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Bioinformatics-Aided Identification, Characterization of Fungal Linalool Synthases and Applications in Linalool biosynthesis

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ABSTRACT: Enzymes empower chemical industries and are the keystone for metabolic engineering. For example, linalool synthases (LSs) are indispensable for the biosynthesis of linalool, an important fragrance used in 60-80% cosmetic and personal care products. However, plant LSs have low activities while expressed in microbes. Aided by bioinformatics analysis, four linalool/nerolidol synthases (LNSs) from various Agaricomycetes were accurately predicted and validated experimentally. Furthermore, we discovered a novel LS with exceptionally high levels of selectivity and activity from *Agrocybe pediades*, ideal for linalool bioproduction. It effectively converted glucose into enantiopure (R)-linalool in *Escherichia coli*, 44-fold and 287-fold more efficient than bacterial and plant counterparts, respectively. Phylogenetic analysis indicated the divergent evolution paths for plant, bacterial and fungal LSs. More critically, structural comparison provided catalytic insights in Ap.LS superior specificity and activity, and mutational experiments validated the key residues responsible for the specificity.

KEYWORDS: biocatalysts, biosynthesis, metabolic engineering, enzyme discovery, terpenoids, terpene synthases, monoterpenes, linalool synthase.

INTRODUCTION

Nature is the best inventor and breeds versatile enzymes. Among various enzymes, terpene synthases represent a unique class of biocatalysts with fascinating capabilities (e.g. introduction of carbon-carbon bonds, facilitation of cyclization and rearrangement of terpenes) ¹. Terpene synthases are pivotal for the biosynthesis and diversity of terpenoids (>80000 different molecules), which constitute the largest group of natural products ¹. Terpenoids have wide applications, including pharmaceuticals, nutraceuticals, flavorings, fragrances, and biofuels ^{2,3}. However, the biosynthesis of most terpenoids has yet to achieve high titers and yields that are vital for commercial production. One major obstacle is that currently identified terpene synthases have low activities and/or low selectivities ^{2,4}.

An example is linalool, a naturally occurring monoterpene alcohol (C₁₀) found in several flowers such as lavender ⁵. With a pleasant floral smell, linalool is an important fragrance ingredient widely used in food, beverage and many personal care products (perfumes, body lotions, etc.). Natural linalool has two stereoisomers with different smells, (S)-linalool and (R)-linalool. (S)-linalool is floral, citrus and petitgrain-like (odor threshold 7.4 ppb) and (R)-linalool is woody and lavender-like (odor threshold 0.8 ppb) ⁶. Natural linalool has higher enantiopurity, thus, superior to synthetic linalool racemates in applications such as high-end perfumes and cosmetics. In 2018, the world consumption of linalool surpassed 11,000 metric tons and its global market is projected to reach 12.3 billion US\$ in 2024 ⁷. Despite great commercial interests, the biosynthesis of linalool has only achieved limited success (mg/L scale) ^{8,9}. This contrasts with the rapidly growing demand for natural linalool. The plant linalool synthases (LSs, converting geranyl pyrophosphate (GPP) into linalool) are relatively abundant, yet proven to have low activities when expressed in microbial hosts (e.g. yeasts and *Escherichia coli*) ¹⁰. Recently, a bacterial bifunctional linalool/nerolidol synthase (LNS) has been identified and characterized ¹¹. However, it produces more nerolidol (a sesquiterpene alcohol, the product of farnesyl pyrophosphate or FPP) than linalool when expressed in microbes. This is because, unlike plants that have special compartments (e.g. plastids) where GPP synthases (GPPSs) are localized ¹², wildtype microbes have neither specialized organelles nor dedicated GPPSs. Rather, GPP is merely an intermediate compound of FPP synthases (e.g. ispA of *E. coli*, ERG20 of *Saccharomyces cerevisiae*) in microbes. Hence, FPP is more abundant than GPP in the cytosol ¹³. As such, a specific and more active LS is desired for microbial linalool production. Besides plants and bacteria, fungi such as the agaric mushroom *Agrocybe aegerita* (recently renamed into *Cyclocybe aegerita*), are also known to produce linalool and several other monoterpenes (α -pinene, *p*-cymene, limonene, and β -ocimene) ¹⁴. However, fungal LSs have not been identified until very recently. In our previous study ⁹, we described the bifunctional role of an LNS from *A. aegerita* that was used to produce linalool or nerolidol in *E. coli* but did not elaborate on the method of identification or its kinetic characterization.

Here guided by bioinformatics predictions and experimental validation, we identified four bifunctional LNSs and a specific LS (no sesquiterpene activity) from four fungal species. We characterized the kinetic parameters of the LNS from *A. aegerita* and

the LS from *Agrocybe pediades*. Phylogenetic analysis indicated clear divergence among the 35 plant enzymes (4 nerolidol synthases, 9 LNs and 22 LNSs), 1 bacterial LNS (D5SL78) and 9 fungal enzymes (LNSs and LSs). Furthermore, *in vitro* and *in vivo* activities, structural and functional comparison were carried out for the plant, bacterial and fungal LSs. It was found that the fungal linalool synthase is highly active in *E. coli* and has an exceptional selectivity, thus ideal for linalool biosynthesis in metabolic engineering and other biocatalytic processes. On top of structural analysis, further mutation experiments identified the key residues responsible for the specificity of the fungal LS, as compared to fungal LNSs. The knowledge is essential for the design of artificial enzymes and interconversion between monoterpene synthases and sesquiterpene synthases.

RESULTS AND DISCUSSION

Plant metabolites are well recognized and explored. In contrast, the biosynthetic capability of fungi is relatively undervalued. Fungi (>3 million species) have enormous diversity and are rich in biocatalysts and secondary metabolites¹⁵⁻¹⁷. In particular, fungi produce a large portfolio of volatile terpenoids, including sesquiterpenes (e.g. β -caryophyllene and cubenol¹⁸) and monoterpenes (linalool¹⁴, limonene¹⁴ and 1,8-cineole¹⁹). To our knowledge, to date, there are only a couple of fungal monoterpene synthases reported in literature (Hyp3, a 1,8-cineole synthase¹⁹ and the very recently identified PpSTS25, a bifunctional myrcene/linalool synthase²⁰). Here, we addressed this gap and specifically explored fungal linalool synthases.

Identification of linalool synthase in agaric mushroom.

As *Agrocybe aegerita* is known to produce linalool and its genome has been recently sequenced²¹, we tried to identify the LS in its genome. In our previous study, we identified 11 putative sesquiterpene synthases and nine of them are functional and produced various sesquiterpenes but not linalool³. A re-evaluation of the raw genomic data led to an additional putative terpene synthase sequence (AAE3_109435). The gene exists in the Illumina sequencing data, whereas absent in the PacBio results. PCR amplification of the AAE3_109435 with subsequent sequencing confirmed the presence of the gene in the genome (Fig. S1).

The subsequent expression of AAE3_109435 in a GPP-accumulating *E. coli* that co-expressed the native enzymes DXS, IDI and ispA_S80F mutant (GPPS). The resulting strain (GPPS+9435) produced the acyclic monoterpene linalool as the main product and small amount of nerolidol (Fig. 1; mass spectra in Fig. S2). Geraniol was detected in GPPS+9435 as well as in its control strain (GPPS_ctrl), indicating that geraniol was not the product of AAE3_109435 but of a native *E. coli* enzyme (such as PhoA, a phosphatase, and NudB, a Nudix hydrolase²²). Furthermore, AAE3_109435 was expressed in the *E. coli* strain that accumulates FPP by overexpressing DXS and IDI (without ispA_S80F), and the strain was named 'FPPS+9435'. Its main product (>96% regarding the peak area of all detected terpenes) was nerolidol and only traces of linalool could be detected (Fig. 1A). The control strain (FPPS_ctrl) with an empty vector produced neither linalool nor nerolidol. The results clearly proved that

the terpene synthase coded by AAE3_109435 is a bi-functional linalool/nerolidol synthase (LNS), which is able to convert FPP into nerolidol and GPP into linalool. Accordingly, it is named Aa.LNS. Furthermore, the linalool produced by Aa.LNS is mainly (*R*)-linalool (95% ee, Fig. 1B), the same stereoisomer as in lavender and sweet basil.

Bioinformatics prediction of other fungal LNSs.

Aa.LNS was used to probe other potential fungal LNSs. The first focus was laid on *Agrocybe pediades*, another sequenced fungal species of the genus *Agrocybe*. Linalool was detected in the headspace of *A. pediades* cultures in our lab. A blast search of Aa.LNS against the *A. pediades* genome using the online tool of the Joint Genome Institute (<https://genome.jgi.doe.gov/Agpped1/Agpped1.home.html>) resulted in 11 terpene synthase homologues (Fig. 1C).

To improve the prediction confidence, we combined two strategies: 1) full sequence alignment (Fig. 1C) and 2) comparison of predicted active sites (Tables S1 and S2, the results were analyzed with 4LXW and 5NX5 (PDB ID) as templates). Four terpene synthase homologues (Agpped1_820868, Agpped1_694262, Agpped1_689671 and Agpped1_689675,) were found to be closely related to Aa.LNS (Fig. 1C). It was hypothesized that the two enzymes Agpped1_820868 and Agpped1_694262 are more similar to Agr8 (a γ -muurolene/ β -cadinene synthase)³ as all three enzymes share almost identical active sites based on our algorithm (Table S1). Hence, we studied the other two enzymes. As predicted, the strain expressing Agpped1_689671 produced linalool and nerolidol (renamed Ap.LNS) similar to Aa.LNS (Fig. 1D). Surprisingly, linalool was the sole terpene product of the *E. coli* clone expressing Agpped1_689675, indicating it is a monofunctional LS (renamed Ap.LS). This attributes to the high specificity of Ap.LS, which is underpinned by the fact that FPP is more abundant in microbial cells than GPP¹³. In addition, Ap.LS produced the enantiopure (*R*)-linalool (Fig. 1B).

Next, we asked if we could use Ap.LS to probe other fungal LSs. We carried out a UniProt BLAST search of Ap.LS and collected those hits with the highest alignment score (score >700, Fig. S3): three from *Galerina marginata* (Galma_223690, UniProt ID A0A067THX9; Galma_63556, A0A067T8I8; Galma_266794, A0A067T571); two from *Hypholoma sublateritium* (Hypsu1_148365, A0A0D2NH86; Hypsu1_148385, A0A0D2NA50) and one from *Hebeloma cylindrosporum* (M413_27416, A0A0C2YLE7).

