Role of the Colletotrichum acutatum sesquiterpene synthase CaTPS in the biosynthesis of sesquiterpenoids

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Colletotrichum acutatum is a major fungal pathogen of fruit crops, which causes severe yield losses in strawberry production. A potential key factor in plant–pathogen interactions is fungal sesquiterpenoids which have mycotoxic and phytotoxic activities. The first committed step in sesquiterpenoid biosynthesis is performed by sesquiterpene synthases (TPS). Only a few TPSs have been functionally characterized from filamentous fungi and none from the genus Colletotrichum. Despite being an important fungal pathogen to agriculture, it is poorly understood at the molecular and chemical levels. The terpenoid biochemistry in Coll. acutatum strain SA 0-1 was studied and one Coll. acutatum TPS (CaTPS) was successfully cloned and characterized in yeast. CaTPS catalyses the biosynthesis of multiple sesquiterpenoids. The two major products are β-caryophyllene and an unidentified sesquiterpenoid along with α-humulene as one of the minor sesquiterpenoid products. These products were also secreted by the fungus in strawberry fruit medium along with several other sesquiterpenoids indicating other TPSs are active during in vitro growth. β-Caryophyllene and α-humulene are known cytotoxic products important for ecological interactions and are produced by SA 0-1. Interestingly, a gene expression analysis using quantitative real-time PCR revealed a significant increase in expression of CaTPS during strawberry fruit infection, thus indicating that it could be involved in fruit infection. This is, we believe, the first characterization of TPS in Colletotrichum spp. and terpenoid profiles of Coll. acutatum, which could facilitate studies on the role of terpenoids in the ecology of Coll. acutatum.

INTRODUCTION

Considerable research interest has been dedicated to sesquiterpenoids, as they are a valuable source of bioactive metabolites with potential industrial use, especially for pharmaceutical and agrochemical applications (Simonsen et al., 2013). They are also known to play central roles in e.g. plant–insect, plant–microbe and microbe–microbe interactions (Cheng et al., 2007; Kramer & Abraham, 2011). Fungi produce a variety of bioactive sesquiterpenoids, but little is known about the genes involved in the biosynthesis (Kramer & Abraham, 2011). The first committed step in the biosynthesis of any sesquiterpenoid is performed by the class I terpene synthases/cyclases referred to as sesquiterpene synthases (TPSs). TPSs catalyse the biosynthesis of a

Abbreviations: CaTPS, Colletotrichum acutatum sesquiterpene synthase; DAI, days after inoculation; EST, expressed sequence tag; FPP, farnesyl diphosphate; NCBI, National Center for Biotechnology Information; PVPP, polyvinylpyrrolidone; qRT-PCR, quantitative real-time PCR; TPS, sesquiterpene synthase.

The Genbank/EMBL/DDBJ accession numbers of the Colletotrichum acutatum sesquiterpene synthase nucleotide and amino acid sequences are KP398851 and AKE33935, respectively.

Three supplementary tables and four supplementary figures are available with the online Supplementary Material.
myriad of structurally very diverse terpenoids from one diphosphate substrate; for sesquiterpenoids, the substrate is farnesyl diphosphate (FPP) (Drew et al., 2013). Some TPSs catalyse the formation of a single product (Proctor & Hohn, 1993), while others form multiple products (Ager et al., 2009; Drew et al., 2015; Pickel et al., 2012). The biosynthesis performed by the TPSs is often followed by subsequent steps catalysed by cytochrome P450s, aldehyde/alcohol reductases and many other enzymes to yield the final myriad of terpenoids (Cardoza et al., 2011; Ikram et al., 2015; Weitzel & Simonsen, 2015).

