding scaffolds (Figs. S3–S5) (Nong et al. 2020). Through alignment of *ASS1–SS3* with the genomic sequence, the *ASS1–ASS3* genes may have originated at the same gene locus but differed in some nucleotides. The causes of the nucleotide differences could be sequencing errors or post-transcriptional editing. Li et al. (2021a) identified 26 AsTPS genes in the *A. sinensis* genome and, considering their AsTPS gene family members, we acquired a total of 32 putative AsTPS genes, of which six were new. The six new AsTPS genes discovered in the present study were designated the names *AsTPS27–32* and submitted to GenBank with accession numbers *AsTPS27* (OM966902), *AsTPS28* (OM933589), *AsTPS29* (OM933590), *AsTPS30* (OM933591), *AsTPS31* (OM933592) and *AsTPS32* (OM933593; Table S2). The names *AsTPS1*, *AsTPS15* and *AsTPS16* were designated for *ASS1–ASS3*, *As-SesTPS* and *AsVS*, respectively.

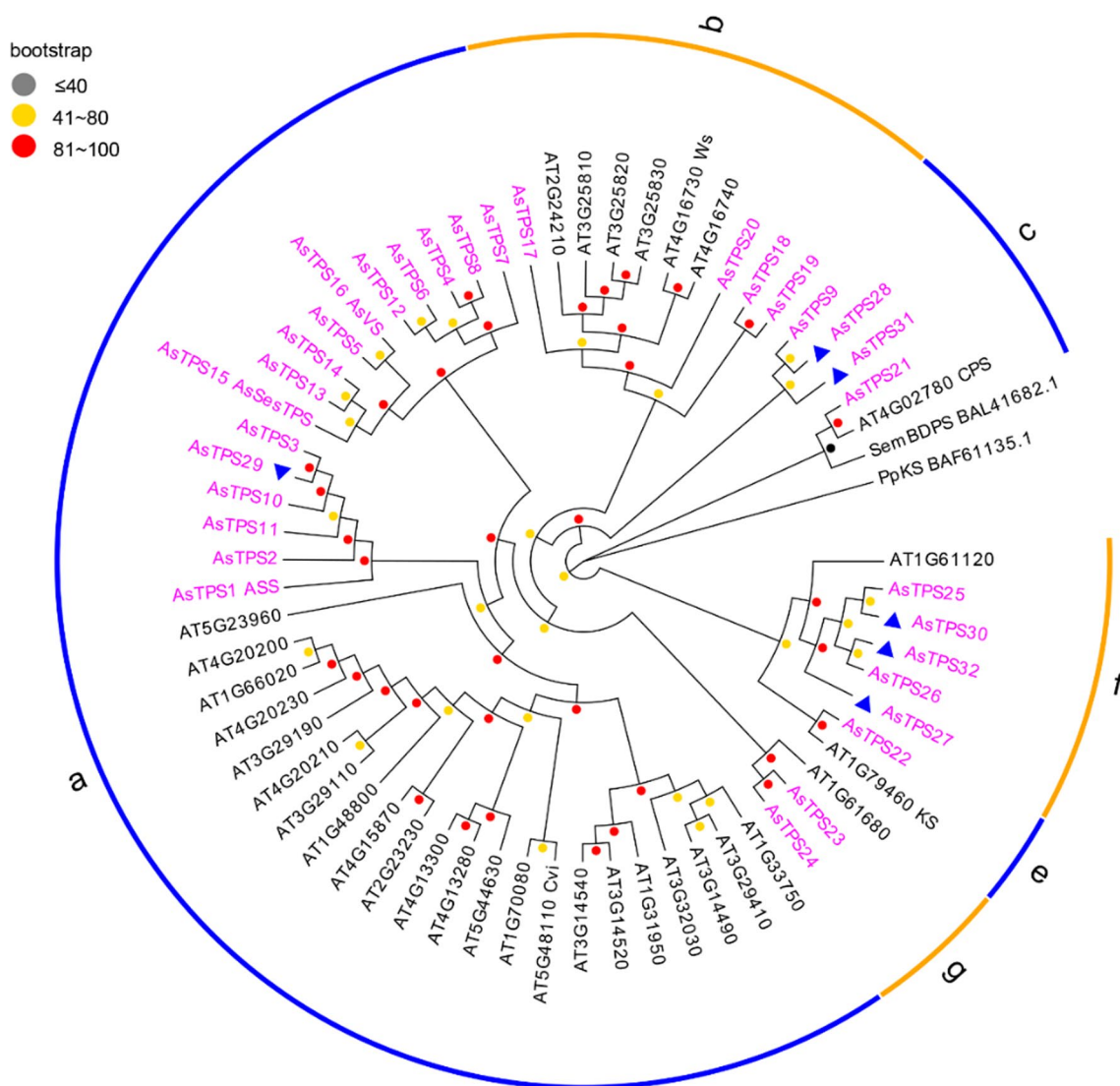
### Phylogenetic analyses of TPSs from *A. sinensis* and other species