Four of them (Galma_223690, Galma_63556, Hypsu1_148365 and Hypsu1_148385) clustered into a branch or subgroup with Ap.LS and Ap.LNS in both analyses using full sequence and active-site alignment (Fig. 2A and B). Overall, the six homologues share >90% identity of active site residues (Tables S1 and S2). Particularly, the active sites of Hypsu1_148385 and Galma_223690 show 95% (36/38) and 97-100% (37-38/38) identity with that of Ap.LS and Ap.LNS, respectively, indicating they are potential fungal LNSs. The other two amino acid sequences (Galma_266794 and M413_27416) were more closely related to Agr8, with as high as 95% active-site identity (Table S1). Thus, it was hypothesized that they were more likely to produce 1,10 cyclization products of FPP, e.g., muurolene, cadinene. This hypothesis was validated by the expression of Galma_223690,

Hypsu1_148385 or Galma_266794 in the FPP-accumulating *E. coli*. As predicted, Galma_223690 and Hypsu1_148385 were found to be bifunctional LNSs (Fig. 2C), thus renamed Gm.LNS and Hs.LNS, respectively. Moreover, like Agr8, the strain expressing Galma_266794 produced germacrene D (1,10 cyclization) as main product and a few minor products (γ -muurolene and (+)- δ -cadinene, Fig. S4), thus validating our hypothesis.

Here, the synergistic use of BLAST search, full-sequence alignment and active-site alignment was explored. As such, we achieved a relatively high predictability of hunting for biocatalysts of the same function (e.g. linalool synthases). BLAST search helps with identifying overall similar enzymes that form our initial screening candidates. Full sequence alignment and phylogenetic tree facilitate the classification of enzymes. Different classes often have distinct catalytic functions (e.g. different cyclization positions). Active-site prediction further supplements the prediction with two main roles. One is to filter out those enzyme candidates of incomplete binding pockets (e.g., Agr11 is missing the NSE triad, Tables S1 and S2). The other is to complement the enzyme classification of full sequence alignment. This is based on the hypothesis that enzymes of the same function may have overall low similarity but more conserved active sites. For example, the Galma_266794 shares comparable full-sequence similarities with Ap.LS (61%) and Agr8 (62%), however it has much higher active-site identity with Agr8 (95%) than with Ap.LS (71%, Table S1). Indeed, the products of Galma_266794 are similar to that of Agr8.

Purification and characterization of fungal LS and LNSs.

To date, a number of plant LSs and LNSs have been identified. However, only one bacterial LNS from *Streptomyces clavuligerus* was recently identified¹¹, which only shares 15.2% identity with Aa.LS (Fig. S5). With the fungal enzymes studied in this work, LSs and LNSs in three kingdoms have been identified. Next, we sought to compare their catalytic activities and mechanisms by *in vitro*, *in vivo* assays, sequence alignments and 3D structural models.

Protein purification is the prerequisite to study the *in vitro* kinetics of the fungal enzymes. Though all the five bacterial strains expressing fungal enzymes produced linalool, their expression levels in *E. coli* were largely different. Aa.LNS had the highest expression level, followed by Gm.LNS, Ap.LNS and Ap.LS. The expression of Hs.LNS (Hypsu_148385) was so low that it was not detectable in a protein gel (Fig. S6A). As Aa.LNS had the highest expression level and Ap.LS is the only specific monoterpene synthase, they were chosen as the representatives of fungal LNS and LS for further studies. However, none of them was soluble based on solubility analysis with B-PER II reagent (Thermo Scientific™) (Fig. S6A). Many approaches were tested but failed to improve their solubility (such as abiotic condition optimization: lowering incubation temperature, tuning inducer dosages, media additives and protein fusion). Refolding of insoluble fraction could be another solution which we did not test because it is very time-consuming to optimize the best conditions. The N-terminal fusion of Aa.LNS with a maltose binding protein or thioredoxin did not help (Fig. S6B). Different chaperone systems (DnaK-dnaJ, GroES-GroEL) and trigger factor (TF) in *E. coli* were further tested. It was found that TF chaperone could slightly improve the solubility of the synthases. With the optimal condition

(3.3 mM arabinose to induce TF chaperone and 0.1 mM IPTG to induce Aa.LNS, Fig. S7) and further separation by size exclusion chromatography, we managed to purify enough soluble Aa.LNS for *in vitro* characterization. Yet its purity was quite low with ~16.3% (Fig. 3A). In contrast, relatively high purity of soluble Ap.LS (~71.2%) was obtained with the same experimental conditions (Fig. 3B). Consistent with the *E. coli* cultures producing the respective synthase, purified enzymes reconfirmed that Aa.LNS can use FPP and GPP to produce nerolidol and linalool, respectively. Whereas Ap.LS was only able to use GPP but not FPP (Fig. 3). The K_m and k_{cat} values of Aa.LNS for FPP were $9.0 \pm 2.3 \mu\text{M}$ and $3.3 \pm 0.3 \text{ min}^{-1}$, respectively; and slightly lower for GPP with $6.7 \pm 4.6 \mu\text{M}$ and $0.5 \pm 0.1 \text{ min}^{-1}$, respectively (Table 1). The K_m value of Ap.LS for GPP with $3.8 \pm 0.7 \mu\text{M}$ was slightly lower than that for Aa.LNS, whereas k_{cat} was much higher with $6 \pm 0.3 \text{ min}^{-1}$. To compare the catalytic efficiencies among the known LSs and LNSs, k_{cat}/K_m value of Ap.LS was the highest, which is about 21-fold, 29-fold, threefold and fourfold higher than that of Aa.LNS, La.LS (Q2XSC5) from *Lavandula angustifolia* (Lavender)⁵, Ma.LS (Q8H2B4) from *Mentha aquatica*²³, and of the bacterial Sc.LNS¹¹, respectively. As for Aa.LNS as a nerolidol synthase, although the k_{cat} value of Aa.LNS for FPP was more than five times lower compared to the bacterial one, the K_m value was similar to that of the bacterial Sc.LNS¹¹ and about half as that of Zm.LNS from *Zea mays* (Maize) (Table 1)²⁴.

***In vivo* activity comparison of LSs and LNSs from three kingdoms.**

Due to potential issues such as poor expression and solubility when expressed in cells and the localization difference (cytosolic/membrane-bound *in vivo* versus a one-pot aqueous reaction), the advantages of *in vitro* enzyme kinetics (Table 1) may not be readily transferable into cellular applications, such as metabolic engineering, where *in vivo* activities are more critical than *in vitro* ones. To test the best candidate for microbial linalool production, our previously engineered *E. coli* strain was used to compare LNs from three kingdoms: Ap.LS, Sc.LNS and Cb.LS from *Clarkia breweri* (Q96376) as representatives for fungi, bacteria and plantae, respectively. They were separately cloned into pET-11a vector (Novagen). Together with a p15A vector carrying the whole mevalonate pathway genes², the bacterial strains grown in ZYM media produced linalool at 381.2, 8.7 and 1.3 mg/L for fungal, bacterial and plant linalool synthases, respectively (Fig. 3C). This means *in vivo* activity of Ap.LS (fungal) is about 44-fold and 287-fold as high as that of Sc.LNS (bacterial) and Cb.LS (plant), respectively. A previous study also supported that the bacterial Sc.LNS is better than plant LSs in terms of *in vivo* activity in TB media¹⁰. In the same TB media, the linalool titre reached 601.2 mg/L for Ap.LS strain, about 65% higher than previously reported using Sc.LNS¹⁰. Our study demonstrated that fungal Ap.LS is even superior to the bacterial one, in both activity and selectivity. Although Sc.LNS has a higher activity than plant Cb.LS, it prefers FPP (lower K_m and higher k_{cat}) to GPP as the substrate¹¹. Therefore, Sc.LNS produced a larger amount of nerolidol than linalool in *E. coli* whose cytosol contained both FPP and GPP, in contrast, Ap.LS produced 100% linalool. The superior activity and selectivity of Ap.LS makes it more suitable for microbial production of linalool than its plant and bacterial counterparts. High activity contributes to high titers, rates and yields (TRYs) of linalool production and low manu-

150 facturing cost. High specificity would greatly simplify the downstream purification process and further reduces the overall pro-
151 duction cost. Thus, this study paves the way for commercialization of linalool bioproduction which is greener, safer, sustainable
152 and of exceptional enantiopurity ((*R*)-linalool), as compared to chemical synthesis.

153 **Structural comparison of LSs and LNSs from three kingdoms (plants, fungi and bacteria).**

154 Next, we generated a phylogenetic tree with 35 plant enzymes (including 4 nerolidol synthases, 9 LNs and 22 LNSs), 1
155 bacterial LNS (D5SL78) and 9 fungal enzymes (Table S3). The enzymes were clearly separated into two major clades (one is
156 plant, clade 1, and the other is microbial, clade 2, Fig. 4). The bacterial LNS was closer to fungal ones, in clade 2. Specifically,
157 the sequence identity among fungal, bacterial and plant LNSs or LSs are only 8%-15%, which includes those metal binding sites
158 (Fig. S5). Overall, plant LSs/LNSs are larger with 500-900 amino acids than microbial ones with 300-400 amino acids. One
159 particular enzyme D8RNZ9, which is a LNS isolated from the nonseed plant *Selaginella moellendorffii* (Spikemoss)²⁵, is more
160 closely related to the bacterial LNS than plant LNSs. It was hypothesized that it could stem from horizontal gene transfer from
161 microbes to plants or that seed plants lost these LNS enzymes during evolution from nonseed plants²⁵. The phylogenetic tree
162 indicates the evolutionary divergence of fungal, bacterial and plant LSs and LNSs.

163 Subsequently, we compared the protein structures of the LSs from the fungus (*A. pediades*), the bacterium (*S. clavuligerus*)
164 and the plant (*M. aquaticus*). The crystal structure of Sc.LNS is 5NX5 (PDB ID)¹⁰. The homologue models of Ap.LS and Ma.LS
165 (Q8H2B4) were built based on the crystal structure of (+)-bornyl diphosphate synthase from *Salvia officinalis* (1N1B/1N21)²⁶
166 and 1,8-cineole synthase from *S. clavuligerus* (5NX7)¹⁰, respectively. As the sequence similarity between Ap.LS and 1,8-cineole
167 is only 20.3% (and active site identity is 49%), the Ap.LS model may have some deviations from its real structure. Nevertheless,
168 their active site regions are highly conserved (Fig. 5A). Fungal LS and bacterial LS are much more similar to each other than to
169 plant LS in both active site regions (Table S4) and overall structures (Fig. 5). As a typical plant monoterpene synthase, Ma.LS
170 has two domains (α and β domains) and, thus, is noticeably larger than the other two synthases with active site residing only in
171 the α domain (catalytic domain, Fig. S8). In contrast, microbial LSs, Sc.LNS and Ap.LS, are similar to typical class I terpene
172 cyclases with a single domain, despite with acyclic products (Fig. S9). Both GPP (its analogue, 2-fluorogeranyl diphosphate)
173 and FPP were docked into the three models. We mainly analyzed the interactions of the three LSs with GPP. With 9 hydrophobic
174 interactions with GPP, Ap.LS had the highest amount, as compared to 7 of Sc.LNS and 6 of Ma.LS (Fig. 5). Except for the
175 negatively charged pyrophosphate (Ppi) head, GPP is largely hydrophobic, thus, these hydrophobic interactions may contribute
176 to the high activity of Ap.LS. The number of hydrogen bonds identified for the three enzymes were similar. In addition, Ap.LS
177 had the highest binding affinity (-7.6 kcal/mol) to GPP, followed by Sc.LNS (-7 kcal/mol) and Ma.LS (-6.4 kcal/mol). The binding
178 affinity inversely correlated with the K_m values of the three enzymes (Table 1), where higher binding affinity contributed to a
179 lower K_m value. As compared for Ap.LS and Ap.LNS, Ap.LS has higher binding affinity to GPP than Ap.LNS (-7.3 kcal/mol), but

180 lower binding affinity (-8.5 kcal/mol) to FPP than Ap.LNS (-9.0 kcal/mol). The binding affinity data are nicely correlated with their
181 difference in monoterpene and sesquiterpene activities.

