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Production of (+)-valencene in the mushroom-forming fungus *S. commune*

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Abstract Production of commercially interesting sesquiterpenes was previously examined in plants and microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. We here investigate the potential of the mushroom *Schizophyllum commune* for the production of sesquiterpenes. Genomic analysis of *S. commune* revealed that the mevalonate pathway required for the synthesis of the farnesyl diphosphate substrate for sesquiterpene production is operational. Introduction of a valencene synthase gene resulted in production of the sesquiterpene (+)-valencene, both in mycelium and in fruiting bodies. Levels of (+)-valencene in culture media of strains containing a mutated RGS regulatory protein gene (*thn*) were increased fourfold compared to those in wild-type transformants. Up to 16 mg L^{-1} (+)-valencene was produced in these strains. In addition, the amount of (+)-valencene containing *n*-dodecane recovered from the culture medium increased sixfold to sevenfold in the *thn* mutant strains due to the absence of schizophyllan.

Keywords Sesquiterpene · (+)-Valencene · Mushroom · Basidiomycete · *Schizophyllum commune*

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Introduction

Isoprenoids (or terpenes, terpenoids) are compounds composed of isoprene units and encompass monoterpenes (C10), sesquiterpenes (C15), and diterpenes (C20). Their synthesis pathway is a branch from the pathway leading to sterols (Ajikumar et al. 2008; Misawa 2011). Terpenes are often found in essential oils of plants and have a wide variety of biological functions such as hormone signaling, attracting pollinators, or repelling and killing pests or biological competitors (Gershenzon and Dudareva 2007; Ajikumar et al. 2008). Sesquiterpenes comprise the largest group of terpenes and are commercially interesting for their aromatic and pharmaceutical properties (Ajikumar et al. 2008). Many sesquiterpenes are produced in very low quantities in their native hosts. Furthermore, chemical synthesis is complex and often results in low yields. Production of sesquiterpenes has been successful in bacteria, yeast, and plants. Introduction of genes involved in their synthesis (Ro et al. 2006; Misawa 2011; van Herpen et al. 2010; Cankar et al. 2011), modification of expression levels of enzymes involved in the metabolic pathways involved in the production of sesquiterpenes (Ro et al. 2006; Asadollahi et al. 2008; Ajikumar et al. 2008; Kirby and Keasling 2008; Misawa 2011), and use of different subcellular compartments for production (Kappers et al. 2005; Wu et al. 2006a; Fahri et al. 2011) have resulted in high levels of

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sesquiterpenes. For example, over 25 g L^{-1} amorpha-4,11-diene, a precursor of the antimalarial drug artemisinin, was produced in *E. coli* after metabolic engineering and optimization of fermentation conditions (Tsuruta et al. 2009).

Among the many platforms tested for production of sesquiterpenes, mushrooms have not been explored. Yet, they are known to produce terpenes and they even may be a source for new members of this class (Abraham 2001; Spiteller 2008; Agger et al. 2009). Mushroom-forming fungi (basidiomycetes) have already been suggested to be a production platform for proteins of interest for industry and for medical use. For instance, a strain of *Schizophyllum commune* was reported to secrete 120 mg L^{-1} of the hydrophobin SC3 (Schuurs 1998), while strains of *Trametes* sp. 420 (Tong et al. 2007) and *Pycnoporus cinnabarinus* (Alves et al. 2004) produce 310 mg L^{-1} and 1 g L^{-1} of laccase, respectively. We decided to examine whether the basidiomycete *S. commune* can be used as a host for the production of terpenes. *S. commune* is a model for mushroom-forming fungi (Ohm et al. 2010a). This mushroom has a relatively short life cycle (10 days) and it grows and fructifies on defined media. Genetic modification of this organism is relatively easy, efficient knockout technology is available, and the genome sequence of *S. commune* is published (Ohm et al. 2010a). Furthermore, *S. commune* has been reported to produce several sesquiterpenes, among which are the aromatic oil components (*Z*,*E*)-α-farnesene, *ar*-curcumene, and α-bisabolol (Ziegenbein et al. 2006).

A disadvantage of *S. commune* as production platform is the secretion of large amounts of the polysaccharide schizophyllan in its culture medium, which hampers isolation and purification of homologous and heterologous products. Therefore, the *thn* mutant of *S. commune* containing a regularly occurring spontaneous mutation (Raper and Miles 1958) was also examined as host for terpene production. The mutation results from the insertion of a transposon, e.g., *scooter-2*, into the *thn* gene that encodes a RGS protein (Fowler and Mitton 2000). RGS proteins are regulators involved in heterotrimeric G protein signaling (Fowler and Mitton 2000). The *thn* mutation causes several morphological changes such as the production of a pungent smell, a higher radial growth rate, and a lower biomass (Schwalb and Miles 1967). In addition, the *thn* mutation causes a significant decrease in secretion of polysaccharides (schizophyllan) and proteins in the culture medium (Schwalb and Miles 1967; Wessels et al. 1991).