Based on previous reports, plant TPSs can be divided into seven groups (Bohlmann et al. 1998; Chen et al. 2011). The TPS-a and -b groups comprise angiosperm sesquiterpenes and monoterpene synthases, and the TPS-c group comprises mostly diterpene synthases involved in primary metabolism, such as copalyl diphosphate synthase. The TPS-d group



comprises gymnosperm TPSs. The TPS-g group comprises mostly acyclic TPSs, such as those in grapevine (Martin et al. 2010; Chen et al. 2011). The TPS-e group comprises kaurene synthase and is always combined with TPS-f, which is probably derived from TPS-e and includes some monoterpenes, diterpenes and STSs, including linalool synthase in *Clarkia breweri* and *Phalaenopsis bellina* (Dudareva et al. 1996; Huang et al. 2021), geranylinalool synthase in *A. thaliana* and *Solanum lycopersicum* (Herde et al. 2008; Falara et al. 2014), and farnesene synthase in kiwifruit (Nieuwenhuizen et al. 2009). By phylogenetic relationship analysis of the 32 TPSs in *A. sinensis*, some 16 TPSs were clustered in group TPS-a, 4 each in TPS-b and TPS-c, 1 in TPS-e, 5 in TPS-f, and 2 in TPS-g (Fig. 1). In the phylogenetic tree,

the 16 AsTPS proteins clustered with the *Arabidopsis* STSs, constituting the largest TPS-a group. The 16 AsTPS proteins are regarded as putative AsSTSs. Recently, Li et al. (2021a) constructed a phylogenetic tree of *A. sinensis* TPSs and also classified 16 proteins in the TPS-a group of AsSTSs. In comparison, our tree placed AsTPS9 in group TPS-c but not in TPS-a. These differences may be due to differences in the tree construction methods because Li et al. used the NJ method and we used the ML method. Additionally, newly discovered AsTPS29 was placed in group TPS-a and is regarded as a new sesquiterpene synthase. Based on previous reports, AsSTSs may also be present in other groups, such as TPS-f and TPS-g. According to sequence alignment, except for AsTPS7, all the remaining 15 AsTPS in the TPS-a



**Fig. 1** Phylogenetic relationships of TPSs from *Aquilaria sinensis* and *Arabidopsis thaliana*. Fuchsia: AsTPS1–AsTPS32, black: *A. thaliana*, blue triangles: the six newly discovered TPSs (AsTPS27–AsTPS32). SemBDPS BAL41682.1, a mitradiene synthase from

*Selaginella moellendorffii* (GenBank BAL41682.1), and PpKS BAF61136.1, an ent-kaurene synthase from *Physcomitrium patens* (GenBank BAF61136.1) were used as outgroups

group had several motifs that are conserved in plant STSs such as RR<sub>x</sub>W, RxR, DDxxD and NSE/DTE (Fig. 2). The AsTPS7 sequence is considered likely a truncated sesquiterpene synthase for it lacks of the N-terminal RR<sub>x</sub>W motif and the C-terminal NSE/DTE motif. Several conserved motifs are important for the catalytic activity of STSs (Gao et al. 2012a). The diphosphate moiety of FPP has been proposed to be captured by the RXR motif and divalent metal ions such as Mg<sup>2+</sup> or Mn<sup>2+</sup>, which are also bound by the motifs DDxxD and NSE/DTE (RxxDDxx(S,T,G)xxxE) at the entrance of the active site (Starks et al. 1997). Arginine-rich RR<sub>x</sub>W motifs were identified in most of the TPS-a and TPS-b proteins but were absent from the other TPS groups in *A. sinensis* (Fig. S6). This result is consistent with a previous study on *Glycine max*, which also identified RR<sub>x</sub>W motifs in groups TPS-a and TPS-b (Liu et al. 2014). However, the function(s) of the “RR<sub>x</sub>W” motif remain unclear.

### Real-time PCR analysis of hormone-induced expression of sesquiterpene genes in *A. sinensis*

In the qPCR expression analysis of the eight sesquiterpene synthase genes in *A. sinensis* suspension cells after short (1 or 2 h) or long (24 or 48 h) treatments with the hormone- and H<sub>2</sub>O<sub>2</sub>-induced sesquiterpene synthesis, 100 μM MeJA (for 1 h and 2 h) upregulated most of the sesquiterpene synthase genes. The expression of *AsTPS2*, *AsTPS3* and *AsTPS10* increased more than 50-fold after a 1-h treatment (Fig. 3). In a previous RNA-seq analysis, the expression of eight *AsTPS* genes encoding STSs (*AsTPS1*, 2, 3, 5, 11, 13, 15, 23) were induced after stems were wounded (Li et al. 2021a). Based on these results, we suggest that *AsTPS2* and *AsTPS3* are major sesquiterpene synthase genes involved in the JA-mediated and wound-induced signaling pathways. After 24 h and 48 h treatment with a higher concentration of MeJA (1 mM), the *AsTPS* genes showed various expression trends. Compared with the shorter treatments with 100 μM hormone or H<sub>2</sub>O<sub>2</sub>, longer treatment with a higher concentration (1 mM) of hormone or H<sub>2</sub>O<sub>2</sub> did not have a stronger effect on increasing the expression levels of some *AsTPS* genes. For example, the expression of *AsTPS10* increased more than 50-fold after the 1-h treatment with 100 μM MeJA, but increased less than fivefold after the 24-h treatment with 1 mM MeJA. After the 2-h treatment with 100 μM H<sub>2</sub>O<sub>2</sub>, the expression of *AsTPS2* and *AsTPS29* increased about 50-fold and that of *AsTPS10* increased about 150-fold, but after the 24- and 48-h treatments with 1 mM H<sub>2</sub>O<sub>2</sub>, the expression of *AsTPS2*, *AsTPS29* and *AsTPS10* did not increase as much. After the short ETh treatments, expression of *AsTPS2*, -3, and -29 was more than 100-fold greater, less than after the longer treatments. After the short ABA treatments, the expression of *AsTPS1* (*ASS1-3*) and *AsTPS2* increased nearly 100-fold, but the expression of *AsTPS1* (*ASS1-3*) and *AsTPS2* did

not increase very much after the long treatments. After the longer treatments with 1 mM MeJA, ABA, ETh or H<sub>2</sub>O<sub>2</sub>, the effect of the signalling molecule on the expression of some sesquiterpene genes was likely unchanged or weakened. However, after the longer treatment with 1 mM of MeSA, the expression of *As-SesTPS*, *AsVS*, *AsTPS3* and *AsTPS12* was significantly higher than after the shorter treatments. The results suggest that 1 mM MeSA may have a longer effect on induce the expression of some sesquiterpene genes in *A. sinensis*.