182 Furthermore, we superimposed the 3D structures of the active sites of the three enzymes (Fig. 6A and Table S4). Residues
183 in the binding pocket of plant LS showed the greatest divergence (green regions, Fig. 6A), although structure folding remains
184 conserved. Overall, as shown in the grey regions (Fig. 6A) and highlighted in Table S4, there were 8 conserved residues among
185 the three enzymes including the aspartate rich motif, D(D/E)XXD, responsible for Mg^{2+} cofactor and substrate binding ¹ and
186 NSE triad, (N/D)Dxx(S/T)xxxE, responsible for the substrate binding and coordination of the diphosphate and trinuclear Mg^{2+}
187 $[PPI-(Mg^{2+})_3]$ cluster ¹. All these structural analyses partially explain the activity difference among the three LSs from different
188 kingdoms. Nevertheless, there are other factors which might also contribute to the high in vivo activity of Ap.LS in *E. coli*, such
189 as, the non-active site residues, protein expression and solubility (Fig. S6) etc.

190 **Mechanism study on the Ap.LS selectivity**

191 Lastly, we attempted to understand the specificity of Ap.LS as compared to other fungal LNSs. Particularly, Ap.LNS and
192 Ap.LS share the highest identity 77.9%, hence, we compared their difference of residues surrounding the substrate binding
193 pocket. In total, five residues were found to be different (Fig. 6B and Table S5): 1) in the Ppi head region (A59:S58, E316:Q315,
194 I153:V152, here, the former and latter residues refer to that of Ap.LS and Ap.LNS, respectively, especially that E316 is expected
195 to have charged interactions with the Ppi group of GPP/FPP); 2) in the FPP tail region (L60:M59, which could affect the interac-
196 tion with FPP); 3) in GPP tail region (G181:A180, which might affect flexibility of helix where the tail end of GPP resides). A
197 series of single mutants were constructed for Ap.LS, A59S, L60M, G181A and E316Q. However, none of these single mutants
198 had effect on the selectivity of Ap.LS (Fig. 6E). We speculated that the specificity might be the synergistic result of multiple
199 residues. The region A59-L60 was particularly interesting, as A59 and L60 are in close proximity to both the Ppi head and
200 hydrocarbon tail of FPP (Fig. 6D). Indeed, the combination of A59S and L60M mutations resulted in the production of a trace
201 amount of nerolidol, ~2% of total amount of linalool and nerolidol produced (Fig. 6C and E), which indicated the two mutations
202 are sufficient to convert Ap.LS from a monofunctional LS to a bi-functional LNS. Adjacent to A59-L60, another residue is also
203 different between Ap.LS (V61) and Ap.LNS (I60). Although single mutation V61I had no effect on the selectivity of the wildtype
204 Ap.LS, the introduction of V61I enhanced the nerolidol production by 12-fold (~40% of total linalool and nerolidol produced) and
205 decreased linalool production by 45% on the basis of the double mutant A59S-L60M (Fig. 6C and E). It seems that the mutation
206 L60M-A59S favors sesquiterpene activity (nerolidol formation) by stabilizing the ligand in a favorable position (A59S) and by
207 promoting the easier leave of the Ppi group from the binding pocket (L60M, Fig. 6D). The third mutation V61I further enhances
208 the effect by pushing M60 and S59 closer to FPP (Fig. 6D).

209 In addition, we also combined the mutations of two regions and obtained the quadruple mutant A59S-L60M-V61I-E316Q,
210 however, it did not further increase nerolidol production. Furthermore, we observed that the wildtype Gm.LNS, whose corre-
211 sponding residues are more similar to those 59-61 of Ap.LS (S59:A59, L60:L60, V61:V61, Fig. S9), produced the highest amount
212 of linalool (15%) among all the wildtype fungal LNSs (Fig. 2C). As such, we concluded that the region residues (A59-V61) play
213 an essential role for the highly specificity of Ap.LS.

214

215 MATERIALS AND METHODS

216 Sequence validation of AAE3_109435 in the genome.

217 AAE3_109435 sequence was analyzed from Illumina sequencing data with manual intron annotation. To verify the presence
218 of the gene AAE3_109435, gDNA of *A. aegerita* was firstly extracted. Briefly, the vegetative mycelia of *A. aegerita* was ground
219 with pestle and mortar using liquid nitrogen. Around 200 mg of ground mycelium was transferred in 1.5 mL E-cups and 500 μ L
220 of lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl, 1% SDS) was added. The solution was vortexed
221 and incubated at ambient temperature for 10 min before the addition of 150 μ L potassium acetate (3 M, pH 4.8). The solution
222 was then vortexed and centrifuged for 10 min at 3000 g. Supernatant containing gDNA was transferred into a new E-cup and
223 the gDNA was obtained by ethanol precipitation method. The gDNA was used as the PCR template to amplify AAE3_109435
224 with primers AAE3_9435f (CCAAGATTGTCGTCACGCC) and AAE3_9435r (CTGTGGGCCATTCTGTCCAT). The amplified
225 region was subsequently sequenced and confirmed (Results in Fig. S1).

226 Bioinformatics prediction of fungal linalool synthases.

227 The fungal LS candidate genes were obtained by the combination of BLAST search in JGI fungal genomics and UniProt
228 databases, full sequence alignment and predicted active site alignment. Those homologues with the highest similarity (usually
229 >50% identity, or in the same cluster of the phylogenetic tree or sequence similarity network) to Aa.LNS (accession number
230 MN954676) or Ap.LS in full sequences and in active sites were selected as the targets for experimental validation.

231 Prediction of active sites of fungal terpene synthases.

232 The full sequence alignment was generated by aligning the complete sequences of LS and LNS proteins from across the
233 three kingdoms by Clustal Omega v1.2.2. In contrast, active-site alignment is proceeded by identifying amino acid residues
234 surrounding the predicted active sites of each enzyme in 3D structures and used to extract the relevant residues from the full
235 alignment. We used an in-house developed algorithm, BioTransformer v0.9, to predict and align the active sites. In brief, the
236 algorithm first searches the PDB for appropriate templates. Next, the user gets to select the most appropriate PDB template or

237 templates with the most appropriate ligands (usually the ligands or combination of ligands that maximize the space within the
238 active site). Using this approach, the PDB structures 4LXW (Epi-isozizaene synthase in complex with inorganic pyrophosphate
239 (Ppi) and benzyl triethyl ammonium from *Streptomyces coelicolor*) and 5NX5 (Linalool/Nerolidol synthase in complex with 2-
240 fluorogeranyl diphosphate from *Streptomyces clavuligerus*) were chosen as templates for the prediction of the active sites. To
241 maximize the number of residues found within the binding pockets of the PDB structures, residues found within 6.0 Angstrom
242 from the substrate are considered as part of the active site, and the union set derived from both structural templates is used as
243 the predicted active site.

244 **Phylogenetic analysis and sequence similarity network of terpene synthases.**

245 Full amino acid sequences were used to build a phylogenetic tree. Alignment was carried out by Clustal Omega program
246 version 1.2.2 and the phylogenetic analysis was conducted with the Neighbor-Joining method or Maximum Likelihood method
247 by MEGA version 7.0.26. For the predicted active sites of terpene synthases, the Enzyme Function Initiative-Enzyme Similarity
248 Tool (EFI-EST, <http://efi.igb.illinois.edu/efi-est/>) was used to generate sequence similarity networks (SSNs) with the database-
249 independent alignment score of 16. The resulting SSNs were visualized using the open source software Cytoscape version
250 3.5.1.

251 **Structural modelling and analysis.**

252 The homologue models of Ap.LS and Ma.LS (Q8H2B4) from *Mentha aquatica* were built based on the crystal structure of the
253 most closely related terpene synthases: Epi-isozizaene synthase from *S. coelicolor* (PDB ID, 4LXW), 1,8-cineole synthase from
254 *S. clavuligerus* (PDB ID, 5NX7)¹⁰ and (+)-bornyl diphosphate synthase from *Salvia officinalis* (PDB ID, 1N1B/1N21)²⁶, using
255 the Modeller software. The binding pockets, consisting of 21-22 residues within 6 angstroms from the substrate, were deter-
256 mined by PyMOL software v2.1.1. Structural alignment of proteins was conducted using MUSTANG²⁷ implemented in YASARA
257 ²⁸. The web implementation of PLIP (Protein-Ligand Interaction Profiler) was used to identify interactions between ligand and
258 the surrounding amino acid residues in the protein²⁹. Docking was performed with AutoDock Vina with an exhaustiveness of
259 200. AutoDock Tools was used to prepare the PDBQT files for the homology models for the enzymes Ap.LS, Ap.LNS, Sc.LNS
260 and Ma.LS and also for the ligands GPP and FPP³⁰. A search space of 24x24x24 Å was used for GPP and a search space of
261 24x28x24 Å was used for FPP.

262 **Plasmid and strain construction.**

263 The fungal terpene synthases (TPSs), Sc.LNS from *Streptomyces clavuligerus* and Cb.LS from *Clarkia breweri* were codon
264 optimized and cloned into pET-11a vector (Novagen) either alone or together with ispA_S80F (GPP synthase) or ispA (FPP
265 synthase) from *E. coli*. For fungal TPS characterization, the chassis *E. coli* strain was used. The strain carried a plasmid p15A-

266 cam-T7-dxs-idi that overexpresses the enzymes DXS and IDI from *E. coli* to enhance the supply of terpene precursors ³. For
267 the monoterpene production study, the mevalonate pathway was overexpressed in p15A vectors under the T7 promoter variants
268 ⁹.

269 **Mutation study of Ap.LS.**

270 The targeted mutations were introduced to Ap.LS using in-house methods as described previously ². Single mutants (A59S,
271 L60M, V61I, G181A and E316Q) and the double mutant (A59S-L60M) were directly introduced to the wildtype. Triple mutants
272 (A59S-L60M-V61I, A59S-L60M-E316Q) were introduced on top of the double mutant A59S-L60M. E316Q was introduced to
273 the triple mutant A59S-L60M-V61I to obtain the quadruple mutant A59S-L60M-V61I-E316Q. Primers used are as listed in Table
274 S6.

