Sesquiterpene Cyclases from the Basidiomycete Cerrena unicolor

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Abstract: Hundreds of terpenoids have been isolated from Basidiomycota, among them are volatile mono- and sesquiterpenes with amazing sensory qualities, representing a promising alternative to essential oils from endangered plant species. Sesquiterpene synthases (STS) appear to be an abundant class of enzymes in these fungi. The basidiomycete Cerrena unicolor, a known sesquiterpene producer, was in silico screened for sesquiterpene cyclases via homology Basic Local Alignment Search Tool searches. Cyclase genes identified were cloned and heterologously expressed in Escherichia coli Bl21 using pCOLD I as the expression vector. Ten cyclases were successfully produced and purified, and their identity was confirmed using amino acid sequencing of tryptic peptides by nano-liquid chromatography-high resolution-electrospray ionization-tandem mass spectrometry. Gas chromatography/mass spectrometry analysis was applied to characterize these cyclases according to the formation of sesquiterpene hydrocarbons and oxidized terpenoids. Bioinformatic characterization and phylogenetic determination allowed for the classification of these diverse fungal enzymes. A representative single and a multi-product STS, respectively, were further analyzed for their dependency from divalent metal cations as a cofactor for the catalytic activity.

Keywords: sesquiterpenes; terpene cyclases; metal ion dependency; phylogenetic analysis; heterologous expression; Cerrena unicolor

1. Introduction

Fungi and, in particular, the division of Basidiomycota, are known to exhibit amazing metabolic diversity. They produce a wide range of complex natural compounds of industrial interest; among them are polyketides and terpenoids [1]. While polyketides have been studied intensively in the past, terpenoids and their biosynthesis have received less attention [2]. Although structurally highly diverse, with more than 95,000 terpenoid compounds known, this group of natural products is based upon the same building blocks [3]. Mono (C<sub>10</sub>)-, sesqui (C<sub>15</sub>)-, di (C<sub>20</sub>)-, sester (C<sub>25</sub>)-, and tri (C<sub>30</sub>)-terpenes result from the chain elongation of five-carbon isoprene starters, namely dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) [4]. The ability to form such a diversity of products out of small linear precursors is largely caused by the catalysis of two types of terpene synthases (TPS), prenyltransferases and terpene cyclases. Whereas prenyltransferases are responsible for chain elongation, cyclases convert linear C<sub>5</sub>-isoprenoid pyrophosphates into structurally complex hydrocarbons containing multiple rings and stereocenters. Cyclic sesquiterpenoids, for instance, originate from C<sub>15</sub> farnesyl pyrophosphate (FPP), which represents the structural core of a multitude of functionalized, bioactive sesquiterpenoids.

Sesquiterpenoids serve as antibiotics [5], antifouling agents, [6] or anti-cancer drugs [7]. Furthermore, they are widely utilized as flavors and fragrances in the food and cosmetic industries [8–10]. Thus far, these sought-after compounds are derived mostly from plant essential oils, but these can be exploited only once a year. As quality and harvest depend on variable factors such as climate, pests, transport, and storage conditions, and the recovery...
from plant raw materials is costly and time consuming [11], alternative bioprocesses are of increasing interest [12]. Cell cultures of plant cells are known to dedifferentiate and stop secondary pathways under typical culture conditions. Whole-cell conversion systems may produce highly desired products, starting from simple sugars and/or nitrogen-sources, but the product yield is typically very low. An alternative to natural sources is chemosynthesis. However, limited stereoselectivity, harsh reaction conditions, and the usage of poisonous transition metals are the main disadvantages. Thanks to modern developments in the field of molecular biology, the identification of suitable STS for terpene production has been simplified [13]. Several whole-genome projects have generated a large pool of uncharacterized, and, therefore, unexploited genes in the kingdom of fungi [14,15]. To explore the potential of fungal enzymes in biotechnological applications, their catalytic mechanisms must be characterized. Heterologous expression and functional characterization will open the gate for industrial implementation.