We here describe the expression of a valencene synthase from *Chamaecyparis nootkatensis* (*CnVS*) in a wild-type strain and in a *thn* strain of *S. commune*. CnVS is an enzyme capable of converting farnesyl diphosphate (FPP) into (+)-valencene (Beekwilder et al. 2013) and was chosen because it is a very robust enzyme which is expressed in a wide range of hosts (Beekwilder et al. 2013). The product of this enzyme, (+)-valencene, is a flavor molecule found in citrus fruit and provides a typical citrus aroma with applications in the flavor and fragrance industry. The production of (+)-valencene was higher in the *thn* strain compared to that in the wild-type strain and reached production levels of 16.6 mg L^{-1}. The absence of schizophyllan in the medium of the *thn* strain increased the efficiency of *n*-dodecane extraction, improving the yield of (+)-valencene from the *thn* strain compared to that from the wild-type strain.

Materials and methods

Analysis of *S. commune* genes

The genome sequence of *S. commune* is available through the interactive JGI genome portal at http://jgi.doe.gov/Scommune (Ohm et al. 2010a). Expression of genes, indicated by protein ID, was examined using previously obtained massively parallel signature sequencing (MPSS) data (Ohm et al. 2010a) and RNA sequencing data (Ohm et al. 2011).

Strains and growth conditions

Cloning was performed in *Escherichia coli* pXLBlue (Promega, Fitchburg, USA). The *S. commune* wild-type strain H4-8 (FGSC 9210; Fowler et al. 1999; Ohm et al. 2010a) and the KS8 *thn* mutant (CBS 137184) were used. KS8 was obtained via a spontaneous event during cultivation of strain 72-3 which contains a deletion of the *sc3* gene (Wösten et al. 1994; van Wetter et al. 1996). The *thn* mutation was previously shown to be caused by a disruption of the *thn* gene (Pubmed AF267870; Fowler and Mitton 2000) by the *scooter-2* transposon (Pubmed AF267872; Fowler and Mitton 2000). PCR analysis using primers flanking the *scooter-2* integration site (AGGCGATG, nucleotide 421-428; Fowler and Mitton 2000; tgcccgccgaccacgaacagg (forward) and tgggtgacgatgatctcgtcg (reverse)) was performed to examine whether the *thn* gene of strain KS8 was disrupted. *S. commune* was grown on minimal medium (MM; Dons et al. 1979) either or not solidified with 1.5 % agar at 25 °C. Liquid cultures were grown in 100 mL liquid MM in 250-mL Erlenmeyer flasks at 225 rpm. For transformation and for schizophyllan and biomass quantification, *S. commune* was grown from a mycelial homogenate for 2 and 7 days, respectively. For analysis of (+)-valencene production, transformants were precultured for 4 days. After addition of 10 mL *n*-dodecane to the culture medium, growth was prolonged for 3–9 days. To analyze production of (+)-valencene in fruiting bodies, transformants derived from the wild-type strain H4-8 were crossed with its compatible mate H4-8B (Ohm et al. 2010b). To this end, these strains were grown next to each other on minimal medium plates for 10–14 days

(Ohm et al. 2010a), resulting in a fructifying dikaryotic strain expressing (+)-valencene. For biotransformation of (+)-valencene with lyophilized mycelium, *S. commune* wild-type strain H4-8 and *Pleurotus sapidus* (CBS 195.92) were grown on MM plates (6 days, 25 °C) on which a polycarbonate membrane (diameter 4.5 cm, pore size 0.1 μm; Poretics, Livermore, USA) was placed. The mycelium was freeze-dried and ground in liquid nitrogen.

Isolation of schizophyllan

Seventy-five milliliters 100 % ethanol (Merck, Readington, USA) was added to 50 mL medium of liquid-shaken cultures and mixed thoroughly. The samples were incubated at 4 °C for 16 h and centrifuged at 3,000*g* in a swing-out rotor. The resulting floating schizophyllan fraction was transferred to preweighed 50-mL Greiner tubes (Sigma-Aldrich, Saint Louis, USA), and excess liquid was removed. The samples were dried at 60 °C and weighed.

Construction of expression plasmids

A 750-bp fragment of the upstream region of the *β-tubulin* 2 *tub2* gene (protein ID 77035) was amplified from genomic DNA of strain H4-8 by PCR using the primers cagtaagctttggaaccgggcctc (forward) and aggtggacgatttcacccatggctgcgg (reverse). This fragment was cloned in the *Hin*dIII and *Nco*I sites of pUC20 resulting in plasmid pKSTub. A 435-bp downstream terminator sequence of *sc3* (protein ID 77028) that had been amplified by PCR from genomic DNA of H4-8 using primers ggatccgcaggtgaacgcgcctatcg (forward) and gaattcggtggtctgggaggtggaag (reverse) was cloned in the *Bam*HI and *Eco*RI sites of plasmid pKSPTub. This resulted in plasmid pKSPTubT3. Previously, it has been shown that introns are necessary for mRNA accumulation of several homologous and heterologous genes in *S. commune* (Lugones et al. 1999; Scholtmeijer et al. 2001). Therefore, an intron was cloned in pKSPTubT3. To this end, two complementary primers encompassing an artificial intron (Lugones et al. 1999) with *Bam*HI compatible ends were annealed and cloned in the *Bam*HI site. This resulted in plasmid pKSPTubiT3. Only ligation of the upstream *Bam*HI compatible end resulted in reconstitution of the *Bam*HI site (Fig. 1a).