275 **Terpenoid production in *E. coli*.**

276 *E. coli* *Bl21-Gold DE3* strain (Stratagene) was used for linalool production. The strains carrying mevalonate pathway and LSs
277 from different species were grown in 1mL of ZYM medium or terrific broth (TB) supplemented with 0.4% glucose in 14 ml BD
278 Falcon™ tube at 28 °C/250 rpm for 3 days ². In addition, 200 µL isopropyl myristate was used for harvesting the linalool and/or
279 nerolidol and antibiotics (34µg/ml chloramphenicol, 50 µg/ml spectinomycin and 100 µg/ml ampicillin) were used to maintain the
280 plasmids. ZYM medium was prepared as previously described ² (1% tryptone, 0.5% yeast extract, 25 mM Na₂HPO₄, 25 mM
281 KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 2 mM MgSO₄, 0.5% glycerol, 0.05% glucose, 15mM of α-lactose). In ZYM medium,
282 the *E. coli* strains were automatically induced with the depletion of glucose and initiation of lactose consumption.

283 **Expression and purification of linalool synthases.**

284 *E. coli* *Bl21-Gold DE3* strains (Stratagene) carrying pET-11a-Aa.LNS or pET-11a-Ap.LS and the chaperone plasmid pTf16
285 (Takara Bio Inc., Japan), were grown in 4L of 2xZYM media with antibiotics (100 µg/ml ampicillin and 34µg/ml chloramphenicol)
286 at 20 °C and 225 rpm. The cells were induced with 0.1 mM IPTG (to express LS) and 3 mM of arabinose (to express chaperone
287 proteins) when OD₆₀₀ reached 0.6-0.8. The cells were further cultured for another 20 hours and harvested. Cell were washed
288 and re-suspended in 100 ml of the solution (50mM Tris of pH 8, 500mM NaCl, 0.1% TritonX100, 5% glycerol) with 2 tablet of
289 protease cocktail inhibitor (Sigma-Aldrich, Singapore). The cells in the suspension were lysed by sonication at 4 °C (5s ON and
290 5s OFF, 40% amplitude). The proteins in the supernatant were then extracted by 1ml x HisTrap column. The column with bound
291 proteins was washed by his-binding buffer (50mM Tris of pH 8, 500 mM NaCl and 20mM imidazole) and eluted by 20 ml of his-
292 elution buffer (50mM Tris of pH 8, 500 mM NaCl and 500mM imidazole). The eluted protein was further concentrated by Vi-
293 vaspin® 10kDa cutoff spin column (Merck, Singapore). In the end, 2.7 mg/ml of Aa.LNS (16.3% purity) and 22.2 mg/ml of Ap.LS
294 (71.2% purity) was obtained.

295 **Kinetic analysis.**

296 Steady-state kinetics of purified Aa.LNS and Ap.LS were determined by measuring PPi release via conversion to phosphate
297 with inorganic pyrophosphatase using the EnzChek® Pyrophosphate Assay Kit (Thermo Fisher Scientific, Singapore). Substrate
298 concentrations of GPP and (E,E)-FPP (Echelon bioscience, USA) were varied between 6.5–40 μ M. Reactions were carried with
299 20 μ g/ml (460 nM) of Ap.LNS or 10 μ g/ml (250 nM) of Aa.LS at 37 °C for 1 hour. PPi concentrations were calculated by linear
300 interpolation of the standard curve using the kit assay (0–60 μ M). The scatter plots of initial rate versus substrate concentration
301 were fitted to the equation $v = v_{\max} [S] / (K_M + [S])$ where $v_{\max} = k_{cat} [E_0]$.

302 **GC-MS detection and quantification of terpenes.**

303 For characterization of fungal terpene synthases, the headspace compounds were sampled at 60 °C for 20 min by SPME
304 with a DVB/CAR/PDMS (50/30 μ m divinylbenzene/carboxen/polydimethylsiloxane) fiber (length 1 cm; Supelco, Steinheim, Ger-
305 many). Subsequently, the compounds were desorbed for 1 min in the split inlet (250 °C; SPME liner, 0.75mm i.d.; Supelco) and
306 analyzed by an Agilent 7980B gas chromatography equipped with an Agilent 5977B MSD. Samples were injected into Agilent
307 DB5ms column with a split ratio of 40:1 at 240 °C. The oven program started at 80 °C for 1 min, was raised up to 210 °C at 10
308 °C/min, then to 310 °C at 60 °C/min and maintained at 310 °C for another 2 min. Mass spectrometer was operated in EI mode
309 with full scan analysis (m/z 33–300, 9 scans/s). In addition to mass spectra, Kovats retention indices of detected compounds
310 were calculated by calibrating the used GC-MS system with a C8–C30 alkane mix and compared with literature data in the
311 National Institute of Standards and Technology database (Fig. S2).

312 For quantification of linalool and nerolidol of different strains, the organic layer with secreted terpenes was separated and
313 diluted into ethyl acetate by 10–100 times. The samples were then analyzed with the same GC-MS program as the characteri-
314 zation method. The concentrations were calculated by interpolation using the standard curve of authentic linalool and nerolidol
315 standards (Sigma-Aldrich, Singapore).

316 **Chiral study of linalool produced by fungal LSs.** The chirality of linalool produced by fungal LSs was analyzed by the
317 GC chiral CycloSil-B column (30m, 0.25mm, 0.25 μ m, Agilent, Singapore) in the same Agilent 7980B gas chromatography
318 equipped with the 5977B MSD. The oven program started at 80 °C for 2 min, was raised up to 210 °C at 5 °C/min, then to 250
319 °C at 20 °C/min and maintained at 250 °C for another 2 min. Mass spectrometer was operated in EI mode with full scan analysis
320 (m/z 33–300, 5.5 scans/s). The retention times and mass spectra of samples were compared with authentic standards of both
321 (*R*)-linalool and a mixture of (*R/S*)-linalool.

322 **SUPPLEMENTARY MATERIALS**

323 Table S1. Predicted active sites of terpene synthases with 4LXW and 5NX5 as the templates.

324 Table S2. Comparison and combination of the predicted active sites of the two templates 4LXW and 5NX5.

325 Table S3. Summary of LSs and LNSs in this study.

326 Table S4. 3D alignment of active-site residues of Ap.LS, Sc.LNS and Ma.LS.

327 Table S5. Comparison of residues in the substrate-binding pockets of Ap.LS and Ap.LNS.

328 Table S6. Primers used for Ap.LS mutation.

329 Fig. S1. Nucleic acid sequence of AAE3_109435 in the genome.

330 Fig. S2. Mass spectra and retention indices for terpenes detected in this study.

331 Fig. S3. The alignment between Agrped_689675 and Galma_223690 and BLAST search results in UniProt database with

332 Agrped_689675 (or Ap.LS).

333 Fig. S4. GCMS chromatograms and spectra for Galma_266794.

334 Fig. S5. Amino acid sequence alignment and identity table of LSs and LNSs from fungi, bacteria and plants.

335 Fig. S6. (A) Expression and solubility analysis of the fungal LNSs and LS.

336 Fig. S7. The optimization of Aa.LNS solubility with the co-expression of chaperone proteins.

337 Fig. S8. The sequence alignment and their secondary structures of plant terpene synthases.

338 Fig. S9. The sequence alignment and their secondary structures of microbial terpene synthases.

339

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348 the project, did the sequence annotation and validation of AAE3_109435. T.C.L and S.M.S. performed the active site prediction

349 of fungal terpene synthases. All authors contributed to the discussion and approved the final manuscript. **Competing interests:**

350 The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the

351 conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may

352 be requested from the authors. **Abbreviations:** GPP: geranyl diphosphate, FPP: farnesyl diphosphate, LS: linalool synthase,
 353 LNS: linalool/nerolidol synthase, TPS: terpene synthases, GPPS: GPP synthase, FPPS: FPP synthase, SSN: sequence simi-
 354 larity network, TRY: titre, rate and yield.

355

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- 422

TABLES

Table 1. Comparison of kinetic parameters for LSs and LNSs from fungi, plant and bacteria.

Sesquiterpene synthase				Monoterpene synthase				
Enzymes	Aa.LNS	Zm.LNS	Sc.LNS	Ap.LS	Aa.LNS	La.LS	Ma.LS	Sc.LNS
Accession no.	/	Q29VN2	D5SL78	/	/	Q2XSC5	Q8H2B4	D5SL78
Product	nerolidol	nerolidol	nerolidol	(R)-linalool	(R)-linalool	(R)-linalool	(R)-linalool	(R)-linalool
Organism	<i>Agrocybe aegerita</i>	<i>Zea mays</i>	<i>Streptomyces clavuligerus</i>	<i>Agrocybe pediades</i>	<i>Agrocybe aegerita</i>	<i>Lavandula angustifolia</i>	<i>Mentha aquatica</i>	<i>Streptomyces clavuligerus</i>
Kingdom	fungi	plantae	bacteria	fungi	fungi	plantae	plantae	bacteria
K_m (μM)	9.04±2.3	18±7.8	9.6±0.8	3.8±0.7	6.7±4.6	42.7	25±6	12.9±1.3
k_{cat} (min^{-1})	3.3±0.3	-	18.7±0.7	6±0.3	0.5±0.1	2.34	14.4±1.2	5.0±0.1
k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)	0.4	-	1.9	1.6	0.1	0.05	0.6	0.4
Reference	This study	²⁴	¹¹	This study	This study	⁵	²³	¹¹

FIGURE LEGEND

Fig. 1. Characterization of fungal linalool and nerolidol synthases (LNSs) and a fungal linalool synthase (LS) in *E. coli*.

(A) GC-MS chromatograms of cultural supernatants of strains GPPS+9435, FPPS+9435 (co-expressing AAE3_109435 and GPP or FPP synthase) and the control strains GPPS_ctrl and FPPS_ctrl (expressing only GPP or FPP synthase). (B) Chiral separation of linalool produced by Aa.LNS and Ap.LS (Agrped1_689675) expressing strains. (C) Phylogenetic tree based on full-sequence alignment of Aa.LNS and terpene synthases (TPSs) in *A. pediades*. AAE3_109435 (a LNS) is marked with “●”, Agr8 (a γ -muurolene/ β -cadinene synthase) with “◆”. (D) GC-MS chromatograms of the supernatant of *E. coli* clones producing Agrped1_689671 and Agrped1_689675.

Fig. 2. Sequence analysis of fungal TPS homologues.

(A) Phylogenetic tree based on full sequence alignment consisting of 12 TPS homologues from *A. aegerita* (Aa.LNS and Agr), 12 from *A. pediades* (Ap.LS, Ap.LNS and Agrped1), three from *Galerina marginata* (Galma), two from *Hypholoma sublateritium* (Hypsu1) and one from *Hebeloma cylindrosporum* (M413_27416). (B) Sequence similarity network (SSN) built on the predicted active sites (Table S1). Agr10 is excluded as it shares limited similarity to the templates (PDB ID: 4LXM and 5NX5). Instead, D5SL78 from *Streptomyces clavuligerus* and D8RNZ9 from *Selaginella moellendorffii* (Spikemoss) are included. The rest of candidates are the same as those of full sequence alignment. LNS and muurolene/cadinene synthase group were highlighted in green and blue, respectively. (C) Experimental validation of predicted fungal TPS homologues. Ap.LS produced exclusively linalool; the other four produced both nerolidol (85-98%) and linalool (15-2%). Here, % refers to the peak area ratios of linalool or nerolidol to the sum of the peak areas of both substances present in each chromatogram.

Fig. 3. In vitro characterization of Aa.LNS and Ap.LS.