*Cerrena unicolor* (Cun), a poroid fungus of the genus *Cerrena*, is classified as a white-rot fungus. So far, this fungus has been studied for the production of manganese peroxidases and lignin-degrading laccases [16,17]. In a preliminary study, Cun attracted attention as a producer of several sesquiterpenoids [18]. The aim of this work was the in silico identification of suitable STS for terpene production has been simplified [13]. Several whole-genome projects have generated a large pool of uncharacterized, and, therefore, unexploited genes in the kingdom of fungi [14,15]. To explore the potential of fungal enzymes in biotechnological applications, their catalytic mechanisms must be characterized. Heterologous expression and functional characterization will open the gate for industrial implementation.

2. Results and Discussion
2.1. Identification of Genes

Two general classes of terpene cyclases are distinguished based on the folds (α, β, γ) and the metal binding motifs. Class I cyclases show catalytic activity in an α domain, where a trinuclear metal binding side (Mg$^{2+}$ A, B, C) is located, which consists of conserved D(D/E)XXD (Mg$^{2+}$ A, C) motifs and an NSE/DTE (Mg$^{2+}$ B) motif. Catalysis by class II cyclases is located in a β-domain, and the aspartic acid responsible for the protonation of a terminal double bond of the isoprenoid is found in the center of the conserved signature sequence motif DXDD [3].

To locate the putative sesquiterpene synthases (STS) in the genome of the basidiomycete *Cerrena unicolor*, Basic Local Alignment Search Tool (BLAST) analyses based on these motifs were performed. Genomic data (Accession number PRJNA207864) were provided by the Joint Genome Institute [19]. A BLAST search within the genome of Cun resulted in 14 putative STS. BLAST searches with consensus sequences have already been used for the identification of potential terpene synthases (TPS) within fungal genomes, but the majority of these putative TPS have not yet been expressed and biochemically characterized [20]. To gain insight into the biochemistry of STS from Cun, all 14 putative cyclases were selected for cloning and expression in *Escherichia coli* (E. coli). As shown in Figure 1, 10 active STS (see also Section 2.2) contained sequences of conserved motifs typical for the TPS family.

The first conserved motif is the aspartate-rich D(D/E)XXD sequence coordinating the Mg$^{2+}$ (A, C) cluster, essential for the catalytic hydrolysis of the pyrophosphate group of the terpenoid substrate, together with a highly conserved NSE triad [21]. This suggests that all STS are class I cyclases. A highly conserved arginine residue, suspected as a pyrophosphate sensor and an arginine-tyrosine dimer close to the C-terminus, involved in pyrophosphate binding, was described for TPS [22,23]. Interestingly, the pyrophosphate sensor R was found to be a single arginine in the upstream position of the NSE motif in *Trichoderma viride* J1-030 [24]. However, for all Cun TPS a highly conserved RR-dimer was observed at the respective position. Furthermore, highly conserved motifs were detected in the multiple sequence alignment (Figure 1). An RF dimer and an AXXR motif were located upstream of the before mentioned pyrophosphate sensor. Their role in forming the active site cavity or the reaction cascade of the respective STS is not yet known and needs to be clarified by X-ray crystallography.
Figure 1. Sequence homology modeling of putative TPS of Cun. Conserved motifs are framed in red. Asterisks indicate conserved residues, colons equivalent residues and dots partial residue conservation.
2.2. Heterologous Production and Functional Characterization

The predicted coding sequences (Figure 1) were used for the design of specific primers. Isolated mRNA was translated into cDNA, an N-terminal 6× His-tag was attached and used for the amplification of the respective STS genes. After cloning into the pCOLD I expression vector, the resulting plasmids were transformed into *E. coli* BL21 (DE3) for recombinant gene expression and enzyme production. In contrast to plant derived STS, *E. coli* is often used as an expression host for fungal STS [25–27]. In total, 10 out of 14 putative STS from Cun were expressed functionally. To confirm the respective cyclases’ identity, the proposed STS were isolated via Ni-NTA and separated using native PAGE. Bands of the overproduced proteins were cut out and subjected to tryptic digestion, followed by peptide mass fingerprinting using nano-LC/QTOF/MS-MS (Table 1). Sequence coverages ranging from 45% to 86% were achieved, thereby confirming—with a different degree of similarity—the identity and successful overexpression of all STS. The generally rather high sequence coverage was remarkable.