A spacer sequence was cloned as a *Nco*I/*Bam*HI fragment in the *Nco*I and *Bam*HI site of pKSPTtubiT3, resulting in plasmid pKSPTubSS3iT3. For selection of transformants, a

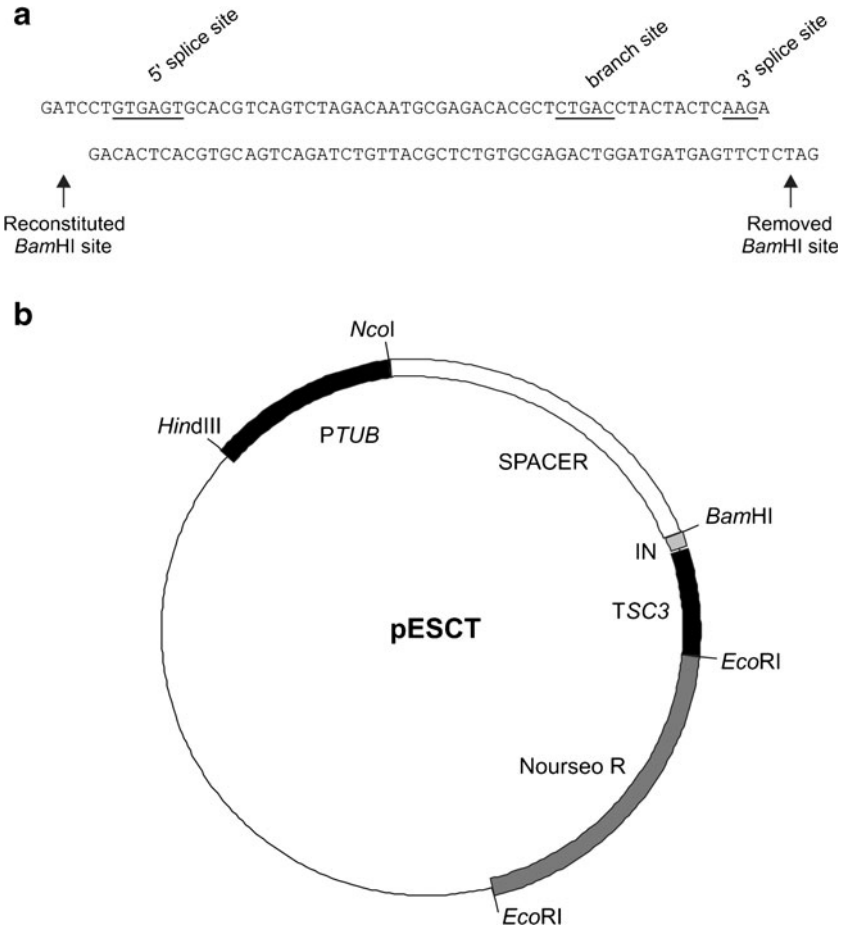

Fig. 1 The designed intron sequence containing conserved 5′-splice, 3′-splice, and branch sites (**a**). Expression vector pESCT, containing the *β-tubulin* promoter (*PTUB*) and *sc3* terminator (*TSC3*) sequences (**b**). In addition, an intron (*IN*) inserted by ligating annealed primers into the *Bam*HI restriction site and the nourseothricin resistance cassette (*Nourseo R*) are present

nourseothricin resistance cassette (van Peer et al. 2009) was cloned in the *Eco*RI site of pKSPTubSS3iT3, resulting in the expression plasmid pESCT (Fig. 1b; Online Resource Fig. S1). The mature valencene synthase (CnVS; GenBank accession number JX040471) protein sequence (Beekwilder et al. 2013) either or not containing the mitochondrial targeting signal (MTS) of the *Saccharomyces cerevisiae Cox IV* gene (Köhler et al. 1997) placed at the N terminus was converted into a corresponding DNA sequence. Only the codon ending with a G or C was used in the case an amino acid was encoded by two or three codons. The two codons ending with a G or C were used in the case of amino acids encoded by four or more codons. The ratio of the two codons was determined by the relative abundance in the codon usage table of *S. commune* (Wu et al. 2006b). The *S. commune* optimized DNA sequences (GenBank accession number KF990996) encoding CnVS and MTS-CnVS were synthesized (GenScript) and cloned between the *S. commune β-tubulin 2* promoter (*β-tub 2*) and *sc*3 terminator sequences in expression vector pESCT. The MTS-CnVS sequence was cloned in *Nco*I/*Bam*HI-digested pESCT using restriction enzymes *Pci*I and *Bam*HI, resulting in plasmid pESCT-MV. The CnVS sequence without the MTS was cloned as a *Sma*I-*Bam*HI fragment in pESCT. To this end, pESCT was cut with *Nco*I, made blunt with the Klenow fragment, and cut with *Bam*HI. The resulting plasmid was named pESCT-V. Restriction enzymes and PCR materials were purchased from Fermentas (Thermo Fisher Scientific, Waltham, USA).