(A) SDS PAGE gel image of purified Aa.LNS and its kinetic analysis. (B) Protein gel of purified Ap.LS and its kinetic analysis (No product was observed for FPP). (C) *In vivo* activity comparison of LSs from a fungus, a bacterium and a plant (Error bars, mean $\pm$ s.d., n=3). Ap.LS, Sc.LNS and Cb.LS from *Clarkia breweri* (Q96376) were selected as the representative of kingdoms fungi, bacteria and plantae, respectively.

Fig. 4. Phylogenetic tree of LNSs and LSs in plants, fungi and bacteria.

Two major clades, plant and microbial, were clearly segmented. (*R*)-linalool synthases were marked with “▲”, (*S*)-linalool synthases with “△”, LNSs with “●” and nerolidol synthases with “■”. PpSTS25 is a myrcene/linalool synthase (MLS) from *Postia placenta*²⁰. For plant and bacterial enzymes, UniProt accession number were used. D8RNZ9, more similar to the bacterial LNS than plant ones, was a LNS isolated from the nonseed plant *Selaginella moellendorffii* (Spikemoss). Details of all the enzymes are in Table S3.

Fig. 5. Structural comparison of LSs in bacteria, fungi and plants. (A) The overlay of 3D structure (grey ribbon) and substrate-binding pocket of Ap.LS. Energy minimized homology model was used with 4LXW as template. GPP and FPP from 2ONG and 6A2C respectively were structurally aligned to 4LXW. Binding pocket surfaces for fungal (B), bacterial (C) and plant (D) LSs. GPP (2-fluorogeranyl diphosphate) and FPP ligands are shown as green and magenta spheres, respectively. The solvent-accessible surface of residues in the binding pocket is colored yellow (hydrophobic), cyan (polar), red (negative) or blue (positive). Best docking model from AutoDock Vina is used to submit to the Protein-Ligand Interaction Profiler (PLIP) server (Salentin et al. NAR 2015). Ap.LS, the most stable protein-ligand complex has the most hydrophobic interactions with the hydrophobic tail of GPP.

Fig. 6. Structure analysis for the understanding of activity and specificity. (A) The aligned 3D active site surfaces of Ap.LS, Sc.LNS and Ma.LS. The residues are listed in Table S4. Gray: conserved residues among the LSs of three kingdoms; green: not conserved in plant LS; blue: not conserved in bacterial LS; yellow: not conserved in fungal LS. (B) Highlight of the key residue difference between Ap.LS and Ap.LNS. Detailed comparison of binding-pocket residues in Table S5. (C) Mutation of the key residues responsible for Ap.LS specificity. (D) The interactions of the selected residues with FPP in Ap.LS wildtype (WT) and its mutants (distance by dash line, unit: Å). (E) Quantitative comparison of Ap.LS and its mutants.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Supporting information

Bioinformatics-Aided Identification, Characterization of Fungal Linalool Synthases and Applications in Linalool biosynthesis

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- Fig. S2. Mass spectra and retention indices for terpenes detected in this study.
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- Fig. S4. GCMS chromatograms and spectra for Galma_266794.
- Fig. S5. Amino acid sequence alignment and identity table of LSs and LNSs from fungi, bacteria and plants.
- Fig. S6. Expression and solubility analysis of the fungal LNSs and LS.
- Fig. S7. The optimization of Aa.LNS solubility with the co-expression of chaperone proteins.
- Fig. S8. The sequence alignment and their secondary structures of plant terpene synthases.
- Fig. S9. The sequence alignment and their secondary structures of microbial terpene synthases.

Table S1. Predicted active sites of terpene synthases with 4LXW and 5NX5 as the templates.

No.	Homologues	Predicted active sites by BioTransformer v0.9 or PyMOL v2.1.1																																						
0	consensus	X	L	X	M	X	F	V	D	E	T	D	V	E	Y	R	T	X	G	X	X	X	N	D	S	Y	X	E	X	X	H	N	W	X	W	S	R	Y		
1	5NX5 ¹	V	I	G	L	T	F	V	D	D	F	D	W	K	H	R	T	I	C	C	L	V	I	N	E	S	F	K	D	-	Q	H	N	F	Y	W	G	R	Y	
2	4LXW ¹	Y	V	M	S	T	F	V	D	D	H	D	Y	E	Y	R	T	F	A	H	W	A	A	N	D	S	L	K	E	-	E	H	N	W	V	F	H	R	Y	
3	D5SL78	V	I	G	L	T	F	V	D	D	F	D	W	K	H	R	T	I	C	C	L	V	I	N	E	S	F	K	D	-	Q	H	N	F	Y	W	G	R	Y	
4	D8RNZ9	I	Y	A	L	V	F	L	D	D	L	E	V	Q	Y	R	T	G	F	M	V	V	I	N	D	S	F	K	E	G	Y	H	N	V	S	W	S	R	Y	
5	Agr6	F	L	A	M	F	F	V	D	E	T	D	V	Q	Y	R	T	I	G	A	S	L	I	N	D	S	Y	V	E	R	D	H	N	W	N	W	S	R	Y	
6	Agr2	F	I	T	H	L	W	F	D	E	T	D	G	E	Y	R	T	S	G	A	C	I	F	N	D	S	Y	M	E	K	N	A	N	W	N	W	S	R	Y	
7	Agr3	S	L	T	L	L	F	H	D	N	S	D	V	Q	Y	R	T	S	G	C	S	V	T	N	D	S	Y	V	E	R	D	H	N	W	S	W	S	R	Y	
8	Agr5	F	S	N	T	L	W	L	D	E	T	D	V	E	Y	R	T	S	A	V	C	V	F	N	D	S	Y	M	E	K	S	A	N	W	N	W	S	R	Y	
9	Agrped1_109003	A	L	T	M	L	F	H	D	N	S	D	V	Q	Y	R	T	S	G	C	C	V	T	N	D	S	Y	V	E	R	D	H	N	W	S	W	S	R	Y	
10	Agrped1_640059	C	L	T	L	L	F	H	D	N	S	D	V	Q	Y	R	T	S	G	C	C	V	T	N	D	S	Y	V	E	K	D	H	N	W	S	W	S	R	Y	
11	Agrped1_665597	F	A	T	M	F	F	V	D	E	T	D	C	Q	Y	R	N	V	G	A	T	V	I	N	D	S	Y	R	E	T	D	H	N	W	N	W	S	R	Y	
12	Agrped1_693394	F	L	A	M	F	F	V	D	E	T	D	C	Q	Y	R	N	I	G	A	S	I	I	N	D	S	Y	K	E	T	D	H	N	W	N	W	N	R	Y	
13	Agrped1_694262	F	L	A	M	Y	F	V	D	E	T	D	V	E	Y	R	T	S	A	G	T	I	I	N	D	S	F	R	E	C	H	N	W	N	W	S	R	Y		
14	Agrped1_749682	N	L	C	V	L	F	V	D	E	S	D	V	E	F	R	N	S	A	V	C	V	C	N	D	S	Y	M	E	K	M	N	N	W	N	W	S	R	Y	
15	Agrped1_804996	-	-	F	M	F	F	V	D	E	T	D	C	Q	Y	R	N	V	G	I	S	I	I	N	D	S	Y	R	E	T	D	H	N	W	N	W	N	R	Y	
16	Agr8	-	L	A	M	Y	F	V	D	E	T	D	V	E	Y	R	T	C	G	A	T	I	I	N	D	S	Y	R	E	C	H	N	W	N	W	S	R	Y		
17	Agrped1_820868	L	L	A	M	Y	F	I	D	E	T	D	V	E	Y	R	T	C	G	A	T	I	I	N	D	S	Y	R	E	C	H	N	W	N	W	S	R	Y		
18	M413_27416	L	L	A	M	Y	F	V	D	E	T	D	V	E	Y	R	T	C	G	A	T	I	I	N	D	S	Y	R	E	C	A	H	N	W	N	W	S	R	Y	
19	Galma_266794	L	L	A	M	Y	F	V	D	E	T	D	V	E	Y	R	T	C	G	A	T	I	I	N	D	S	Y	R	E	C	A	H	N	W	N	W	S	R	Y	
20	Galma_63556	-	-	F	M	Y	F	A	D	E	T	D	I	E	Y	R	T	C	G	A	S	I	A	N	D	S	Y	L	E	R	D	H	N	C	Y	W	S	R	Y	
21	Hypsu1_148365	-	-	F	M	Y	F	A	D	E	T	D	V	E	Y	R	T	C	G	G	S	I	A	N	D	S	Y	L	E	R	D	H	N	C	Y	W	S	R	Y	
22	Hypsu1_148385	F	L	A	M	Y	F	A	D	E	T	D	V	E	Y	R	T	C	G	A	S	I	A	N	D	S	Y	L	E	R	D	H	N	C	Y	W	S	R	Y	
23	Agrped1_689671	F	L	G	M	Y	F	A	D	E	T	D	V	E	Y	R	T	C	G	A	S	I	A	N	D	S	Y	L	E	R	D	H	N	C	Y	W	S	R	Y	
24	Agrped1_689675	F	L	G	M	Y	F	A	D	E	T	D	I	E	Y	R	T	C	G	G	S	I	A	N	D	S	Y	L	E	R	D	H	N	C	Y	W	S	R	Y	
25	Galma_223690	F	L	G	M	Y	F	A	D	E	T	D	V	E	Y	R	T	C	G	A	S	I	A	N	D	S	Y	L	E	R	D	H	N	C	Y	W	S	R	Y	
26	Agrped1_804989	-	-	-	M	F	L	V	D	K	T	D	C	Q	Y	R	N	V	G	A	A	I	T	N	D	S	Y	K	E	T	D	H	N	W	N	W	N	R	Y	
27	Agr11	T	L	A	M	F	F	V	D	E	T	D	V	E	Y	R	T	S	G	A	M	T	-	-	-	-	-	-	R	S	A	H	N	A	E	W	A	R	Y	
28	Agr1	C	L	T	L	L	F	H	D	N	S	D	V	Q	Y	R	T	S	G	C	C	V	T	N	D	S	Y	V	E	K	H	H	N	W	S	W	S	R	Y	
29	Agr9	F	L	A	M	F	Y	V	D	E	T	D	V	E	Y	R	S	S	G	C	S	I	A	N	D	S	Y	M	E	R	E	H	N	W	N	W	T	R	Y	
30	Agr4	N	L	C	V	L	F	V	D	E	S	D	V	E	F	R	N	S	A	V	C	V	C	N	D	S	Y	M	E	K	S	N	N	W	N	W	S	R	Y	
31	Agr7	F	L	A	M	F	F	V	D	E	S	D	V	Q	Y	R	T	I	G	A	S	L	C	N	D	S	Y	V	E	R	D	H	N	W	N	W	S	R	Y	
32	AAE3_109435	T	L	A	M	F	F	V	D	E	T	D	V	E	Y	R	T	S	G	A	M	Y	C	N	D	S	Y	S	E	S	A	H	N	A	E	W	A	R	Y	
33	Agr10	T	L	A	M	F	Y	V	D	E	A	D	W	K	-	-	-	-	-	T	I	G	N	D	S	Y	M	K	-	-	-	R	W	N	W	T	R	Y		
34	Agrped1_705454	P	G	H	Y	I	V	M	D	D	T	G	L	Q	F	Y	T	T	K	G	A	V	L	T	N	P	G	F	Y	E	R	N	A	E	W	K	W	A	M	Y
35	Q8H2B4 ²	-	-	R	W	I	I	T	D	D	Y	D	Y	E	Y	A	T	I	G	A	V	L	R	D	D	F	R	K	L	-	Y	-	-	-	-	-	-	-	-	

Murolene/cadinene synthase cluster and LS/LNS cluster predicted or characterized in this study were shown in blue and green, respectively (consistent with Fig. 2). The non-functional synthase Agr10 and Agr11 were highlighted in red.