### Determination of the activity of sesquiterpene synthase AsHS1

Since we failed to clone novel sesquiterpene synthase genes in the *A. sinensis* cell suspension, the transition area between white wood and brown agarwood was cut from the branches of a 5-year-old *A. sinensis* tree from Hainan and used as another material for gene amplification. A 1,671-bp cDNA sequence encoding *AsTPS2* was isolated. The coding sequence of *AsTPS2* was cloned into vector pET-28a (+) and transferred to *E. coli* BL21(DE3)plysS cells. His-tag recombinant protein was purified and incubated with FPP in assay buffer according to the method of Kumeta and Ito (2010). Products of the reaction were then analyzed by GC-MS. The recombinant enzyme generated a major sesquiterpene product, identified as  $\alpha$ -humulene by comparison of its retention times and mass spectra with authentic standards (Fig. 4). Since the *AsTPS2* produced a major amount of  $\alpha$ -humulene, it was also named AsHS1.

### Sequence comparison and structural analysis of sesquiterpene synthases

Generally, terpenoid cyclases fall into two main classes depending on the chemical strategy involved in the initial carbocation formation (Wendt and Schulz 1998). The sesquiterpene synthases belong to Class I-type terpenoid cyclases and utilize a trinuclear metal cluster to trigger the ionization of an isoprenoid diphosphate substrate to yield an allylic cation and inorganic pyrophosphate. To investigate the structural properties of AsHS1 that affect carbocation formation and product cyclization, homology modelling was used via the SWISS-MODEL webserver (Waterhouse et al. 2018). Using the structure of the tobacco 5-*epi*-aristolochene synthase M4 mutant as the template, the predicted structure model of AsHS1 had a high level of confidence. Through calculation and classification, the secondary structure of AsHS1 was found to contain 29  $\alpha$ -helices and two  $\beta$ -folds. The 22 free amino acids at the N-terminal were not shown due to their unpredictable conformation within the template. Based on the predicted space conformation of the AsHS1 model, the small ligand molecule may bind to the free amino