**Table 1. Results of peptide mass fingerprinting.**

<table>
<thead>
<tr>
<th>STS</th>
<th>Molecular Mass (kDa)</th>
<th>Sequence Coverage (%)</th>
<th>Score</th>
<th>Matches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cun3157</td>
<td>40.4</td>
<td>70</td>
<td>40,853</td>
<td>603</td>
</tr>
<tr>
<td>Cun7050</td>
<td>38.8</td>
<td>67</td>
<td>15,743</td>
<td>256</td>
</tr>
<tr>
<td>Cun3574</td>
<td>38.8</td>
<td>86</td>
<td>30,151</td>
<td>568</td>
</tr>
<tr>
<td>Cun3158</td>
<td>38.2</td>
<td>58</td>
<td>12,608</td>
<td>229</td>
</tr>
<tr>
<td>Cun0759</td>
<td>43.0</td>
<td>69</td>
<td>18,445</td>
<td>339</td>
</tr>
<tr>
<td>Cun9106</td>
<td>45.7</td>
<td>62</td>
<td>8345</td>
<td>188</td>
</tr>
<tr>
<td>Cun5155</td>
<td>38.8</td>
<td>72</td>
<td>10,039</td>
<td>213</td>
</tr>
<tr>
<td>Cun3817</td>
<td>46.4</td>
<td>45</td>
<td>5341</td>
<td>102</td>
</tr>
<tr>
<td>Cun0773</td>
<td>38.2</td>
<td>46</td>
<td>4622</td>
<td>100</td>
</tr>
<tr>
<td>Cun0716</td>
<td>38.3</td>
<td>65</td>
<td>12,289</td>
<td>288</td>
</tr>
</tbody>
</table>

1 Calculated based on the AA sequence.

For functional characterization, the purified cyclases were incubated with FPP and the resulting volatile products were analyzed by GC-MS. Adjusted for control incubation with heat inactivated enzymes, products exhibiting a molecular ion at *m/z* 204 and fragment ions at *m/z* 91, 105, 119, and 161 were indicative of an enzymatic formation of cyclic C15 hydrocarbons. These products were identified by comparing both their electron impact mass spectra and linear retention indices with the published data (NIST spectral library; NIST online data base) (Table 2).
Table 2. Identified products of heterologous STS from *C. unicolor*. Product ratios are given to highlight the main products.

<table>
<thead>
<tr>
<th>STS</th>
<th>Cyclic Terpene Products</th>
<th>RI Polar (Lit.)</th>
<th>RI Non-Polar (Lit.)</th>
<th>Product Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cun3157</td>
<td>β-Cubebene A</td>
<td>1547 (1540)</td>
<td>1385 (1388)</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>Germacrene D A</td>
<td>1720 (1719)</td>
<td>1478 (1476)</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>γ-Amorphene A</td>
<td>1488 (1491)</td>
<td>1515 (1521)</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>δ-Cadinene A</td>
<td>1764 (1762)</td>
<td>1625 (1625)</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Epicubebenol A</td>
<td>2084 (2072)</td>
<td>1644 (1647)</td>
<td>14.1</td>
</tr>
<tr>
<td>Cun7050</td>
<td>δ-Cadinol A</td>
<td>2212 (2205)</td>
<td>1644 (1647)</td>
<td>100</td>
</tr>
<tr>
<td>Cun3574</td>
<td>α-Copaene A</td>
<td>1495 (1502)</td>
<td>1372 (1375)</td>
<td>100</td>
</tr>
<tr>
<td>Cun3158</td>
<td>β-Cubebene A</td>
<td>1546 (1540)</td>
<td>1390 (1388)</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Germacrene D A</td>
<td>1720 (1719)</td>
<td>1477 (1476)</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>γ-Amorphene A</td>
<td>1488 (1491)</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>δ-Cadinene A</td>
<td>1765 (1762)</td>
<td>1515 (1521)</td>
<td>61.6</td>
</tr>
<tr>
<td>Cun5765</td>
<td>none</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Cun0759</td>
<td>α-Muurolene A</td>
<td>1729 (1711)</td>
<td>1495 (1500)</td>
<td>100</td>
</tr>
<tr>
<td>Cun9106</td>
<td>unknown</td>
<td>1512</td>
<td>1372</td>
<td>100</td>
</tr>
<tr>
<td>Cun5155</td>
<td>Aromadendrene B</td>
<td>1632 (1608)</td>
<td>1432 (1442)</td>
<td>100</td>
</tr>
<tr>
<td>Cun6114</td>
<td>none</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Cun3817</td>
<td>γ-Cadinene A</td>
<td>1767 (1745)</td>
<td>1511 (1513)</td>
<td>100</td>
</tr>
<tr>
<td>Cun0773</td>
<td>Germacrene D A</td>
<td>1718 (1707)</td>
<td>1478 (1476)</td>
<td>100</td>
</tr>
<tr>
<td>Cun0716</td>
<td>α-Muurolene A</td>
<td>1730 (1727)</td>
<td>1497 (1500)</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>δ-Cadinol A</td>
<td>2209 (2205)</td>
<td>1646 (1647)</td>
<td>92.1</td>
</tr>
<tr>
<td>Cun7487</td>
<td>none</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Cun0802</td>
<td>none</td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