Transformation of *S. commune*

Wild-type *S. commune* and the *thn* strain KS8 were transformed as described (van Peer et al. 2009) and selected on minimal medium (Dons et al. 1979) containing 8 or 20 μg mL^{-1} nourseothricin (Jena Bioscience, Jena, Germany), respectively. Transformants were transferred to fresh selection plates after 4 days to confirm stable integration of the constructs.

GC-MS analysis of (+)-valencene production in culture media

For analysis of (+)-valencene levels in liquid-shaken cultures, the *n*-dodecane (Sigma-Aldrich, Saint Louis, USA) fraction of the cultures was separated from the culture medium and the mycelium by centrifugation (1,200*g*, 30 min). The *n*-dodecane fraction was diluted threefold in ethyl acetate, water was removed from the solution using anhydrous Na_2SO_4 (VWR, Amsterdam, The Netherlands), and 1 μl was analyzed by gas chromatography-mass spectrometry (GC-MS) analysis. Analytes were separated using a gas chromatograph (5890 series II, Hewlett-Packard, Palo Alto, USA) equipped with a 30 m×0.25 mm, 0.25-mm film thickness column (ZB-5, Phenomenex, Torrance, USA). Helium (>5.0 grade) was used as carrier gas at a flow rate of 1 mL min^{-1}. The inlet temperature was set to 250ºC and injections were performed in the splitless mode. The initial oven temperature of 45ºC was increased after 1 min to 300ºC at a rate of 10ºC min^{-1} and held for 5 min at 300ºC. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard, Palo Alto, USA). (+)-Valencene and (+)-nootkatone were identified by comparison of mass spectra and retention times with those of the authentic standards (Fluka, Buchs, Switzerland). Absolute concentration of (+)-valencene was calculated from the peak area (TIC) by comparison to a standard curve prepared by measuring a dilution series of authentic standards with a known concentration.

Analysis of volatiles in fruiting bodies of *S. commune* by thermo-desorption GC-MS

(+)-Valencene production in fruiting bodies (see above) was analyzed by headspace trapping followed by thermo-desorption GC-MS analysis. In addition, the fruiting bodies were analyzed after separation of the fruiting bodies from the supporting mycelium. For each construct, three MM plates or three batches of isolated fruiting bodies were analyzed. Samples were placed in a 0.5-L sealed glass container. A vacuum pump was used to draw air through the glass container at 100 mL min^{-1}, with the incoming air being purified through stainless steel cartridges containing 200 mg Tenax TA 20/35 (Camsco, Houston, USA). At the outlet, the volatiles emitted by the fruiting bodies were trapped on a stainless steel cartridge containing 200 mg Tenax TA 20/35 (Camsco, Houston, USA). Volatiles were collected for 24 h. Headspace samples were analyzed with a Thermo Trace GC connected to a Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Before thermo-desorption, cartridges were flushed with helium (>5.0 grade) at 50 mL min^{-1} for 20 min to remove moisture and oxygen. After flushing, the collected volatiles were desorbed from the cartridges at 220 °C (Ultra, Markes, Llantrisant, UK) for 5 min with a helium (>5.0 grade) flow of 30 mL min^{-1} with a split ratio of 1:1. The released compounds were focused on an electrically cooled sorbent trap (Unity, Markes, Llantrisant, UK) at a temperature of 10 °C. Volatiles were injected on the analytical column (ZB-5MSi, 30 m×0.25 mm ID, 1.0-μm film thickness, Zebron, Phenomenex, Torrance, USA) with a split flow of 40 mL min^{-1} by ballistic heating of the cold trap to 250 °C for 3 min. The temperature program started at 60 °C (3-min hold) and rose by 10 °C min^{-1} to 280 °C (3-min hold). The column effluent was ionized by electron impact (EI) at 70 eV. Mass scanning was done from 33 to 280 *m/z* with a scan time of five scans per second. The ion source temperature was set to 250 °C and the transfer line was set to 275 °C. The eluted compounds were identified using Xcalibur software (Thermo Fisher Scientific,

Waltham, USA) by comparing the mass spectra with those of authentic reference standards.

Biotransformation of (+)-valencene with lyophilized mycelium of *S. commune* and *P. sapidus.* Two milliliters 20 mM Tris–HCl (pH 7.5, Merck, Readington, USA) and 5 μl (+)-valencene (>70 %, Fluka, Buchs, Switzerland) were added to 50 mg ground, lyophilized mycelium. The samples were incubated horizontally on an orbital shaker at 90 rpm for 24 h at 24 °C and sequentially extracted with 1 mL pentane (Biosolve, Valkenswaard, The Netherlands) and 1 mL ethyl acetate (Biosolve, Valkenswaard, The Netherlands). The pentane and ethyl acetate fractions were combined and centrifuged (10 min, 300*g*), and water was removed from the top phase using a Na_2SO_4 column (VWR, Amsterdam, The Netherlands). One microliter of the samples was analyzed via GC-MS.

Glucose levels in the medium

Glucose determination was performed using a glucose assay kit (GAGO-20; Sigma-Aldrich, Saint Louis, USA). Volumes were downscaled to an end volume of 200 μl, and a calibration curve ranging between 0 and 3.2 μg glucose per well was used. Absorbance at 490 nm was measured using a BioRad (Hercules, USA) microtiter plate reader, model 550.