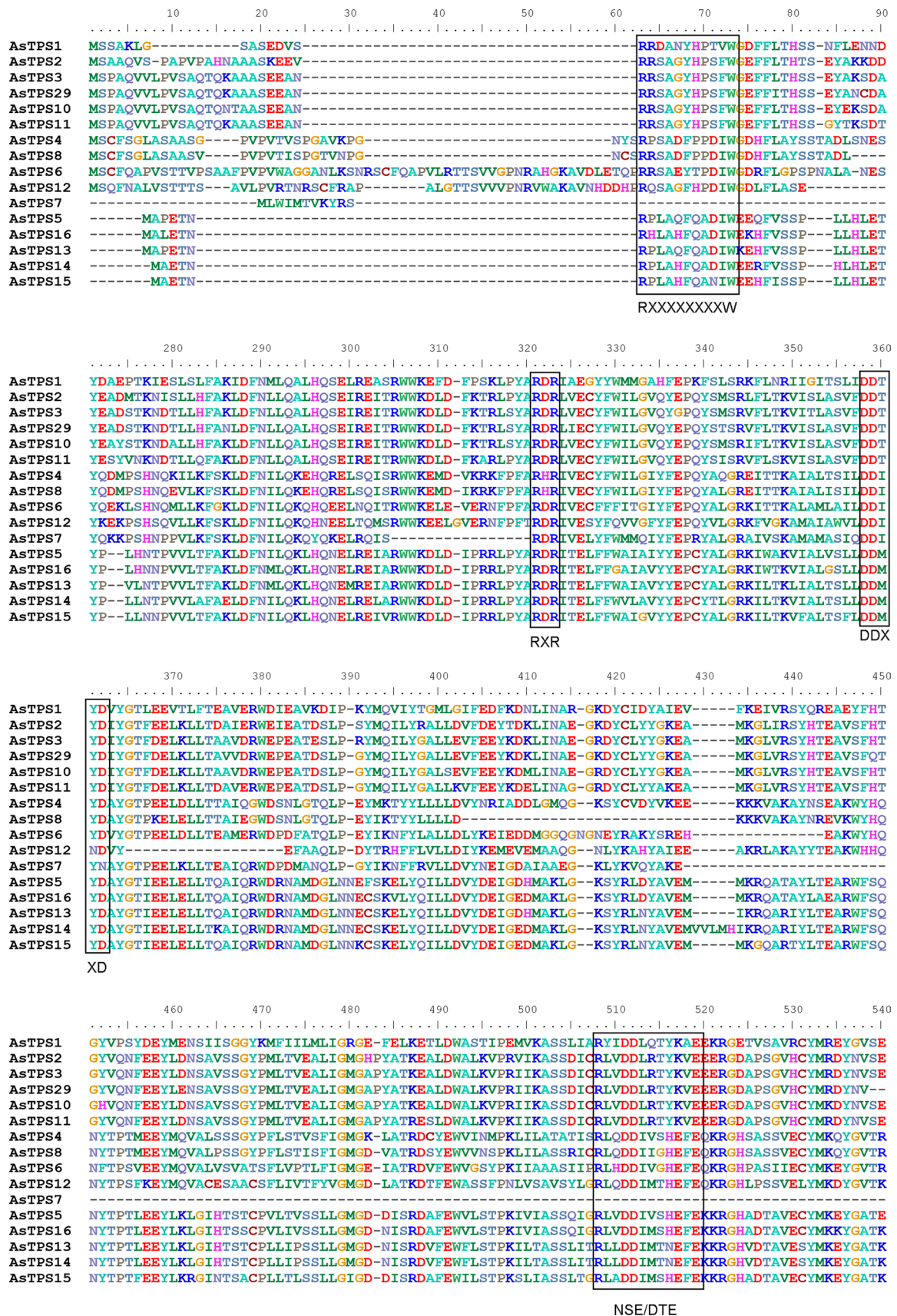
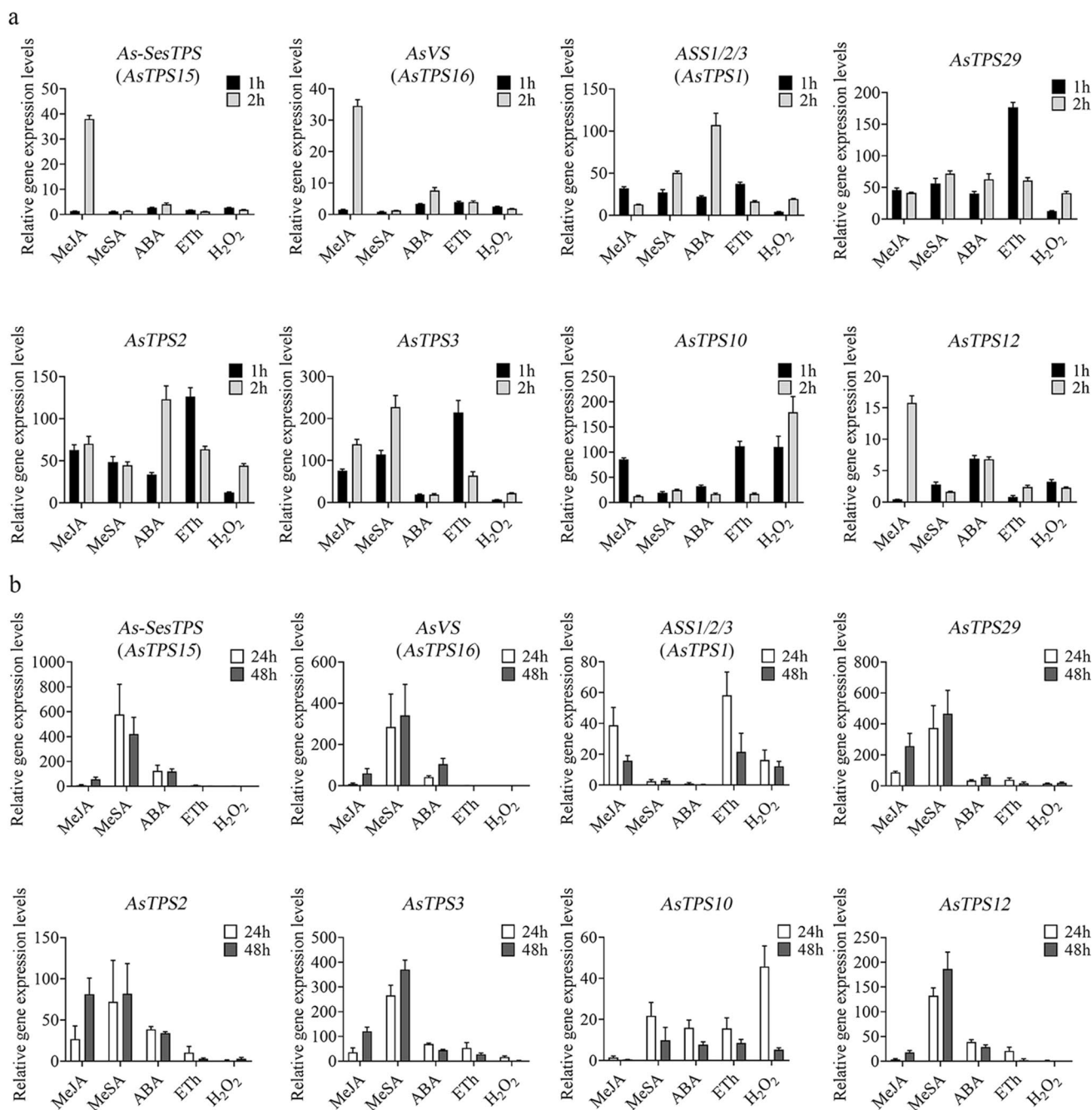


Fig. 2 Sequence alignment for the TPS-a group of proteins in *Aquilaria sinensis*. Some sequences without conserved motifs are omitted