A = Matched with library spectrum (NIST database); B = matched with EI-MS of an authentic standard; Lit. = compared with RI from NIST online database.

Cun3157 produced β-cubebene as the main product and germacrene D, γ-amorphene, δ-cadinene, and epicubebenol as the side products, whereas Cun7050 and Cun0716 produced δ-cadinol respectively, with α-muurolene as a side product for the latter STS. Cun5765, Cun6114, and Cun7487 were found to be inactive and, therefore, were not further addressed. The missing activity might be explained by difficult intron prediction [28]. Despite the usage of a web application such as Augustus, some recombinant STS were found inactive [29]. For example, Cop5, an STS from *Coprinus cinereus* remained inactive, although a defined splicing model was used for gene prediction [30]. The remaining STS catalyzed the formation of a single product: Cun3574 α-copaene, Cun0759 α-muurolene, while Cun5155, Cun3817 and Cun0773 catalyzed the formation of aromadendrene, γ-cadinene, and germacrene D, respectively. The cloning of Cun0802 was not successful. The gene clusters of secondary metabolism are tightly regulated, which may have resulted in low transcript levels [31,32]. Cun3158 produced the same sesquiterpenes as Cun3157, except epicubebenol, but with significantly less activity. Deduced from its EI-MS spectrum, the product of Cun9106 was most likely a cyclic sesquiterpene as well, but it did not match with any database entry and thus remains unidentified.

2.3. Phylogenetic Determination

Until now, 86 basidiomycota and 240 ascomycota have already been analyzed for homologous STS in silico, totaling to 1133 putative STS [20]. Among them, only 40 STS were functionally characterized. After performing a phylogenetic analysis of basidiomycetous STS, four clades (clade I–IV) were distinguished (Figure 2).
Figure 2. Phylogenetic determination of sesquiterpene synthases from basidiomycota. Unrooted neighbor-joining phylogram of 37 experimental characterized STS. Included are STS protein sequences from *Antrodia cinnamomea* (AcTPS) [33], *Armillaria gallica* (ArmGa) [34], *Boreostereum vibrans* (BvCS) [35], *Coprinopsis cinereus* (Cop) [30], *Coniophora puteana* (Copu) [26], *Formitopsis pinicola* (Fompi) [25], *Lignosus rhinoceros* (GME) [36], *Stereum hirsutum* (Stehi) [27], *Omphalotus olearius* (Omp) [25], and *Cerrena unicolor* (Cun). Different cyclization mechanisms were found for the clades. Clades 1 and 2 follow an initial 1,10-cyclization, clade 3 follows a 1,6-cyclization, and clade 4 follows a 1,11-cyclisation. Modified after [26].

This clustering leads to the presumption that the STS within one clade are catalyzing similar cyclization reactions. Comparing the main reaction products of respective cyclases within one clade, four different underlying cyclization mechanisms became obvious. Clade one contains the STS Cop4, Omp4, Omp5a, and Omp5b, all known for the transformation of FPP via nerolidyl diphosphate (NPP) 1,10 ring closure; a 1,11 H-shift; and a 1,6-ring closure, ending up with the bicyclic cadinyl cation. Cun3157 and Cun3158 are part of this clade.