Only few TPSs have been functionally characterized within the filamentous fungi important to agriculture and the food industry, including species of Fusarium, Trichoderma, Botrytis, Aspergillus and Penicillium. The TPSs often play an important role in fungal virulence in the regulation of mycotoxin and phytotoxin biosyntheses. In Fusarium and Trichoderma species, the trichodiene synthase generates the sesquiterpene scaffold of different trichothecene mycotoxins (Cardoza et al., 2011; Hohn & Desjardins, 1992; Proctor et al., 1995; Rynkiewicz et al., 2001; Trapp et al., 1998) and several gene clusters have been described (Brown et al., 2001; Cardoza et al., 2011). More recently, the main products from the two sesquiterpene synthases Ffs6c and Ffs4 of Fusarium fujikuroi have been identified as (−)-α-acoreno- and koraiol, respectively (Brock et al., 2013). In Botrytis cinerea, the product presilphifolan-8-β-ol, the precursor of botrydial phytotoxin, is synthesized by presilipherfolan-8β-ol synthase (Pinedo et al., 2008). Finally, the aristolochene synthases from Penicillium roqueforti and Aspergillus terreus catalyse the formation of aristolochene, the first committed step in the biosynthesis of several mycotoxins including PR toxin (Cane & Kang, 2000; Jelen & Wasowicz, 1998; Proctor & Hohn, 1993).

Anthracnose fruit rot caused by the fungus Colletotrichum graminicola and Colletotrichum higginsianum expresses several putative TPSs during plant infection, supporting their importance in the infection process (O’Connell et al., 2012). None of these sesquiterpene synthases has been functionally characterized.

Here we identify and functionally characterize, we believe for the first time, one sesquiterpene synthase (CaTPS) from Coll. acutatum and gain insight into the production of sesquiterpenoids in this fungi. We show that CaTPS catalyses the biosynthesis of multiple sesquiterpenoids and its kinetic parameters are established. CaTPS is significantly induced during fruit infection and the first volatile sesquiterprenoid profile of Coll. acutatum is obtained in different culture media of the fungus.

**METHODS**

**Coll. acutatum growth media and conditions.** The Coll. acutatum strain 1A 0-1 was originally isolated from a Danish strawberry field (Sundelin et al., 2005) and a stock culture was stored in 10% glycerol at −80°C. Coll. acutatum was propagated for 14 days at 28°C on potato dextrose agar (PDA) prior to preparation of mycelial plugs and spore suspensions for inoculation. PDA plates were inoculated with mycelial plugs and incubated at room temperature for 10 days and fungal biomass for DNA extraction and genome walking was harvested. For terpenoid metabolite profiling of Coll. acutatum, the fungus was cultured in 500 ml gas washing bottles containing 60 ml potato dextrose broth (PDB) and inoculated with 1.2 ml spore suspension to obtain a final concentration of 10^6 spores ml⁻¹. Four biological replicates of inoculated PDB cultures and two mock-inoculated cultures were incubated for 14 days in darkness at 25°C with agitation at 150 r.p.m. The experiment was set up with three biological replicates, including mock-inoculated controls.

Coll. acutatum was grown in a liquid strawberry medium for the identification of differentially expressed fungal genes and for terpenoid metabolite profiling. Lyophilized green strawberry fruits (cv. Elsanta) were ground in liquid nitrogen using a mortar and pestle. MilliQ water was added to the strawberry powder (1:10 w/v) and the pH was adjusted to pH 7 using 5 M NaOH. The medium was subsequently sterilized by autoclaving. Coll. acutatum was cultivated in 250 ml Erlenmeyer shake flasks containing 100 ml strawberry medium inoculated with Coll. acutatum spores to a final concentration of 10^6 spore ml⁻¹. Submerged cultures for terpenoid profiling were incubated for 7, 12, 17 and 22 days as described for the PDB cultures. The experiment was set up with three biological replicates for each treatment, including mock-inoculated controls.