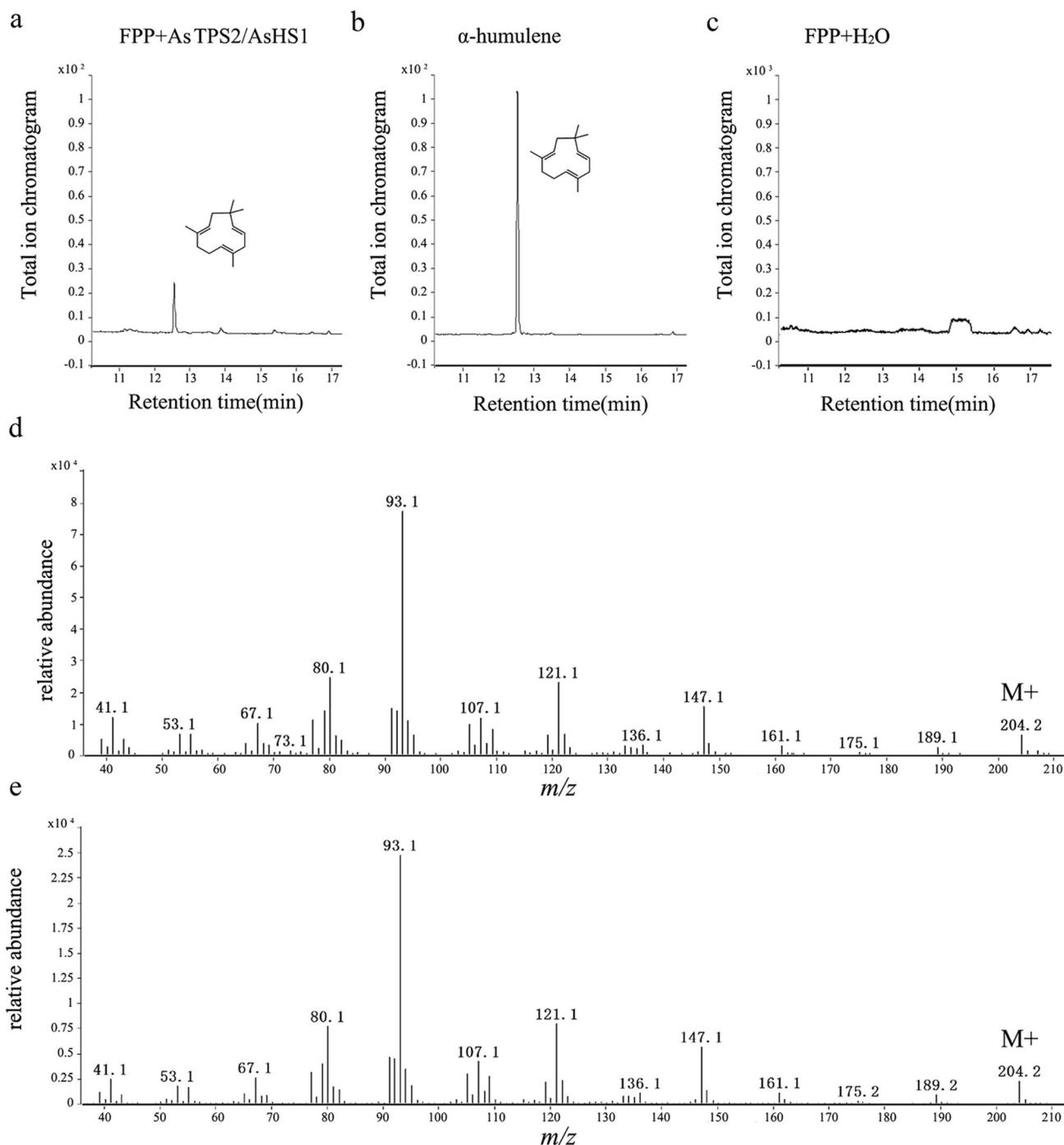
**Fig. 3** Real-time PCR analysis of fold-changes in expression of eight genes encoding *Aquilaria sinensis* TPS-a group sesquiterpene synthases after treatment with various hormones or H<sub>2</sub>O<sub>2</sub> at 100  $\mu$ M for 1

and 2 h (Fig. 3a) and 1 mM for 24 and 48 h (Fig. 3b). Expression for each gene before the treatments was arbitrarily set to 1. MeJA, methyl jasmonate; MeSA, methyl salicylate; Eth, ethylene

acid by hydrogen bonding to complete the function of the enzyme, while the substrate farnesyl diphosphate (FPP) was probably bound with the free amino acid at one side of the RxR motif (Fig. 5a, b). Generally, the conserved catalytic mechanism of sesquiterpene synthase is to stabilize the binding of FPP through the formation of an enzyme-Mg<sup>2+</sup>-ligand ternary complex (Singh et al. 2021). Accordingly, the positions of the amino acid residues around the two

Mg<sup>2+</sup> clusters that may form polar interactions with them in the range of 3.5 Å were calculated. They are R450, D453, D454, T457 and E461 from the NSE/DTE motif and D307 and D311 from the DDxxD motif. These amino acids are highly conserved in terpenoid synthases. The Mg<sup>2+</sup>-bound DDxxD and NSE/DTE motifs and the free RXR motif are jointly directed into the catalytic pocket structure (Fig. 5b). For further identifying the key positions that determine the