An initial 1,10-cyclization of FPP forms an *E,E*-germacradienyl cation with cyclases of clade two. AcTPS5, Cop1, and Omp1 share this clade with Cun0716, Cun0759, Cun3574, Cun0773 and Cun 7050. In contrast, clade three follows a 1,6 cyclization, forming an intermediate bisabolyl cation. None of the active heterologous cyclases from Cun fit into this clade. STS grouped in clade four share a 1,11-cyclisation mechanism. Six cyclases of this clade produce the tricyclic Δ6-protoilludene. In contrast, GME9210 produces 2H-2,4a-ethanonaphtalene and Cun5155 catalyzes the production of the tricyclic aromadendrene.
In Scheme 1, the initial cyclization steps performed by STS from the four clades are shown. The three types of initial cyclization’s all include the loss of diphosphate and result in a primary carbocation intermediate. From there, several reaction steps such as H-shifts and additional cyclization’s take place to form the final sesquiterpene products.

Scheme 1. Initial cyclization reactions of STS. FPP: farnesyl diphosphate.

2.4. Temperature Optimum and Metal Ion Dependence of Cun3157 and Cun7050

In the past, large differences between single product and multi-product STS have been observed. Therefore, two cyclases, Cun3157 (multi-product) and the high-yielding Cun7050 (single product), were selected for further biochemical characterization to disclose the differences. The same experimental conditions as for STS product identification were used to evaluate the impact of the incubation temperature on enzyme activity as a function of product formation. For visualization, the total peak area was plotted against the incubation temperature (Figure 3).

Figure 3. Temperature optimum of Cun3157 and Cun7050.

For Cun7050, solely producing δ-cadinol, the activity increased steadily until maximal activity was reached at 40 °C incubation temperature. At higher temperatures, the activity decreased, with 20% remaining activity at 60 °C. Compared to that, Cun3157 produced five different sesquiterpenes and was found to be less affected by the temperature. Activity fluctuated within a range of ±10% between 20 °C and 50 °C, with a maximum at 40 °C. By increasing the temperature up to 60°C the activity remained at approximately 65%. For the following experiments, incubations were carried out at 40 °C.

The effect of metal ions on the catalytic efficiency of terpene synthases is gaining more and more interest. It is well-known that STS require Mg²⁺ coordination in the active site
The effect of metal ions on the catalytic efficiency of terpene synthases is gaining prominence. Reported high-resolution X-ray crystal structures show the mechanistic role of metal cofactors, for example, epi-isozizaene synthase from *Streptomyces coelicolor* [37]. Another study showed that Mn$^{2+}$ is a suitable cofactor too [38]. Monoterpene synthases were shown to be promiscuous in terms of the required divalent cations [39]. Furthermore, it was shown that the presence of different metal ions altered the product ratio of the prenyl diphosphate synthase from *Phaedon cochleariae* significantly [40]. In the presence of Mg$^{2+}$, farnesyl diphosphate was the major product with a yield of 82%, whereas in the presence of Co$^{2+}$ ions, geranyl diphosphate was the preferred product with a yield of 96%. Additionally, plant STS showed activity in the presence of different metal ions. However, STS from fungi have rarely been investigated for their acceptance of metal ions other than Mg$^{2+}$.

Cun3157 and Cun7050 were incubated with FPP in the presence of different metal ions. Activity was monitored as product compounds detected in the GC-MS chromatograms and plotted against the metal ions used (Figure 4).

As expected, controls (without enzyme or metal ion) showed no product formation. For the multi-product STS, Cun3157, incubation in the presence of 20 mM Mn$^{2+}$, Co$^{2+}$, or Ni$^{2+}$, 40–60% relative activity was detected compared to 20 mM Mg$^{2+}$ (100%). Supplementation with Ca$^{2+}$, Cu$^{2+}$, or Zn$^{2+}$ depleted almost all activity. By using Mn$^{2+}$, a slight change from $\beta$-cubebene to germacrene D as the main product was observed. Supplementation with Co$^{2+}$ and Ni$^{2+}$ resulted in the formation of the sesquiterpene alcohol epicubenol as the main product. For Cun7050, chosen as a representative for a single-product STS because of its high catalytic efficiency, out of all divalent metal ions tested, only Mn$^{2+}$ showed a relative activity of merely 3% compared to Mg$^{2+}$. For all other divalent cations, no activity was detected.