Results

S. commune expresses genes predicted to be involved in the mevalonate pathway. Homologues of the *S. cerevisiae* mevalonate (MEV) pathway genes were found in the genome sequence of *S. commune* using a BLAST algorithm. This revealed homologues of all genes of the pathway (*erg10*, *erg13*, *hmgr*, *erg12*, *erg8*, *erg19*, and *erg20*) that leads to farnesyl diphosphate (FPP), the direct precursor of sesquiterpenes. Moreover, four putative terpene synthases were identified in the *S. commune* genome sequence in addition to squalene synthase (*erg9*), the first dedicated step toward sterol formation from FPP. These putative terpene synthase genes show no homology with *CnVS*. Recently, a mono-oxygenase protein from *P. sapidus* was identified, which is capable of oxidizing sesquiterpenes such as (+)-valencene (Fraatz et al. 2009a). A homologue of this gene could not be found in *S. commune*. MPSS and RNA sequencing data showed expression of genes involved in all steps in FPP synthesis in wild-type *S. commune*.

Analysis of the disruption of the *thn* gene in strain KS8

Strain KS8 was obtained via a spontaneous event during cultivation and showed the phenotype typical for a *thn* mutant. It produced a pungent smell, the submerged hyphae were wavy or corkscrew shaped, and the strain showed the typical fluffy appearance of the aerial hyphae. The *thn* mutation was previously shown to be caused by a disruption of the *thn* gene by the *scooter-2* transposon (Fowler and Mitton 2000). PCR analysis of genomic DNA of the wild-type strain resulted in a product of the expected size (665 bp; data not shown), while with genomic DNA of strain KS8, a fragment of 1,300 bp was obtained (data not shown). This confirmed the disruption of the *thn* gene of strain KS8.

Quantification of schizophyllan production in wild-type and strain KS8

To determine whether our spontaneously obtained *thn* strain produces less schizophyllan in its culture medium as was described for other *thn* strains (Schwalb and Miles 1967; Wessels et al. 1991), the amount of schizophyllan produced by the wild-type strain and strain KS8 was quantified. The dry weight of schizophyllan produced by the wild-type was 2.9±0.7 g L^{-1}, while the dry weight of the mycelial biomass was 9.0±1.5 g L^{-1}. The dry weight of the biomass of KS8 was 3.9±0.2 g L^{-1}, but schizophyllan could not be detected in the medium of strain KS8.

Production of (+)-valencene in *S. commune* mycelium

Plasmids pESCT-MV and pESCT-V contain the codon-optimized *CnVS* sequence with and without the sequence for the *S. cerevisiae CoX IV* mitochondrial targeting signal, respectively. The open reading frames are under control of the *S. commune β-tubulin 2* promoter (*β-tub 2*) and the *sc*3 terminator sequence. Both constructs were introduced into *S. commune* H4-8 and KS8. For analysis of (+)-valencene production, eight transformants of each type were grown in liquid-shaken cultures, H4-8 and KS8 serving as a control. For the wild-type derivatives, 10 mL *n*-dodecane was added to the culture medium at day 4. The KS8 derivatives revealed slower growth rates and therefore, *n*-dodecane was added at day 5. The cultivation was in both cases continued until day 6. The *n*-dodecane was separated from the medium and mycelium by centrifugation and analyzed via GC-MS.

In strains harboring the *CnVS* gene, valencene could be detected as a dominant compound in the dodecane layer (peak 1, Fig. 2.). Comparison of the chromatograms and corresponding mass spectra of (+)-valencene produced by *S. commune* with a (+)-valencene standard are shown in Online Resource Fig. S2. The standard curve for (+)-valencene quantification is shown in Online Resource Fig. S3. The transformants for each of the constructs (pESC-V or pESC-MV) produced different levels of valencene with a maximum of 16.6 mg L^{-1} culture medium (Fig. 3.). In general, the wild-type transformants produced fourfold less (+)-valencene than the KS8 strain (Fig. 3.). Mitochondrial targeting did not result in an increased