**Identification of differentially expressed genes.** Subtractive suppression hybridization was performed on RNA isolated from strawberry cultures inoculated with Coll. acutatum, which were prepared and incubated as described above. Fungal biomass was harvested 2 and 6 days after inoculation (DAI) by centrifugation at 18,000 RCF for 10 min and the pellets were stored at −80°C. Total RNA was isolated from the pellets as previously described (Sundelin et al., 2011) with the following modification: the mRNA was purified from the total RNA using the Dynabeads mRNA purification kit (Dynal) following the manufacturer’s instructions. The cDNA was synthesized from mRNA representing an early 2 DAI and a late 6 DAI time point and both forward and reverse subtractions were performed between the two mRNA populations. In total, 300 clones were sequenced by MWG-Biotech (Germany) or StarSEQ (Germany) and used for BLAST and BLAST similarity searches of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).
Gene expression of CaTPS during infection of strawberry fruits. Green strawberry fruits (cv. Elsanta) were carefully chosen based on similar size and fruit ripening stage. The fruits were disinfected in 70% ethanol for 1 min and for 30 s in 1% NaOCl followed by washing fruits in distilled water for 1 min and air-drying. The inoculation with *Coll. acutatum* isolate SA 0-1 was performed by dipping fruits three times in spore suspensions of 10^6 spores ml^-1, and controls were dipped in distilled water. The fruits were placed in sterile plastic boxes with five fruits in each. Controls and inoculated fruits were placed in separate boxes and placed in darkness and incubated at 25 °C for 7 days. At day 4 and day 5, one box with water-inoculated (controls) and one with *Coll. acutatum*-inoculated (infected) fruits were removed from the incubator and used for further real-time PCR analysis. Of the five berries in each box, three were chosen at similar ripening and infection stages for real-time PCR analysis. The strawberry samples were peeled using a sterile scalpel. Strawberry peels (2 g) were analysed separately of samples from both infected and control berries. The total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich). This was performed following the manufacturer’s instructions, with the following modifications: to lyse material samples, we used a mixture of lysis solution/ME supplemented with 0.01 g ml^-1 polyvinylpyrrolidone (PVPP; Sigma-Aldrich) solution. The first column wash was repeated twice and continued with on-column DNA digestion (Qiagen). The incubation time was extended to 30 min at room temperature. The second column wash was repeated three times before elution. The concentration of the purified total RNA sample was determined using a NanoDrop ND-1000 instrument. The first-strand cDNA was generated by iScript cDNA synthesis (Bio-Rad) from 1 µg total RNA with random primers. The gene expression analysis was set up for each biological replicate in two technical replicates on an Mx3000p quantitative PCR system (Strategene) with 150 ng of cDNA and Maxima SYBR Green quantitative PCR Master Mix (Fermentas). Expression of the selected gene CaTPS at 4 and 5 DAI was normalized with the β-tubulin gene previously evaluated as housekeeping gene of *Coll. acutatum* (Brown et al., 2008). The fold-changes in target genes were determined by the 2^{ΔΔCt} method, where the ΔΔCt = (CTarget - Cβ-tubulin)treatment - (CTarget - Cβ-tubulin)control (Livak and Schmittgen, 2001). The expression data of β-tubulin were used as calibrator at 4 DAI and were statistically analysed together with CaTPS using the REST software (Pfaffl et al., 2002). The primers used for quantitative real-time PCR (qRT-PCR) analysis of CaTPS during strawberry fruit infection are listed in Table S1 (available in the online Supplementary Material). A melting curve analysis of each primer pair was used to confirm the identity and absence of primer dimers. The specificity of the PCR products was confirmed by agarose gel electrophoresis. PCR products were separated on a 1.0% agarose gel (TAE buffer), excised and purified using the MinElute (Qiagen) gel extraction kit. The PCR products were cloned into pET12/Blunt vector (Fermentas) and thereafter sequenced by MWG-Biotech. A full-length sesquiterpene synthase gene designated CaTPS was obtained and the nucleotide (KP398851) and amino acid sequence (AKE33935) were submitted to NCBI.

**Alignment of CaTPS and other sesquiterpene synthases.** A funnel gene sequence alignment analysis of the biochemically characterized and putative sesquiterpene synthase sequences was extracted from NCBI’s non-redundant protein database. These sequences were aligned using default options in MUSCLE (Edgar, 2004) and implemented in the software GENEIOUS 6.1.8 (www.geneious.com). Putative aristolochene synthases extracted from *Colletotrichum* spp. genomes were only included if they contain the two conserved metal binding domains called DDxxD/E (motif i) and NSE/DYE (motif ii) and are of similar size to CaTPS and experimentally characterized aristolochene synthases (Cane & Kang, 2000; Proctor & Hohn, 1993). In addition, the latter domain has a joined H–α loop region, which has been characterized in *Coprinus cinereus* by López-Gallego et al. (2010), which was extracted with motif i and motif ii from the multiple sequence alignment analysis. These were concatenated into a single global alignment and used for a subsequent neighbour-joining method to generate the phylogenetic tree. This was performed by using matrix pairwise distances estimated under the Jones–Thornton–Taylor model for amino acid sequences in MEGA6 (Tamura et al., 2013). The alignment of the motifs, the joined H–α loop region and phylogenetic tree are shown in Fig. 1(a–c) and the full alignment and amino acid sequence information are given in Fig. S1.