In summary, the multi-product cyclase Cun3157 showed a distinctly broader acceptance for metal ions. A study by Ronnebaum et al. proposed a correlation between the volume of the active cavity and product promiscuity of STS, which might also be an explanation for the higher metal ion acceptance of Cun3157 [37]. Another unsolved question is whether the formation of sesquiterpene alcohols is part of enzyme catalysis, or a subsequent, merely chemical attack of water on a cationic intermediate. In the future, more X-ray data of crystallized fungal STS or AI based 3D-structure models (Alpha Fold 2) will allow for deeper insight into the correlation of the geometry of the active site and its surroundings, and the resulting reaction mechanisms and sesquiterpene products.
3. Materials and Methods

3.1. Chemicals, Reagents, and Strains

All chemicals were purchased from Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany) if not otherwise indicated. Enzymes for molecular biology were purchased from Thermo Fisher Scientific (St. Leon-Roth, Germany). The pUC57 vector and the pCOLDI vector were purchased from Merck KGaA (Darmstadt, Germany). The E. coli strains BL21 (DE3) and TOP10 were maintained in our laboratory. Oligonucleotides were synthesized using Microsynth Seqlab GmbH (Göttingen, Germany).

3.2. Gene Identification

To identify the presence of the STS sequences in the genome of Cerrena unicolor, the Basic Local Alignment Search Tool (BLAST) and the sequence of 33 known and functionally characterized STS from Basidiomycota were used. Based on this bioinformatic approach, 14 possible STS sequences could be identified.

3.3. Cloning of pCOLD I_STS Constructs

For the generation of cDNA encoding for the predicted STS, C. unicolor was cultivated and submersed in SNL medium (30 g/L D-glucose monohydrate, 3 g/L yeast extract, 4.5 g/L L-asparagine monohydrate, 0.5 g/L MgSO_4_, 1.5 g/L KH_2PO_4, 1 mL of trace element solution, pH 6.0 [0.08 g/L FeCl_3, 0.09 g/L ZnSO_4·7H_2O, 0.005 g/L CuSO_4·5H_2O, 0.027 g/L MnSO_4, and 0.4 g/L Titriplex III]). RNA was isolated using the plant and tissue RNA extracting kit from Analytic Jena (Jena, Germany). Afterwards, genomic DNA was digested with DNAse I (Thermo Fisher, Waltham, MA, United States), and cDNA was produced using FastGene® Scriptase II (Nippon Genetics, Düren, Germany). The generated cDNA was used for amplification with Phusion High Fidelity Polymerase (Thermo Fisher). Specific primers were designed based on the BLAST search (see Section 3.2) with restriction sites for FastDigest SacI and BamHI (Thermo Fisher). Chemocompetent E. coli TOP10 cells were transformed with the finished constructs by heat shock. After incubation at 37 °C, clones were selected for overnight incubation and plasmids were isolated with an innuPrep Plasmid Mini Kit 2.0 (Analytik Jena, Jena, Germany). Sequencing was performed by Microsynth Seqlab GmbH (Göttingen, Germany). E. coli BL21 (DE3) were transformed with correct constructs for expression. Glycerol stocks of cells were stored at −80 °C.

3.4. Cultivation and Expression

The open reading frames of each STS were re-amplified from the cloning vector by adding endonuclease restriction sites to the sequences. The amplified genes were ligated into the expression vector pCOLDI I and transformed into the expression host E. coli BL21 (DE3) using chemical competence. Transformed clones of each construct were cultivated on Luria/Miller media (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl), supplemented with the appropriate antibiotic (ampicillin, 100 µg/mL) at 37 °C and a shaking rate of 150 rpm. Precultures were growing in 5 mL cultures. Main cultures were inoculated to an OD_600 of 0.05 and grown until an OD_600 of 0.6–0.8 was reached. After induction with IPTG, the cultures were cold shocked and incubated at 17 °C, with a shaking rate of 150 rpm for another 20 h. Depletion of cells was performed at 5000 rpm at 4 °C for 30 min.