¹ The two proteins were used as templates: D5SL78, Sc.LNS from *Streptomyces clavuligerus*, (PDB ID: 5NX5) and Q9K499, Epi-isozaene synthase from *Streptomyces coelicolor* (PDB ID: 4LXW). For 4LXW, the ligands BTM, POP and MG were used for prediction of active sites. For 5NX5, the ligands 0FV and MG were used for prediction of active sites.

² Analyzed by PyMOL software version 2.1.1. The homologue model of Q8H2B4 was built on the structure of (+)-bornyl diphosphate synthase from *Salvia officinalis* (PDB entry ID, 1n1b/1n21) with Modeller software.

Table S2. Comparison and combination of the predicted active sites of the two templates 4LXW and 5NX5.

Index Position	52	55	56	76	79	80	81	83	84	86	87	153	156	171	175	178	179	180	181	184	217	218	221	222	225	226	228	229	232	235	237	238	299	303	306	307	312
4LXW_position		72	73	92	95	96		99	100	102	103	172	175	190	194	197	198	199		203	236	237	240	241	244	245	247	248	253	255	256	325	329	332	333	338	339
4LXW_residue		V	M	S	F	F		D	D	H	D	Y	E	Y	R	T	F	A		W	A	A	N	D	S	L	K	E	E	H	N	W	V	F	H	R	Y
5nx5_position	49	52		72	75	76	77	79	80		83				172	175	176	177	178	181	214		218		222		225	226	229	231			295	299	302		
5nx5_residue	V	I		L	T	F	L	D	D		D				R	T	I	C	C	L	V		N	S		K	D	Q	H			F	Y	W			
5NX5_m ¹	V	I	G	L	T	F	L	D	D	F	D	W	K	H	R	T	I	C	C	L	V	I	N	E	S	F	K	D	Q	H	N	F	Y	W	G	R	Y
4LXW_m ¹	Y	V	M	S	F	F	V	D	D	H	D	Y	E	Y	R	T	F	A	H	W	A	A	N	D	S	L	K	E	E	H	N	W	V	F	H	R	Y

¹ Here, individual active sites of 4LXM and 5NX5 were predicted with BioTransformer v0.9, which are identical to PyMOL software prediction. Furthermore, to cover more broadly in our model, the active sites of the two templates were merged with each other to obtain 4LXW_m and 5NX5_m, respectively.

Table S3. Summary of LSs and LNSs in this study.

No.	Accession no.	Entry/Gene name	Protein function	Organism	Length
1	Q84ZW8	ACSS_MAIZE	Nerolidol synthase	<i>Zea mays</i> (Maize)	590
2	P0CV94	NES1_FRAAN	Nerolidol synthase	<i>Fragaria ananassa</i> (Strawberry) (<i>Fragaria chiloensis</i> x <i>Fragaria virginiana</i>)	519
3	P0CV95	NES2_FRAAN	Nerolidol synthase	<i>Fragaria ananassa</i> (Strawberry) (<i>Fragaria chiloensis</i> x <i>Fragaria virginiana</i>)	578
4	P0CV96	NES1_FRAVE	Nerolidol synthase	<i>Fragaria vesca</i> (Woodland strawberry) (<i>Potentilla vesca</i>)	580
5	Q9SPN0	LLOS1_ARTAN	R-linalool synthase	<i>Artemisia annua</i> (Sweet wormwood)	567
6	Q84UV0	LINS_ARATH	S-linalool synthase	<i>Arabidopsis thaliana</i> (Mouse-ear cress)	569
7	Q6ZH94	LINS_ORYSJ	S-linalool synthase	<i>Oryza sativa</i> subsp. <i>japonica</i> (Rice)	595
8	Q8H2B4	LLOS_MENAQ	R-linalool synthase	<i>Mentha aquatica</i> (Water mint)	606
9	Q9SPN1	LLOS5_ARTAN	R-linalool synthase	<i>Artemisia annua</i> (Sweet wormwood)	583
10	Q5SBP3	LLOS_OCIBA	R-linalool synthase	<i>Ocimum basilicum</i> (Sweet basil)	574
11	Q96376	LIS_CLABR	S-linalool synthase	<i>Clarkia breweri</i> (Fairy fans) (<i>Eucharidium breweri</i>)	870
12	Q2XSC5	LALIN_LAVAN	R-linalool synthase	<i>Lavandula angustifolia</i> (Lavender)	564
13	R4I6S7	R4I6S7_9MAGN	S-linalool synthase	<i>Cinnamomum osmophloeum</i>	585
14	Q29VN2	TPS2_MAIZE	Nerolidol linalool synthase	<i>Zea mays</i> (Maize)	581
15	H6WBC5	H6WBC5_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	577
16	A0A068B0N9	A0A068B0N9_9ROSA	Nerolidol linalool synthase	<i>Prunus cerasoides</i> var. <i>campanulata</i>	618
17	A0A068B6B6	A0A068B6B6_9ROSA	Nerolidol linalool synthase	<i>Prunus cerasoides</i> var. <i>campanulata</i>	561
18	A0A072UZ75	A0A072UZ75_MEDTR	Nerolidol linalool synthase	<i>Medicago truncatula</i> (Barrel medic) (<i>Medicago tribuloides</i>)	570
19	B1NA84	B1NA84_ANTMA	Nerolidol linalool synthase	<i>Antirrhinum majus</i> (Garden snapdragon)	596
20	B1NA83	B1NA83_ANTMA	Nerolidol linalool synthase	<i>Antirrhinum majus</i> (Garden snapdragon)	566
21	Q5UB06	Q5UB06_MEDTR	Nerolidol linalool synthase	<i>Medicago truncatula</i> (Barrel medic) (<i>Medicago tribuloides</i>)	573
22	G5CV39	G5CV39_SOLLC	Nerolidol linalool synthase	<i>Solanum lycopersicum</i> (Tomato) (<i>Lycopersicon esculentum</i>)	563
23	G7KNU1	G7KNU1_MEDTR	Nerolidol linalool synthase	<i>Medicago truncatula</i> (Barrel medic) (<i>Medicago tribuloides</i>)	519
24	E5GAH7	E5GAH7_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	545
25	A0A1N7T9S5	A0A1N7T9S5_GOSHI	Nerolidol linalool synthase	<i>Gossypium hirsutum</i> (Upland cotton) (<i>Gossypium mexicanum</i>)	586
26	A0A061GH69	A0A061GH69_THECC	Nerolidol linalool synthase	<i>Theobroma cacao</i> (Cacao) (Cocoa)	583
27	E5GAH4	E5GAH4_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	545
28	G7INZ1	G7INZ1_MEDTR	Nerolidol linalool synthase	<i>Medicago truncatula</i> (Barrel medic) (<i>Medicago tribuloides</i>)	806
29	E5GAH1	E5GAH1_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	584
30	E5GA11	E5GA11_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	820
31	G7INZ4	G7INZ4_MEDTR	Nerolidol linalool synthase	<i>Medicago truncatula</i> (Barrel medic) (<i>Medicago tribuloides</i>)	819
32	E5GA10	E5GA10_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	840
33	A0A061GEP1	A0A061GEP1_THECC	Nerolidol linalool synthase	<i>Theobroma cacao</i> (Cacao) (Cocoa)	809
34	E5GAH3	E5GAH3_VITVI	Nerolidol linalool synthase	<i>Vitis vinifera</i> (Grape)	577
35	D8RNZ9	MTS22_SELML	Nerolidol linalool synthase	<i>Selaginella moellendorffii</i> (Spikemoss)	368
36	D5SL78	SCLAV_p1185	Nerolidol linalool synthase	<i>Streptomyces clavuligerus</i>	333
37	MN146034	AAE3_05024	Not functional	<i>Agroclybe aegerita</i> (<i>Cyclocybe aegerita</i>)	355
38	MN954676	AAE3_109435	Nerolidol/linalool synthase	<i>Agroclybe aegerita</i> (<i>Cyclocybe aegerita</i>)	384
39	JGI ID: Agrped1_689671	Agrped1_689671	Nerolidol/linalool synthase	<i>Agroclybe pediades</i>	343
40	JGI ID: Agrped1_689675	Agrped1_689675	R-linalool synthase	<i>Agroclybe pediades</i>	344
41	A0A067THX9	Galma_223690	Nerolidol/linalool synthase	<i>Galerina marginata</i>	343
42	A0A067T8I8	Galma_63556	Possible nerolidol/linalool synthase	<i>Galerina marginata</i>	330
43	A0A0D2NA50	Hypsu1_148365	Possible nerolidol/linalool synthase	<i>Hypholoma sublateritium</i>	337
44	A0A0D2NH86	Hypsu1_148385	Nerolidol/linalool synthase	<i>Hypholoma sublateritium</i>	344
45	A0A348B793	PpSTS25	Myrcene/linalool synthase	<i>Postia placenta</i>	332

Table S4. 3D alignment of active-site residues of Ap.LS, Sc.LNS and Ma.LS. Converted residues among the three enzymes are highlighted in green.

Yellow		Blue		Green	
Ap.LS_pos	Ap.LS_res	Sc.LNS_pos	Sc.LNS_res	Ma.LS_pos	Ma.LS_res
54	F				
56	L	49	V		
77	M	72	L	360	T
78	N	73	G	361	A
79	F	74	W	362	L
80	Y	75	T	363	D
81	F	76	F	364	D
82	A	77	L	365	V
83	F	78	F	366	Y
84	D	79	D	367	D
85	E	80	D	368	I
		82	F		
88	D	83	D		
149	Y				
153	I	150	W	438	Y
156	E	153	K	441	E
160	R	157	R	445	Y
171	Y				
175	R	172	R	460	A
178	T	175	T	463	T
179	C	176	I	464	I
		215	I	504	R
219	N	218	N	507	D
223	S	222	S	511	T
227	E	226	D	515	E
230	R			518	R
235	H				
309	Y	309	Y	579	A
315	L	316	G	586	G

Table S5. Comparison of residues in the substrate-binding pockets of Ap.LS and Ap.LNS. The different residues of the same position are highlighted in green.