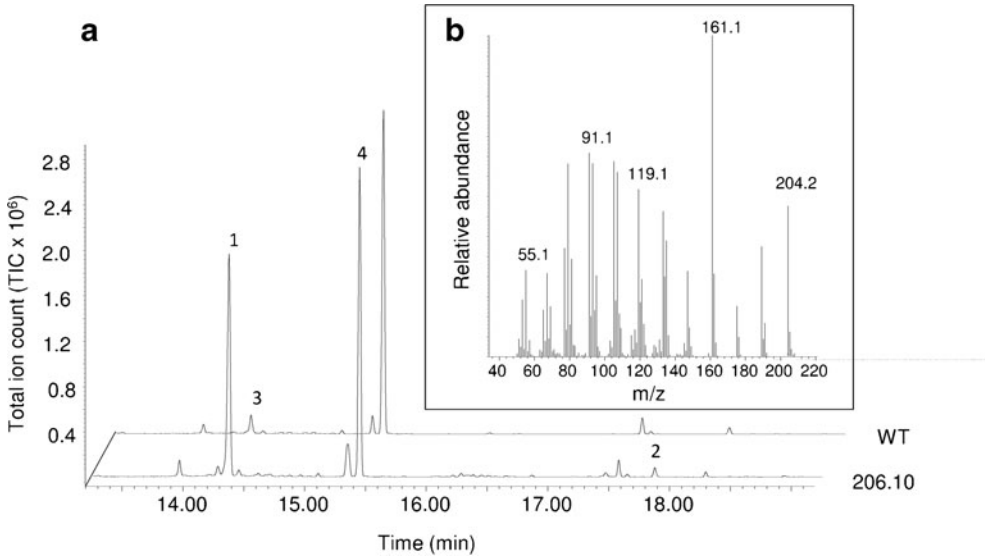

Fig. 2 Production of valencene by transgenic *S. commune* in minimal medium. GC-MS analysis of the *n*-dodecane layer from a H4-8 strain transformed with *CnVS* (strain WT206.10) is compared to that of H4-8 (**a**). Two additional products were formed in the transformant compared to the wild-type strain and were identified as (*1*) (+)-valencene at a retention time (rt) of 14.316 min and (*2*) (+)-nootkatone at a rt of 17.817 min. A small overlaying peak (*3*) at a rt of 14.303 min was identified as 2,4-di-tert-butyl-phenol that is present in the solvent. A solvent peak of hexadecane (*4*) at a rt of 15.394 min was seen in all samples. The mass spectrum of (+)-valencene is shown in the *insert* (**b**)

production level. In fact, production of transformants containing plasmid pESCT-V reached 3.5- and 4.5-fold higher levels compared to plasmid pESCT-MV in strains H4-8 and KS8, respectively. Notably, separation of the *n*-dodecane fraction from the culture medium of the wild-type transformants was difficult due to the production of schizophyllan. Only 10 % of the added *n*-dodecane fraction could be recovered. In contrast, separation of the *n*-dodecane fraction from the medium of the KS8 strain resulted in recovery of 60–70 % of the *n*-dodecane fraction.

Production of (+)-valencene was followed over time in the KS8 transformant, named T206.2, with the highest production of the sesquiterpene. The *n*-dodecane was added at day 4 and *n*-dodecane samples were taken from day 5 to 12. At the same time, medium samples were analyzed for glucose levels (Fig. 4.). Glucose was depleted from the medium at day 7; however, (+)-valencene levels continued to increase until day 10. A maximum level of 9.5±0.4 mg L^{-1} was reached.

(+)-Nootkatone formation in *S. commune* mycelium

During GC-MS analysis of *n*-dodecane fractions obtained from shaken cultures of CnVS-expressing strains, small amounts of (+)-nootkatone (10–15 μg mL^{-1}) were detected in the strains with the highest (+)-valencene levels (Fig. 2, peak 2). Comparison of the chromatograms and corresponding mass spectra of (+)-nootkatone in the *S. commune* samples with a (+)-nootkatone standard are shown in Online Resource Fig. S2. To examine whether the conversion of (+)-valencene into (+)-nootkatone occurred via an enzymatic reaction, lyophilized mycelium of *S. commune* H4-8 was incubated with (+)-valencene. As control, heat-inactivated mycelium (10 min, 100 °C) of *S. commune* was used. Since *P. sapidus* expresses a mono-oxygenase capable of converting (+)-valencene into (+)-nootkatone (Fraatz et al. 2009a), lyophilized mycelium (either heat inactivated or not) of *P. sapidus* was used as control. GC-MS analysis revealed similar levels of (+)-nootkatone in both untreated and heat-treated samples of *S. commune*, while in heat-treated *P. sapidus* samples, the (+)-nootkatone levels were significantly lower compared to those in the untreated samples. This indicates that the (+)-nootkatone observed in *n*-dodecane fractions of *S. commune* is formed via a non-enzymatic conversion.

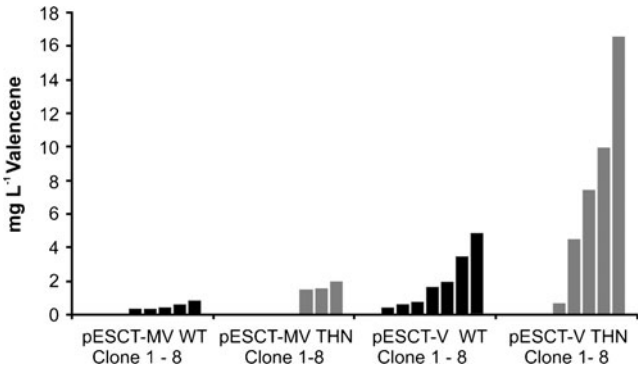

Fig. 3 (+)-Valencene levels produced by the wild-type (WT) and KS8 transformants of *S. commune* transformed with the *CnVS* gene preceded by the *Cox IV* mitochondrial targeting signal (MTS; *pESCT-MV*) or the *CnVS* gene without MTS (*pESCT-V*). (+)-Valencene levels indicated are extrapolated to milligrams per liter of culture medium