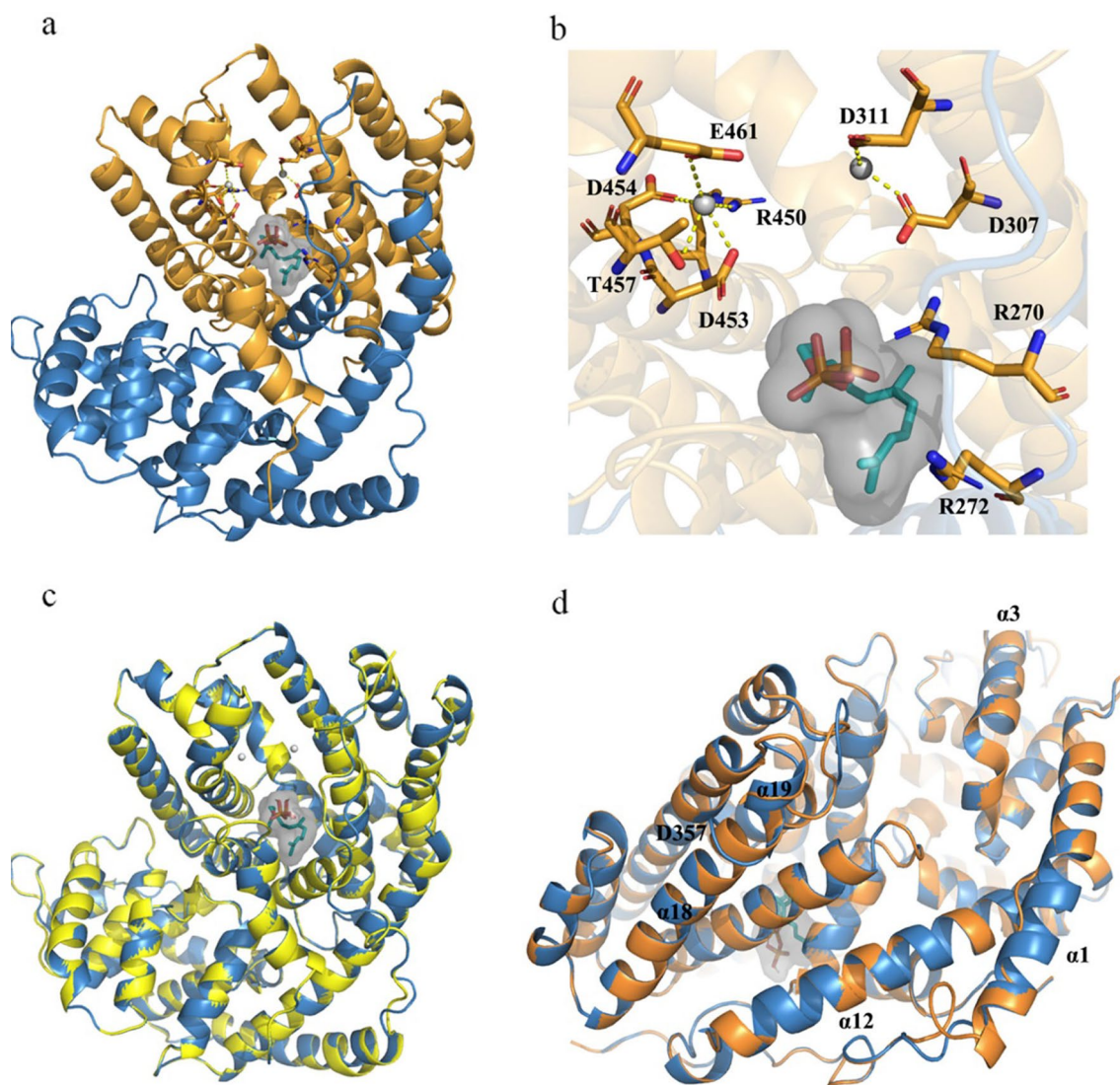


**Fig. 4** GC–MS spectra of sesquiterpene products generated by AsTPS2/AsHS1 from *Aquilaria sinensis*. **a**  $\alpha$ -Humulene produced by incubation of farnesyl diphosphate (FPP) with AsHS1; **b** authentic  $\alpha$ -humulene; **c**  $\alpha$ -humulene produced by incubation of FPP with

H<sub>2</sub>O; **d** total ion chromatogram of the product of AsHS1 enzyme with FPP as a substrate with the major product identified as  $\alpha$ -humulene; **e** total ion chromatogram of  $\alpha$ -humulene standard. M<sup>+</sup> represents the molecular ion of  $\alpha$ -humulene

activity of AsHS1, the structure of AsHS1 was compared with the  $\alpha$ -humulene synthase AcHS1 from *A. crassna* and  $\delta$ -guaiene synthase ASS1 from *A. sinensis* (Xu et al. 2013; Kumeta and Ito 2016). As shown by Fig. 5c, the model of AcHS1 was highly similar to that of AsHS1; their structures

in the catalytic cleft were almost identical, which may result in their similar catalytic products. As shown by Fig. 5d, the structures of ASS1 and AsHS1 have some differences. Their RRX<sub>8</sub>W motifs were all localized in the free amino acids before the  $\alpha$ 1-helix, and these free amino acids are very



**Fig. 5** Tertiary structural models of AsHS1 from *Aquilaria sinensis* and comparison with other STSs. **a** Structural model of AsHS1 with farnesyl diphosphate (FPP) and three  $Mg^{2+}$ . The C-terminal domain is in bright orange, with three bound  $Mg^{2+}$  atoms (dark-gray spheres), the N-terminal domain is in light blue, and the hydrophobic structure is the active site. **b** Detail of the AsHS1 catalytic cleft, showing the

known TPS motifs RXR, DDxxD and NSE/DTE. Dashed gold lines represent polar interactions between  $Mg^{2+}$  atoms and D311, D307, D453, D454, R450 or E461 residues in each amino acid. N is in blue; O is in red. Predicted tertiary structures of AsHS1 (light blue) and AchS1 (yellow) (**c**) and (**d**) AsHS1 (light blue) and ASS1 (orange)

close to the structure of the  $Mg^{2+}$ -binding catalytic pocket. Therefore, we speculated that differences in the N-terminal free amino acids may be the cause for the specific catalytic products. Additionally, AsHS1 and ASS1 are not completely the same at the first  $\alpha$ -helix; ASS1 has some free amino acids in the first  $\alpha$ -helix, but the first  $\alpha$ -helix of AsHS1 is not destroyed. In the middle of the 18 and 19  $\alpha$ -helices, D357 was predicted as a free amino acid in AsHS1, while ASS1 does not have a free amino acid in this position (Fig. 5d). Moreover, two catalytic pockets in the tertiary structures of AsHS1 and ASS1 were predicted by the online tool POCASA 1.1. The first larger catalytic pocket, where

the predicated FPP binding sites are located, is highly conserved, but the second catalytic pocket is less conserved. Some amino acid sequences surrounding this second catalytic pocket of ASS1 and AsHS1 showed different secondary structures, which may also contribute the product specificity of the sesquiterpene synthase (Fig. S7).