LS Ap pos	LS Ap res	LNS Ap pos	LNS Ap res	LS Ap pos	LS Ap res	LNS Ap pos	LNS Ap res
54	F	53	F	215	I	214	I
56	L	55	L	216	A	215	A
57	L	56	L	219	N	218	N
58	G	57	G	220	D	219	D
59	A	58	S	223	S	222	S
60	L	59	M	224	Y	223	Y
73	S	72	S	226	L	225	L
74	C	73	C	227	E	226	E
75	D	74	D	230	R	229	R
76	L	75	L	233	D	232	D
77	M	76	M	235	H	234	H
78	N	77	N	236	N	235	N
79	F	78	F	295	C	294	C
80	Y	79	Y	299	Y	298	Y
81	F	80	F	302	W	301	W
82	A	81	A	304	Y	303	Y
83	F	82	F	305	E	304	E
84	D	83	D	306	T	305	T
85	E	84	E	309	Y	308	Y
87	T	86	T	310	Y	309	Y
88	D	87	D	313	N	312	N
149	Y	148	Y	315	L	314	L
153	I	152	V	316	E	315	Q
156	E	155	E	332	Y	331	Y
157	A	156	A				
160	R	159	R				
171	Y	170	Y				
175	R	174	R				
178	T	177	T				
179	C	178	C				
180	G	179	G				
181	G	180	A				
184	S	183	S				

Table S6. Primers used for Ap.LS mutation. The mutational regions are highlighted in red and caps.

Mutations	Forward primers	Reverse primers
A59S	tgctgggtTCGctggttggtccgctgggtac	ccagCGAaccagcaggttgaaatcacatgc
L60M	tgggtgcaATGgttggtccgctgggtaccaa	caacCATtgcaaccagcaggttgaaatcaca
V61I	tgactgATTggtccgctgggtaccaaggaa	ggaccAATcagtgcaccagcaggttgaaat
G181A	ctgtggtgCgaaaccgtcgttctcgttctt	ggtttcGcaccacaggtgtcacggc
E316Q	aatggcctgCaaatccagaaaactcgtcaga	tctggatttGcaggccattttaccgtaata
A59S-L60M	gggtTcaAtggttggtccgctgggtac	gaccaaccaTtgAaccagcaggttgaa
A59S-L60M-V61I	ggTcaAtgAttggtccgctgggtacca	cggaccaaTcaTtgAaccagcaggttga

Fig. S1. Nucleic acid sequence of AAE3_109435 in the genome. The two primers (AAE3_9435f and AAE3_9435r) used for PCR amplification were highlighted here.

Terpenes	Calculated RI	Literature RI
linalool	1099	1086
nerolidol	1567	1564
geraniol	1259	1256
γ -muurolene	1486	1472
germacrene D	1498	1481
(+)- δ -cadinene	1527	1516

Fig. S2. Mass spectra and retention indices for terpenes detected in this study. Retention indices (RIs) were calculated by calibrating with GC-MS with a C8–C30 alkane mix and compared with literature data in National Institute of Standards and Technology database.

Names in this study	Entry	E-value	Score	Identity	Gene names	Organism
Galma_223690	A0A067THX9	0	1,461	77.20%	GALMADRAFT_223690	Galerina marginata (strain CBS 339.88)
Galma_63556	A0A067T8I8	0	1,324	71.60%	GALMADRAFT_63556	Galerina marginata (strain CBS 339.88)
Hypsu1_148365	A0A0D2NH86	3.10E-171	1,257	65.90%	HYPsudRAFT_148385	Hypholoma sublateritium FD-334 SS-4
Hypsu1_148385	A0A0D2NA50	1.30E-159	1,180	64.90%	HYPsudRAFT_148365	Hypholoma sublateritium FD-334 SS-4
Galma_266794	A0A067T571	3.60E-94	750	45.50%	GALMADRAFT_266794	Galerina marginata (strain CBS 339.88)
M413_27416	A0A0C2YLE7	6.90E-92	738	43.10%	M413DRAFT_27416	Hebeloma cylindrosporum h7

Agrped1_689675	1	MSSQIYIPDLLITWPQKVRNPLLQEVQDEANEWVKSFLVFEPEQFEKFKACDFNLLGAL	60
Galma_223690	1	MPSQFTIPDLLITWPQKEITNPMLHEVDAEANEWVQSLNLFEPKQFEKFKACNFNLLGSL	60
		* ** : ***** : ** : * : * : * : * : * : * : * : * : * : * : * : *	
Agrped1_689675	61	VGPLGTKEELRISCDLMNFYFAFDEYTDLASADEAKVIARDVMESFRHTDKPSHNKITEM	120
Galma_223690	61	VGPLPSRDHLRVSCDLMNFYFAFDEYTDMAKDEAMRIARDVMQAFRNTDTPSNSKITEM	120
		**** : : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Agrped1_689675	121	ARQFFERTINTVGNDPTGIEQFIADFDAYTTSIQEADDRASGHIRSVEDYFILRRDTCG	180
Galma_223690	121	ARQFFKRTIEVVGEDLPGIERFIADFDAYTRSVIQEADDRVAGHIRNVEDYFILRRDTCG	180
		***** : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
Agrped1_689675	181	GKPSFSFFGLGLNIPKEVFAHPMFISMATESATDLIAITNDMHSYNLEQSRGLDGHNVITA	240
Galma_223690	181	AKPSFSFYGLGLNIPTEVFEHPLLISMVESATDLIAVTNDMHSYGLEHSRGLDGHNVITA	240
		***** : ***** : * : * : * : * : * : * : * : * : * : * : * : *	
Agrped1_689675	241	IMHEYKINLQGALYWLSGYATKTIKFIKSDRKNLPSWGPVVDRAVEQYFDRVGRGVRGYD	300
Galma_223690	241	IMHEYQLDLQGALYWLSGYATKTIKFLTDKRNLPWGPFTIDKALEIYLDRLGRCVRGYD	300
		***** : : * : * : * : * : * : * : * : * : * : * : * : * : *	
Agrped1_689675	301	AWSYETKRYYGKNGLEIQKTRQITLRPLDPAYVTKEQLQVSMKA	344
Galma_223690	301	AWSYSTRYYGKNGLKVKQKTRITLKPDAAYITKDQLQVSI-	343
		***** : ***** : * : * : * : * : * : * : * : * : * : * : *	

Fig. S3. The alignment between Agrped_689675 and Galma_223690 and BLAST search results in UniProt database with Agrped_689675 (or Ap.LS). The search was done in March-2018 with the top 6 proteins listed here. The results may change today as new proteins are being deposited into UniProt database.

Fig. S4. GCMS chromatograms and spectra for Galma_266794. Retention indices (RIs) were listed with the calculated values on the left and literature values on the right. Germacrene D and γ -murolene are further verified by Cubeb essential oil. Cedrela woods oil was used as the authentic standard of (+)- δ -cadinene¹.

1. Aa.LS (fungal)
2. Sc.LNS, D5SL78(bacterial)
3. Zm.LNS, Q29VN2 (plant)
4. Ma.LS, Q8H2B4 (plant)

	1	2	3	4
1		15.2%	13.4%	10.0%
2	15.2%		7.8%	10.4%
3	13.4%	7.8%		24.0%
4	10.0%	10.4%	24.0%

Fig. S5. Amino acid sequence alignment and identity table of LSs and LNSs from fungi, bacteria and plants. The two conserved regions, aspartate-rich motif and NSE triad were highlighted in blue boxes. The sequence identity table indicated the large difference of LSs among different kingdoms. The overall sequence identity is very low, and the fungal LS is slightly more similar to the bacterial LNS than to the plant LS and LNS. Alignment was done with Clustal Omega program v1.2.4.

Fig. S6. (A) Expression and solubility analysis of the fungal LNSs and LS. (B) Solubility analysis for the fusion Aa.LNS with maltose binding protein (MBP) or thioredoxin (TrxA). M: protein ladder, TP: total proteins, SF: soluble fraction, In: induced cells, No: not induced as control.

Fig. S7. The optimization of Aa.LNS solubility with the co-expression of chaperone proteins. Two chaperone systems were used here: pGro7 expressing groES-groEL proteins, pTf16 expressing the trigger factor protein (tig). IPTG and arabinose were used to induce the expression of Aa.LNS and chaperones, respectively. It was found arabinose concentration (or chaperone expression levels) has invisible effect on the solubility of Aa.LNS. Hence, 3.3mM arabinose and 0.1 mM IPTG were used for the large-scale expression and purification experiments. M: protein ladder, TP: total proteins, SF: soluble fraction, In: induced, No: not induced as control.

Fig. S8. The sequence alignment and their secondary structures of plant terpene synthases. The three regions, arginine effector motif, aspartate-rich motif and NSE triad, were highlighted in black boxes. The four proteins were (1) O81192, PDB ID 1n1b/1n24, (+)-bornyl diphosphate synthase from *Salvia officinalis*; (2) Q8H2B4, (R)-linalool synthase from *Mentha aquatica* (Water mint); (3) Q29VN2, linalool/nerolidol synthase from *Zea mays* (Maize); (4) Q96376, S-linalool synthase from *Clarkia breweri* (Fairy fans). The figure was prepared with the ESPrnt 3.0 ².

Q9K499 1 10 20 30 40 50 60
 Q9K499 MHAFPTGTTATPTAIVP...LRFVTEAATF...RQLHRYWPKQCEITRT...LEKRLM...A
 D5SL78 MQEFE...FAV...FAP...SRVSDLARARARBLD...HAMDLVR...G
 D8RNZ9 MKD...LFRISGVTHFELLLFNH...FACHFQSGISLKIDK...FLGKMRIA...D
 Agrped1_689675 ...MSQIYIFDILLTWPWQKVRNELLQENQDEANE...VKSFV...JFE
 Agrped1_689671 ...MSQIYIFDILLTWPWQKVRNELLQENQDEANE...VKSFV...JFE
 AAE3_109435 ...STQLFTSTPTMQLI...DILLCSWQKGFENHYGAKAENT...RPLVVK...JFE
 Galma_223690 ...MPSQITFDILLTWPWQETTFMHLHEDAEANE...VQSLH...JFE
 Hypsul_148385 ...MSAQITFDILLAHNFWQRTVISMLEDNRDEANE...VMSLG...JFE
 consensus>70p.l...p.....n.wv.....e

Aspartate-rich motif

Q9K499 60 70 80 90 100 110 120
 Q9K499 DKVEETADGLCYTDIMAGYYGAFDF...QRIADYSANTY...DRHDEIVHSGAG...ANR
 D5SL78 EEARRRYEFSCVADIGAYGPHATGAD...DLCDVDLGT...DDQFADSGRERDALAVCA
 D8RNZ9 ETSEKKVLESRIGLYACMMHFAHRRERLVLAGKHINAVFL...DLLESSSKHEMPOIN...
 Agrped1_689675 ...PEQFEKPKACDFNLGLALVGLGTKEELRISCDLMNF...DEYTDASADEARVIA...
 Agrped1_689671 ...PEQFEKPKACDFNLGLALVGLGTKEELRISCDLMNF...DEYTDASADEARVIA...
 AAE3_109435 ...ERQKAFPAIDYITGLASMTYHHNKEELCVACDMMNLF...DEYTDIFPETAQRLA...
 Galma_223690 ...FQFEKPKACDFNLGLALVGLGTKEELRISCDLMNF...DEYTDANKDEAMRIA...
 Hypsul_148385 ...FQFEKPKACDFNLGLALVGLGTKEELRISCDLMNF...DEYTDANKDEAMRIA...
 consensus>70f...l...p...e.l...d.m...yf...e...f...e