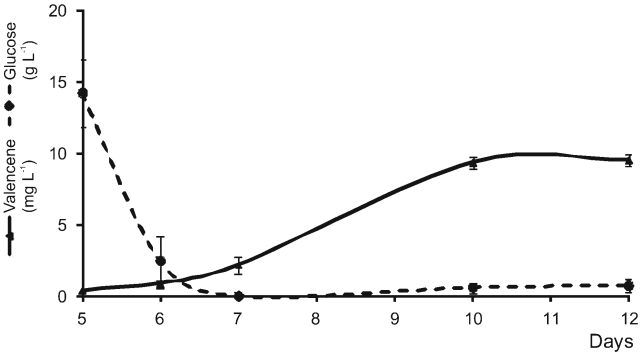

Fig. 4 (+)-Valencene production and glucose consumption over time in KS8 transformant T206.2. Curves were fitted using the line style smoothed line in Microsoft Excel

Analysis of (+)-valencene levels in fruiting bodies

Levels of (+)-valencene were also determined in the fruiting bodies. To this end, the highest-expressing H4-8 transformant of pESCT-V, named WT206.10, was crossed with a compatible wild-type strain to obtain a dikaryon. As a control, a wild-type dikaryon was analyzed. Plates containing mushrooms of ±0.5 cm in diameter were obtained and used for headspace trapping. Several sesquiterpenes were detected in the volatile profile of the wild-type *S. commune* fruiting bodies, but no (+)-valencene or (+)-nootkatone was detected. In the dikaryon containing the CnVS gene, an additional predominant peak was found at the retention time of 18.33 min and was confirmed to be (+)-valencene (Fig. 5). Another smaller peak at the retention time of 22.19 min was identified as (+)-nootkatone (Fig. 5). Finally, a peak at the retention time of 16.84 min was identified as β-elemene (Fig. 5). β-elemene is the heat-induced cope rearrangement product of germacrene A. Germacrene A in the headspace of WT206.10 was identified by the comparison of the mass spectra with the NIST08 (http://www.nist.gov/) and Wiley7 (http://www.sisweb.com/software/ms/wiley.htm) mass spectral libraries. In addition, the retention time and mass spectra were compared to germacrene A produced by the previously characterized *Tanacetum partenium* germacrene A synthase (Liu et al. 2011; Online Resource Fig. S4). In addition to the new peaks observed, some minor changes in the intensity of endogenous sesquiterpene peaks were observed. The concentration of (+)-valencene in the headspace of the recombinant fruiting bodies was high (4.6±2.7 μg per plate per 24 h). Fruiting bodies were separated from the supporting mycelium, and emission of (+)-valencene was measured to be 16.9±5.5 μg per gram fruiting bodies per 24 h.

Discussion

We here show the potential of the mushroom-forming basidiomycete *S. commune* as a platform for the production of terpenes. The genome sequence of *S. commune* revealed the presence of homologues of genes involved in the mevalonate (MEV) pathway, and expression of these genes was observed in MPSS and RNA sequencing data. Introduction of a heterologous gene encoding valencene synthase (*CnVS*; Beekwilder et al. 2013) in *S. commune* resulted in production levels of (+)-valencene up to 16.6 mg L^{-1}. Clones obtained from the expression cassettes with or without the *S. cerevisiae Cox IV* mitochondrial targeting signal showed variation in their (+)-valencene expression levels. This is the result of differences in copy number of integrated expression cassette and/or the position in the genome of the integrated expression cassettes.

In both plants and yeast, targeting of terpene synthases to mitochondria resulted in increased levels of terpenes (Kappers et al. 2005; Fahri et al. 2011). However, adding the Cox IV mitochondrial targeting signal to valencene synthase in *S. commune* did not result in higher levels of (+)-valencene.

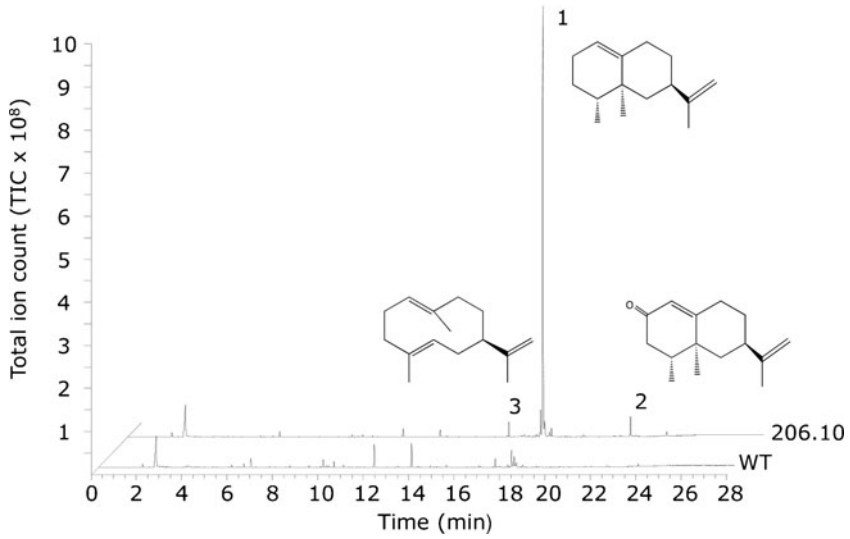

Fig. 5 Volatile profile of *S. commune* fruiting bodies. The analysis of volatiles in WT *S. commune* fruiting bodies is compared to fruiting bodies of *S. commune* transformed with *CnVS*. Three predominating new peaks in the volatile profile of CnVS fruiting bodies were identified as (+)-valencene (*peak 1*, rt=18.33 min), (+)-nootkatone (*peak 2*, rt=22.19 min), and germacrene A (*peak 3*, rt=16.84 min; germacrene A was detected as its cope rearrangement, β-elemene)

Possibly, the Cox IV MTS does not mediate targeting to *S. commune* mitochondria or processes such as transcription, translation, or protein folding may be affected by the presence of the N-terminal sequence. Since efficient (+)-valencene production was obtained without the MTS, we decided to focus on strains transformed with plasmid pESCT-V.