## Discussion

The genome-wide identification of TPS families has been reported for various plant species such as pineapple (Chen

et al. 2017b), *Citrus sinensis* (Alquezar et al. 2017), tomato (Zhou and Pichersky 2020), and *Cannabis sativa* (Booth et al. 2020). In the first comprehensive analysis of the TPS family in *A. sinensis*, we discovered six new *AsTPS* genes. We classified the putative *AsTPS* proteins based on their phylogenetic relationships and revealed 16 *AsSTS* genes that encode *AsTPS* proteins that belong to group TPS-a. We also analyzed the expression of eight *AsSTS* genes and identified a  $\alpha$ -humulene synthase using an *in vitro* catalytic experiment. Our results provide new experimental evidence for the diversity of terpene synthases in *A. sinensis*.

Zhang et al. (2010) proposed that agarwood is induced by a defence reaction in *A. sinensis* for which  $H_2O_2$  and jasmonate (JA) are signal transducers (Zhang et al. 2014; Xu et al. 2016).  $H_2O_2$  can induce vessel occlusions and stimulate sesquiterpene accumulation in the pruned stems of *A. sinensis* (Zhang et al. 2014).  $H_2O_2$  can also promote programmed cell death and salicylic acid (SA) accumulation during the induction of sesquiterpene production in cultured cell suspensions of *A. sinensis* (Liu et al. 2015; Siah et al. 2016). JA is a crucial signal transducer for the formation heat-shock-induced sesquiterpene in *A. sinensis*, and exogenous MeJA affects the production of agarwood sesquiterpenes (Xu et al. 2016; Sun et al. 2020). Fungal endophytes can also induce the production of agarwood in *Aquilaria* species, with the quality similar to that of naturally produced agarwood (Chen et al. 2017a; Chhipa et al. 2017; Monggoot et al. 2017; Sen et al. 2017; Subasinghe et al. 2019). Based on these studies, we think other hormones could act as secondary signalling transducers for the defence reaction in *A. sinensis* and initiate the gradual formation of agarwood. In this study,  $H_2O_2$ , MeJA, MeSA, ABA or ETH are the respective signalling transducers for reactive oxygen species and stress from wounding, biotrophs, drought and senescence. In the qPCR analysis, the expression of selected eight *AsSTS* genes varied in the short and longer treatments. These differences in expression may lead to the varied sesquiterpene profiles in *A. sinensis* under abiotic or biotic stress and may also reflect redundant or specific roles of the genes. The greater expression of some *AsSTS* genes, such as *As-SesTPS*, *AsVS* and *AsTPS12* after 24 or 48 h of treatment with 1 mM MeSA compared with levels after 1 or 2 h with 100  $\mu$ M MeSA implies that 1 mM MeSA may have a long-term role for inducing expression of some sesquiterpene synthases and thus change the profiles of sesquiterpenes, which may lead to differences in the perfumes of agarwood. Further studies on the signalling pathways of MeSA-induced sesquiterpene biosynthesis are needed to reveal the fungus-induced mechanism of agarwood formation.

Sesquiterpenes  $\alpha$ -humulene,  $\delta$ -guaiene and  $\alpha$ -guaiene were previously detected from *A. sinensis* cell suspensions using GC-MS analyses (Xu et al. 2016; Lv et al. 2019), and the  $\delta$ -guaiene synthase *AsTPS1* (ASS1–3) has