Q9K499 120 130 140 150 160 170
 Q9K499 RIRGLHTALD...PGDHLHEDTVA...FQSDSR...LYAF...FA...TMAAFARHHT...
 D5SL78 ELTDLLWKG...TAAT.AASPFIVVAFSDC...MRAGMS...AWRRRTVHEWVDY...
 D8RNZ9 ...LITISLANNSDE.DYTNPLALALYDVRERTRAEAP...FLDRTYQVSGS...
 Agrped1_689675 ...RDVMEFRNT.DKP...SHNKLTEMAROFFERTINTVQND...TGIEQFIADFDAY...
 Agrped1_689671 ...RDVMEFRNT.EKP...AHNQLTEMAROFFERTINTVQND...TGIEQFIADFDAY...
 AAE3_109435 ...KIVVAMRNFEIAALGEDS...GTMTQFMRRAMTLLFNGCNSSES...CHIFIDYTEEY...
 Galma_223690 ...RDVMEFRNT.DTP...SNSKLTEMAROFFERTIEVYGED...FGIERFIADFDAY...
 Hypsul_148385 ...QGMHAFKRAEP...SSSKLTEMAROFFERTIVDVYGED...PALNLTIDEDY...
 consensus>70i.m.f...r.....e.f...y

Arginine effector motif

Q9K499 170 180 190 200 210 220
 Q9K499 IEANDRETHN...RGRIVPGVEEY...RLTFANHINTDLEPSS...GELPDARVHFAFYR
 D5SL78 LACWPTKILADRAHGAVIDPFAHRL...RRTTCR.FLFALAERVCGYEFPRANWSESLDG
 D8RNZ9 LEAVKQVHRAEMKQIFGVVEYKL...RAACGFHEAVGGIMTEFCIGIRLSQAOIQSPIFRE
 Agrped1_689675 ...TISILQEAODRASGHIRGVVEYFT...RRCCTGGK.PSF...SFFGILMIPKEVAFPMFIS
 Agrped1_689671 ...TISILQEAODRASGHIRGVVEYFT...RRCCTGGK.PSF...SFFGILMIPKEVAFPMFIS
 AAE3_109435 ...TAMTIRACD...SGGIVHRAVMDYLA...RACCTGAG.LMV...GLLEF...LDLPEVHKEVITQE
 Galma_223690 ...TAMTIRACD...SGGIVHRAVMDYFT...RRCCTGAG.PSF...SFFGILMIPKEVAFPMFIS
 Hypsul_148385 ...TAMTIRACD...SGGIVHRAVMDYFT...RRCCTGAG.PSF...SFFGILMIPKEVAFPMFIS
 consensus>70 ...v.e.dR.g.i.vedy...Rr.T...G.n.p.v.hp...

NSE triad

Q9K499 230 240 250 260 270 280
 Q9K499 RALLSSEFAAY...NDLC...DPR...AGDEVHNLGLSLITH...HSDT...EATISEVRRNHEEC
 D5SL78 RRTTISDAVIM...NELS...TEK...A...QHANLVLSLVH...GLTG...PEAVTFRCDLVQGS
 D8RNZ9 LLENSVSDV...NDLLS...RKE...Y...QDYHNNWISVLH...HSPR...TSFQVVDRLCEMIOAE
 Agrped1_689675 ...MIEGATDLTA...TNDMS...NL...ERGLDGHNVITAMHXYQLD...QCALYWLSGYATKT
 Agrped1_689671 ...MIEGATDLTA...TNDMS...NL...ERGLDGHNVITAMHXYQLD...QCALYWLSGYATKT
 AAE3_109435 ...LSTGALDMYC...TNDMS...ASE...SSGASHNVITVVMHERNLSL...QCAFONLASYAAGV
 Galma_223690 ...MVEGATDLTA...TNDMS...VGL...ERGLDGHNVITAMHXYQLD...QCALYWLSGYATKT
 Hypsul_148385 ...LVERATDLTA...TNDMS...VGL...ERGLDGHNVITAMHXYQLD...QCALYWLSGYATKT
 consensus>70 m...f...i...N\$shsy...g.havi...imh...l...q.a...

Q9K499 290 300 310 320 330
 Q9K499 RTFFAVRDRDTRFADEAD...GTVRKKE...S...AVRANVGNRRN...F...VYVNFHES
 D5SL78 IESFALRLGGLFELGERALGV...EGAVLDRYADALSAFCRGYH...GRGASRYTTRD
 D8RNZ9 ELSDIALARKKIAO...E...EGGS...DSE...UTKEFARFFMVASGSLV...GYVTRVHGYG
 Agrped1_689675 ...IARFISDPKRLPSMGVVD...AVEQVDFVGRGVGYDA...GYVTRVHGYG
 Agrped1_689671 ...IARFISDPKRLPSMGVVD...AVEQVDFVGRGVGYDA...GYVTRVHGYG
 AAE3_109435 ...ISKFVLDKRLHSMGFTDOK...ALEIYLDRLGRGVGYDA...GYVTRVHGYG
 Galma_223690 ...IARFISDPKRLPSMGVVD...AVEQVDFVGRGVGYDA...GYVTRVHGYG
 Hypsul_148385 ...IARFISDPKRLPSMGVVD...AVEQVDFVGRGVGYDA...GYVTRVHGYG
 consensus>70 i...f...p...e.y.d...vrg...W.y.v.r.v.g...

Q9K499 340 350 360
 Q9K499 GRVVD...WDDRS...F...F...YVNNH...AGEK...
 D5SL78 HFGLGL...ENLVARSSG...
 D8RNZ9 HELLTGEIFSGTWLLHFMATVVL...FSKFRMOTMRFLAPKKRDSFP
 Agrped1_689675 ...GLEIQXTR...QITLRLDPAYVITEQLQVSMKA...
 Agrped1_689671 ...GLEIQXTR...RITLRLDPAYVITEQLQVSMKA...
 AAE3_109435 ...GPQIKLTK...MLTIRPQVKDNYAKQAPRVEAPERPMLLEV...RS
 Galma_223690 ...GLKVGQXTR...RITLRFDAAYITKQQLQVSA...
 Hypsul_148385 ...GLQVQXTR...QITLRLDPAYVITEQLQVSMKA...
 consensus>70 g...t...p...

Fig. S9. The sequence alignment and their secondary structures of microbial terpene synthases. The three regions, arginine effector motif, aspartate-rich motif and NSE triad, were highlighted in black boxes. The eight proteins were (1) Q9K499 (PDB ID 4LXW), Epi-isozizaene synthase from *Streptomyces coelicolor*; (2) D5SL78, Sc.LNS from *S. clavuligerus*; (3) D8RNZ9, LNS from Spikemoss; (4) Ap.LS; (5) Ap.LNS; (6) Aa.LNS (7) Gm.LNS; (8) Hs.LNS. The figure was prepared with the ESPrnt 3.0 ².

Reference

1. Agger, S., Lopez-Gallego, F. & Schmidt-Dannert, C. Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. *Molecular microbiology* **72**, 1181-1195 (2009).
2. Robert, X. & Gouet, P. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Research* **42**, W320-W324 (2014).

Figures

Figure 1

Characterization of fungal linalool and nerolidol synthases (LNSs) and a fungal linalool synthase (LS) in *E. coli*. (A) GC-MS chromatograms of cultural supernatants of strains GPPS+9435, FPPS+9435 (co-expressing AAE3_109435 and GPP or FPP synthase) and the control strains GPPS_ctrl and FPPS_ctrl (expressing only GPP or FPP synthase). (B) Chiral separation of linalool produced by Aa.LNS and Ap.LS (Agrped1_689675) expressing strains. (C) Phylogenetic tree based on full-sequence alignment of Aa.LNS and terpene synthases (TPSs) in *A. pediades*. AAE3_109435 (a LNS) is marked with "=", Agr8 (a g-murolene/b-cadinene synthase) with "⬢". (D) GC-MS chromatograms of the supernatant of *E. coli* clones producing Agrped1_689671 and Agrped1_689675.

Figure 3

In vitro characterization of Aa.LNS and Ap.LS. (A) SDS PAGE gel image of purified Aa.LNS and its kinetic analysis. (B) Protein gel of purified Ap.LS and its kinetic analysis (No product was observed for FPP). (C) In vivo activity comparison of LSs from a fungus, a bacterium and a plant (Error bars, mean $\pm$ s.d., $n=3$). Ap.LS, Sc.LNS and Cb.LS from *Clarkia breweri* (Q96376) were selected as the representative of kingdoms fungi, bacteria and plantae, respectively.

Figure 4

Phylogenetic tree of LNSs and LSs in plants, fungi and bacteria. Two major clades, plant and microbial, were clearly segmented. (R)-linalool synthases were marked with “◻”, (S)-linalool synthases with “◻”, LNSs with “=” and nerolidol synthases with “◻”. PpSTS25 is a myrcene/linalool synthase (MLS) from *Postia placenta* 20. For plant and bacterial enzymes, UniProt accession number were used. D8RNZ9, more similar to the bacterial LNS than plant ones, was a LNS isolated from the nonseed plant *Selaginella moellendorffii* (Spikemoss). Details of all the enzymes are in Table S3.

Figure 5

Structural comparison of LSs in bacteria, fungi and plants. (A) The overlay of 3D structure (grey ribbon) and sub-strate-binding pocket of Ap.LS. Energy minimized homology model was used with 4LXW as template. GPP and FPP from 2ONG and 6A2C respectively were structurally aligned to 4LXW. Binding pocket surfaces for fungal (B), bacterial (C) and plant (D) LSs. GPP (2-fluorogeranyl diphosphate) and FPP ligands are shown as green and magenta spheres, respectively. The solvent-accessible surface of residues in the binding pocket is colored yellow (hydrophobic), cyan (polar), red (negative) or blue (positive). Best docking model from AutoDock Vina is used to submit to the Protein-Ligand Interaction Profiler (PLIP) server (Salentin et al. NAR 2015). Ap.LS, the most stable protein-ligand complex has the most hydrophobic interactions with the hydrophobic tail of GPP.

Figure 6

Structure analysis for the understanding of activity and specificity. (A) The aligned 3D active site surfaces of Ap.LS, Sc.LNS and Ma.LS. The residues are listed in Table S4. Gray: conserved residues among the LSs of three kingdoms; green: not conserved in plant LS; blue: not conserved in bacterial LS; yellow: not conserved in fungal LS. (B) Highlight of the key residue difference between Ap.LS and Ap.LNS. Detailed comparison of binding-pocket residues in Table S5. (C) Mutation of the key residues responsible for Ap.LS specificity. (D) The interactions of the selected residues with FPP in Ap.LS wildtype (WT) and its mutants (distance by dash line, unit: Å). (E) Quantitative comparison of Ap.LS and its mutants.

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