**Heterologous expression of CaTPS for product analysis in Saccharomyces cerevisiae.** Fungal biomass was harvested at 5 DAI after centrifugation for 10 min at 18 000 RCF, 4 °C. Total RNA was extracted from pellets using the Spectrum Plant Total RNA kit (Sigma-Aldrich). The manufacturer’s instructions were followed using this modification: a mixture of lysis solution/ME supplemented with 0.01 g ml^-1 PVPP (Sigma-Aldrich) solution was used for lysis. The first column wash was repeated twice and continued with on-column DNA digestion (Qiagen). The incubation time was extended to 30 min at room temperature. The second column wash was repeated three times before elution. The cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer’s instructions. The ORF of CaTPS was PCR amplified using Phusion High-Fidelity polymerase (Thermo Scientific) with the cDNA library as template together with primers CaTPS forward BamHI (CTGTGATCCATGCTTCGCAAGATGAGGGGCATTG) and CaTPS reverse XhoI (CAGCTCGAGCTACCTAAGATGAGGGGCATTG). The full length of CaTPS was cloned into pET12 vector. The gel-purified PCR amplicons were digested by BamHI and XhoI and were ligated into the respective sites in pESC-Leu2d. After both cloning steps, the CaTPS was verified by sequencing. The pESC-Leu2d vector with CaTPS was transformed into S. cerevisiae strain EBY300 based on the Liac/sdDNA/PEG method (Gietz & Schiestl, 2007a, b). The growth conditions were as described in Nguyen et al. (2012) with a few modifications. Yeast suspensions of subcultures were transferred to 20 ml solid phase microextraction (SPME) headspace vials with 5 ml medium consisting of His, Leu and Met dropout synthetic complete (SC) medium with 2% galactose and 1.8% bacterial agar. Chemotyping was thereafter performed for S. cerevisiae strain EBY300 containing pESC-Leu2d vector with or without CaTPS gene insert. The recombinant yeast strains were incubated at 30 °C for 2 days. Three biological replicates of metabolically engineered yeast with either empty vector or insert were used. The divinylbenzene/carboxymethylcellulose-coated SPME fibre was injected into the 10 ml vial to collect the volatile metabolite with 1 cm of the fibre exposed for 30 min at 25°C and, after absorption, was inserted into the injection port of a GC-MS for thermal desorption (see ‘Terpenoid profiling of CaTPS expressed in S. cerevisiae’, below).