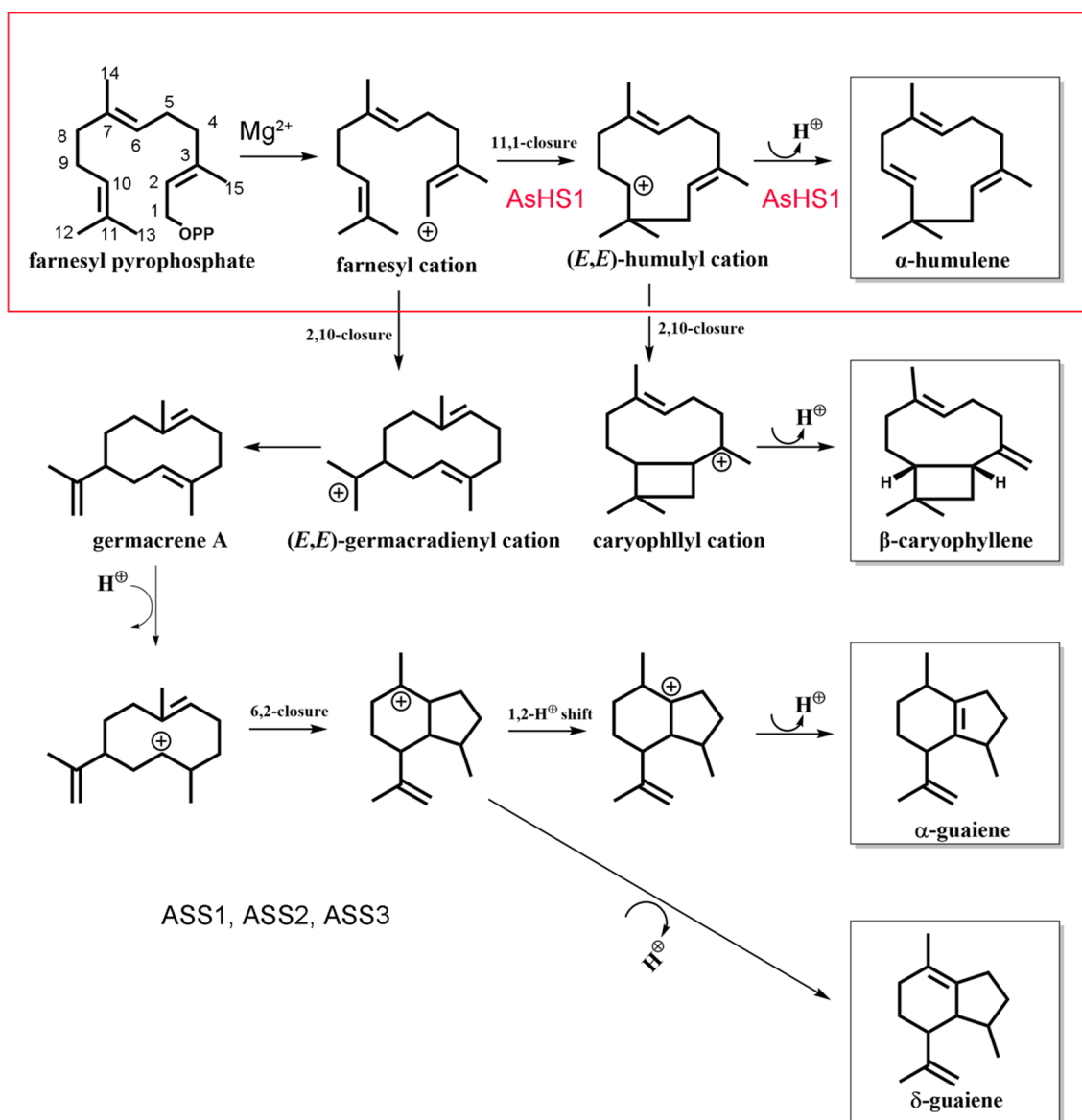
been identified by Xu et al. (2013). In the present study, an  $\alpha$ -humulene synthase *AsHS1/AsTPS2* has also been identified as part of the terpene biosynthesis pathways in *A. sinensis*. Based on the results, the putative biosynthesis pathways for  $\delta$ -guaiene and  $\alpha$ -humulene in *A. sinensis* have been proposed, which are similar to those in *A. crassna* proposed by Kumeta and Ito (2010, 2016) (Fig. 6). Since the *AsHS1* gene is expressed at the transition area between white wood and brown agarwood, this gene may be related to the mechanism of agarwood formation. *AsHS1* (*AsTPS2* in Fig. 3) had greater expression after the 1-h treatment with 100  $\mu$ M ETH compared with the 2-h exposure and after the 24-h treatment with 1 mM MeSA compared with 48 h. Because ETH is involved in senescence and MeSA is related to fungal stress, *AsHS1* may be involved in agarwood formation induced by senescence and endophytic fungi (Halim et al. 2008; Li et al. 2013).

The structural analysis of *AsHS1* revealed its amino acids, and the structural comparisons of *AsHS1* with *AcHS1/ASS1* showed specific amino acid positions in *AsHS1* that probably contribute to its enzymatic activity. Humulene is widely distributed among the plant kingdom and has anti-inflammatory and anticancer activities (Fernandes et al. 2007; Rahman et al. 2014; Chia et al. 2016; Yan et al. 2017). It has been used widely in aromatherapy and has vast potential for medical applications. Currently,  $\alpha$ -humulene synthases have been identified in several plant species, such as *A. crassna* (Kumeta and Ito 2016), *Zingiber zerumbet* (Yu et al. 2008), *Humulus lupulus* (Wang et al. 2008), *Santalum austrocaledonicum* (Jones et al. 2011), *Picea glauca* (Keeling et al. 2011) and *Solanum habrochaites* (Bleeker et al. 2011). Here, the new humulene synthase *AsHS1* produces humulene as the major product when expressed in *E. coli*. The identification of *AsHS1* and its catalytic sites will be helpful to produce humulene with a high purity via bioengineering.

## Conclusions

Through a bioinformatics analysis, we identified 32 *AsTPS* genes and 16 *AsSTS* genes and the conserved motifs in 16 putative *AsSTS*s in group TPS-a in *A. sinensis*. Compared with the shorter treatments with 100  $\mu$ M hormone or  $H_2O_2$ , the longer treatments with 1 mM hormone or  $H_2O_2$  did not always have a stronger effect on increasing the expression levels of specific *AsTPS* genes, such as *AsTPS10*. A novel sesquiterpene synthase gene, *AsHS1*, was isolated and characterized as encoding an  $\alpha$ -humulene synthase. Differences in the predicted tertiary structures of *AsHS1* with those of *AcHS1* and *ASS1* may contribute to the specific product generated by *AcHS1* and *ASS1*. Our results provide novel insight into sesquiterpene biosynthesis and regulatory pathways in *A. sinensis*, and the putative





**Fig. 6** Proposed humulene and guaiene biosynthetic pathways in *A. sinensis*. ASS1, ASS2 and ASS3 are the  $\delta$ -guaiene synthases identified by Xu et al. (2013)

biosynthetic pathways for some specific sesquiterpenes including  $\alpha$ -humulene in *A. sinensis* that we propose are a foundation for work to synthesize high purity sesquiterpenes. The discovery of structural differences between AsHS1 and ASS1 will aid further study of the variations and specificities of STSs in *A. sinensis*.

**Author contributions** Jiadong Ran and Yuan Li have contributed equally to this work and share co-first authorship. Zheng Zhang and Yuan Li conceived and designed the study. Jiadong Ran, Xin Wen, Xin Geng, Xupeng Si performed the experiments. Yimian Ma and Jiadong Ran wrote the paper. Yuan Li, Zheng Zhang and Liping Zhang reviewed and edited the manuscript.

**Funding** The work was supported by the National Natural Science Foundation of China (81773844). The online version is available at <http://www.springerlink.com>

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