The *thn* mutant KS8 was shown to contain a disruption of the RGS protein-encoding gene (the *thn* gene). Production of (+)-valencene in the *thn* strain KS8 was higher compared to that in the wild-type strain. This may be explained by the fact that mutations in RGS proteins of fungi affect several aspects of fungal growth and development, including secondary metabolite production (Yu and Keller 2005; Li et al. 2007). It would be of interest to analyze whether there is a difference in expression of MEV pathway genes between the wild-type strain and strain KS8. In addition, (+)-valencene was isolated more efficiently from the culture medium of the KS8 strains via a *n*-dodecane extraction. This was due to the fact that the *thn* mutation results in a significant decrease in secretion of schizophyllan in the culture medium (Schwalb and Miles 1967; Wessels et al. 1991, this manuscript).

A time-course experiment showed that (+)-valencene production increased during 3 days after depletion of glucose from the medium. This may be caused by more efficient extraction of (+)-valencene from the fungal mycelium due to the autolysis of the mycelium (Demain 1986; White et al. 2002). Furthermore, conditions of nutrient limitation often stimulate production of secondary metabolites such as terpenes (Demain 1986; Calvo et al. 2002). Thus, genes involved in the MEV pathway may be upregulated during nutrient limitation.

Besides (+)-valencene, small amounts of (+)-nootkatone were detected in both H4-8 and KS8 strains expressing the *CnVS* gene. (+)-Nootkatone is characterized by a grapefruit flavor and is a relevant product for the flavor and fragrance industry. Several enzymes are known to be capable of converting (+)-valencene to (+)-nootkatone (Sowden et al. 2005; Seifert et al. 2009; Cankar et al. 2011; Fraatz et al. 2009a, b). However, no difference in the level of (+)-nootkatone was detected using fresh or heat-inactivated mycelial extracts in in vitro (+)-valencene conversion experiments. This suggests a chemical instead of enzymatic conversion. This is in line with the absence in the genome of *S. commune* of a homologue of the mono-oxygenase from another basidiomycete, *P. sapidus*, capable of oxidizing sesquiterpenes such as (+)-valencene to (+)-nootkatone (Fraatz et al. 2009a). Introduction of chicory P450 mono-oxygenase (Cankar et al. 2011) or the mono-oxygenase identified in *P. sapidus* (Fraatz et al. 2009a) in (+)-valencene-producing *S. commune* strains may result in increased production of (+)-nootkatone in *S. commune*.

Production of (+)-valencene and small amounts of nootkatone was also detected in fruiting bodies. In addition, β-elemene was detected in the volatile profile of fruiting bodies. β-Elemene is a heat-induced cope rearrangement product of germacrene A (de Kraker et al. 2001). Previously, it was identified as a side product of the CnVS enzyme in bacteria and yeast (Beekwilder et al. 2013). Comparing the peak area of β-elemene (germacrene A) with that of (+)-valencene suggests that the level of this side product is very low.

Comparing CnVS levels obtained in *S. commune* with those of the yeast *S. cerevisiae* and the bacterial system *Rhodobacter spaeroides* (1.36±0.05 and 57.5±3.5 mg L^{-1}, respectively) (Beekwilder et al. 2013) indicates that *S. commune* may be an efficient production system for (+)-valencene. Increase of (+)-valencene expression in *S. commune* may be achieved by reducing the amount of FPP used for production of endogenous terpenes or ergosterol by decreasing expression of endogenous terpene synthases and squalene synthase. In yeast downregulation of the *erg9*, squalene synthase gene resulted in a 2.5-fold increase in sesquiterpene production (Asadollahi et al. 2008). Furthermore, the amount of FPP substrate for (+)-valencene synthase may be increased by overexpressing homologous or heterologous components of the MEV pathway such as HMGR or ERG20. In yeast, these approaches have resulted in an increase of sesquiterpene production fourfold (Fahri et al. 2011). Targeting of (+)-valencene synthase to the mitochondrion using a homologous MTS, in combination with targeting of farnesyl diphosphate synthase (FDS), may alleviate FPP limitation in the mitochondrion as was observed in humans, plants, insects, and yeast (Martin et al. 2007; Fahri et al. 2011). In combination with cytosolic expression, this approach may also increase (+)-valencene production in *S. commune*. Finally, mushroom-forming basidiomycetes produce endogenous terpenes (Abraham 2001; Spiteller 2008; Agger et al. 2009) and may thus be a source for new terpene products. The expression of endogenous genes usually leads to higher expression levels, and in combination with engineering, *S. commune* for optimized terpene production may result in high expression levels of these new compounds.

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