3.5. Enzyme Purification

The cells were re-suspended in binding buffer (50 mm MOPS, 500 mM NaCl, pH 7) and disrupted via sonification using an UP100 (Hielscher, Germany) at amplitude 50 and cycle 0.5 for 5 min. Produced STS were purified using metal affinity chromatography, desalted using 10 kDA MWCO columns (Vivaspin 500, Sartorius, Göttingen, Germany), and used for biocatalysis right after purification.
3.6. Biocatalysis

Transformation of farnesyl pyrophosphate was performed in reaction buffer (50 mM MOPS, 20 mM MgCl₂, and 25% glycerol) with a reaction volume of 1 mL containing 30 µM of FPP and incubation at room temperature for 15 min. Reactions were overlaid with 500 µL Hexane. The reaction was stopped by shaking. After centrifugation at maximum speed and 4 °C for 15 min, the hexane fraction was collected, dried with sodium sulfate, and analyzed via GC-MS for product identification.

3.7. Product Analysis

GC-MS analysis was performed on an Agilent GC-7890B coupled to a 5977A mass selective detector (Agilent Technologies, Santa Clara, CA, USA). The following conditions were set: interface: 230 °C, ion source: 200 °C, quadrupole: 100 °C, electron impact ionization: 70 eV, and scan range m/z: 33-330 amu. For the identification of sesquiterpenes, two stationary phases of different polarities were installed: DB-WAX MS (30 m; 0.25 mm i.d.; 0.25 µm film; Agilent J&W GC Columns, Santa Clara, CA, USA) and VF-5 MS (30 m; 0.25 mm i.d.; 0.25 µm film; Agilent J&W GC Columns). Helium with a volumetric flow rate of 1 mL/min was used as a carrier gas. The following temperature programs were set: 40 °C for 3 min, 10 °C/min until 230 °C, 230 °C for 10 min (DB-WAX MS), and 40 °C for 3 min, 10 °C/min until 300 °C, 300 °C for 10 min. For both stationary phases, a MS hold time of 5 min was set. Structural determination of sesquiterpenes was carried out by comparison of reference spectra from the NIST Standard Reference Database. Alkanes from C₈ to C₃₀ (VF-5 MS) and C₈ to C₃₀ for the calculation of retention indices.

3.8. Phylogenetic Analysis

For the classification of the cyclases derived from Cun, the protein sequences were compared with the sequences of all functionally characterized basidiomycetic cyclases known so far via Clustal Omega (https://www.ebi.ac.uk/Tools, accessed on 30 September 2021). The phylogenetic tree dataset was formerly edited using the tool iTOL (https://www.itol.embl.de, accessed on 9 October 2021). For the phylogenetic tree, the maximum likelihood method was used.

3.9. Semi-Native PAGE

Recombinant samples were prepared by adding a sample buffer 1:1 (v/v) (0.05 M Tris/HCl; pH 6.8, 10% glycerol, 0.1% bromophenol blue). Polyacrylamide gel electrophoresis was performed at 10 mA per gel, at 4 °C, using 12% gels.

3.10. Peptide Mass Fingerprinting

Purified protein bands of STS from Cun were excised from native acrylamide gel, dried, and digested with trypsin (sequencing grade modified trypsin, Promega, Madison, WI, USA). The resulting peptides were analyzed via a nano-HPLC (EASY-nLC II, Bruker, Bremen, Germany), coupled with a maXis quadrupole time of flight (QTOF) mass spectrometer (Bruker, Bremen, Germany) using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Peptides were analyzed via Mascot search matrix and protein scrape software.

Author Contributions: Conceptualization, N.P. and R.G.B.; methodology, N.P.; validation, N.P.; formal analysis, N.P.; investigation, N.P.; writing—original draft preparation, N.P.; writing—review and editing, R.G.B. and U.K.; visualization, N.P.; supervision, R.G.B., F.E. and U.K.; project and funding administration, R.G.B. and F.E. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by the Open Access fund of the Gottfried Wilhelm Leibniz Universität Hannover.

Data Availability Statement: Data are contained within the article.
Acknowledgments: We thank Carmen Theobald for skillful nLC-ESI-QTOF-MS/MS sequencing.

Conflicts of Interest: The authors declare no conflict of interest.

References