**His tag purification of CaTPS expressed in yeast.** An N-terminal His<sub>6</sub>-tagged version of CaTPS was constructed as described previously for non-tagged CaTPS but using primers CaTPS forward BamHI-His<sub>6</sub> and reverse CaTPS XhoI. The resulting vector CaTPS-His-pESC-Leu2d was sequenced for validation and transformed into yeast wild-type.
Fig. 1. The multiple sequence alignments of the two conserved metal binding motifs, the aspartate-rich DDxxD/E motif i (a) and NDxxS/TxxE/D (NSE/DTE) motif ii with H-α1 loop (b) from functionally characterized or putative fungal sesquiterpene synthases (full alignment and sequence information are found in Fig. S1). For better visualization, only conserved regions with functions important to the cyclization reaction are displayed in the alignments and selected for the phylogenetic analysis. The unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree (c) was made on the basis of the CaTPS and putative Colletotrichum spp. and experimentally characterized aristolochene synthases. In addition, fungal sesquiterpene synthases that have the H-α1 loop characterized (i.e. Cop1–4) or have a similar product profile (i.e. Ff Ffs4) to CaTPS are included. The numbers at the branches indicate the confidence values as percentage and are calculated based on the bootstrap method (10 000 replicates). Only bootstrap values above 50% are shown next to branches. Abbreviations: CfACS,
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BY4742 grown overnight (O/N) in liquid 2× YPD. Transformants were plated on SC-Leu plates and grown for 4 days, and thereafter stored at 4°C. All yeast strains were cultivated at 28°C at 120 r.p.m. in baffled Erlemeyer flasks (containing 4 parts air and 1 part culture) and centrifuged at 4°C. For expression, one large transformed colony was selected and grown O/N in 20 ml SC-Leu-GLU. This starter culture was transferred to 400 ml SC-Leu-GLU and grown O/N. The medium was exchanged with 21 SC-Leu-GLU, transferred to an appropriate flask and grown O/N. Finally, the medium was exchanged with 21 SC-Leu-GAL expression medium and grown for 7 h. We harvested 1 l culture and stored the pellet at −80°C until further processing. The remaining culture was allowed to grow for an additional 10 h before harvesting. These two time points were expected to represent a useful time window for assessing the optimal expression. For purification of His-tagged CaTPS, yeast pellets were thawed on ice and suspended in 50 ml binding buffer (BB: 50 mM Tris/HCl pH 7.5, 25 mM imidazole pH 7.5, 50 mM NaCl, 10 % glycerol and 1 mM PMSF). The cell suspension was lysed on a tissue lyser (Constant Systems) at 38 kpsi. We pooled 30 ml rinse using BB with the first fraction. This lysate was centrifuged at 4000 RCF for 30 min, followed by 18000 RCF for 1 h. The supernatant was filtered through a 0.45 µm syringe filter and incubated with Nickel NTA Agarose resin (Qiagen) on a rotator at 6°C for 2 h or O/N. The resin was washed with 5 column volumes BB, then eluted with imidazole gradient elution buffers 1–8 (5 mM Tris/HCl pH 7.5, 50–500 mM imidazole pH 7.5, 500 mM NaCl, 10 % glycerol and 1 mM PMSF). Each eluate was analysed by SDS-PAGE: the most concentrated and pure were pooled and concentrated further using a 10 kDa centrifugal spin column. The concentrate was desalted against 25 mM Tris/HCl pH 7.5, 10 % glycerol and 100 mM NaCl and was flash frozen and stored at −80°C.

Biochemical characterization of purified CaTPS. For each reaction, 1 µg enzyme was diluted to 20 µl using 5 mM Tris/HCl pH 7.5. To start the reaction, we added 80 µl substrate mix to make a final concentration of 50 mM Tris, 100 mM MgCl₂ and 1.625–200 mM FPP pH 7.5 spiked with tritium labelled FPP to a final specific activity of 0.082 mCi mmol⁻¹ (1 mCi = 3.7×10⁸ Bq). Reactions were swiftly overlaid with 200 µl hexane and tubes were sealed. Each substrate concentration was assayed in triplicate for 1, 2 and 5 min, including controls (reaction mix only, boiled enzyme). Reactions were stopped by addition of 100 µl of 0.5 M KOH and 0.25 M EDTA, followed by rescaling and shaking the tubes thoroughly. Then, the tubes were centrifuged for 1 min at 1500 RCF and either frozen for later analysis or analysed directly. For analysis, 50 µl hexane overlay or control was thoroughly mixed with 200 µl Eco-Scint A scintillation fluid in a 96-well plate and sealed. Plates were counted on a Microbeta 1450 scintillation counter for 1 min. Analysis was performed using SigmaPlot 12. The overlaid hexane extract was analysed by GC-MS analysis (see the next section).

Terpenoid profiling of CaTPS expressed in S. cerevisiae. The analyses were performed as described previously (Andersen et al., 2015; Drew et al., 2012), with minor modifications, on a Shimadzu GCMS-QP2010 with a CTC AOC-5000 autosampler. The injection temperature was set at 250°C. The column was an Agilent HP5ms, fused silica capillary column 30 m, 0.25 mm diameter×0.1 µm film thickness. The carrier gas was H₂ (99.9999 %). Using direct injection and pressure control mode, we maintained the pressure at 20 kPa resulting in an initial flow rate of 1.22 ml min⁻¹. The oven temperature was 45°C for 3 min, increased to 230°C with a rate of 10° min⁻¹ and held for 3 min. The ion source temperature was 230°C with an interface temperature of 250°C. The ionization electron energy was 70 eV and the mass range scanned was m/z in full scan acquisition mode. All data were analysed using the Shimadzu software LabSolutions, GCMS Solutions ver. 2.50 SU3, using the latest libraries provided by NIST (8.01) and Wiley (8.0) including our own data references. Obtained spectra were compared with the spectra in the mass spectral libraries. Compounds were identified by comparing the data with library mass spectra and retention indices (RI). All reference RI were drawn from www.pherobase.com, and based on reference within; references were checked for all identified compounds. Only RI from the well-established DB5 columns (non-polar) were used for identification. The RI of the identified compounds were determined using alkane standards (Table S2) and the chromatogram of EYP300 without expression of exogenous TPS is shown in Fig. S4. α-Humulene and β-caryophyllene were identified using standard compounds (Sigma-Aldrich).

Terpenoid profiling of Coll. acutatum during in vitro growth. The terpenoid profiles of PDB and strawberry medium with or without fungus were determined by dynamic headspace GC-MS. For each biological replicate of the strawberry cultures, two technical replicates of 20 ml were transferred to 150 ml gas washing bottles and 4-methyl-1-pentanol solution (5 mg l⁻¹) was added as internal standard to washing bottles with strawberry and PDB cultures. The bottles were equilibrated to 50±1°C in a circulating water bath and then purged with nitrogen (200 ml min⁻¹) for 60 min. Volatile compounds were collected in Tenax TA traps. The traps contained 250 mg of Tenax TA with mesh size 60/80 and a density of 0.37 g ml⁻¹ (Buchem). The trapped volatiles were desorbed using an automatic thermal desorption unit (ATD 400; Perkin Elmer). Primary desorption was carried out by heating the trap to 250°C with a flow (60 ml min⁻¹) of carrier gas (H₂) for 15.0 min. The stripped volatiles were trapped in a Tenax-TA cold trap (30 mg held at 5°C), which was subsequently heated at 300°C for 4 min (secondary desorption, outlet split 1:1). This allowed rapid transfer of volatiles to a gas chromatograph—mass spectrometer (7890A GC-system interfaced with a 5975C VL MSD with triple-axis detector from Agilent Technologies) through a heated (225°C) transfer line. The column used was a DB-Wax capillary column 30 m long×0.25 mm internal diameter, 0.50 µm film thickness. The column pressure was held constant at 2.4 psi. (1 p.s.i.=6895 Pa), resulting in an initial flow rate of 1.2 ml min⁻¹. The carrier gas was H₂ (99.9999 %). The oven temperature was 40°C for 10 min, from 40°C to 240°C at 8°C min⁻¹, and finally 240°C for 5 min. The mass spectrometer was operating in the electron ionization mode at 70 eV. Mass-to-charge ratios between 15 and 300 were scanned. Terpenoids were identified by probability-based matching of their mass spectra with those of a commercial database (Wiley275.L), and the full list is given in Table S3. The software program MSD ChemStation (version E02.00. Agilent Technologies) was used for data analysis. The concentrations of identified metabolites are presented as relative areas calculated as peak area of the volatile compound divided by the peak area of internal standard. α-Humulene and β-caryophyllene were identified using standard compounds (Sigma-Aldrich).

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RESULTS

Identification and gene expression profile of CaTPS in infected strawberry fruits

From subtractive suppression hybridization clones of *Coll. acutatum* grown on strawberry medium, one gene was found to be similar to terpene synthases. In a BLAST search towards NR on NCBI, this 289 bp EST clone had similarity to an aristolochene synthase and was designated CaTPS. None of the other clones showed similarity to terpene synthases. Genome walking revealed that the full-length DNA sequence of the CaTPS gene was 1088 bp and had three exons and two introns with a corresponding RNA transcript and amino acid sequence size of 981 bp (Fig. S2a) and 327 aa (Fig. S2b), respectively. The full-length CaTPS transcript from *Coll. acutatum* grown on both PDB and strawberry media was investigated by PCR and a single band on gel electrophoresis was observed. The protein signature of CaTPS identified the classic sesquiterpene domains (i.e. motif i and motif ii) using the software program InterProScan 4. In addition, a qRT-PCR analysis was performed to test the importance of the gene during strawberry fruit infection as disease symptoms start to occur 4 DAI. The gene expression levels from qRT-PCR showed that CaTPS is expressed at significantly higher levels (the REST software program estimated the *P* value of 0.001 by factor 79.76) in comparison to the fungal housekeeping gene β-tubulin at 5 DAI (severe black spot symptoms). The housekeeping gene was constitutively expressed at similar levels at 4 and 5 DAI. Thus, the expression of CaTPS was induced during the infection of the strawberry.

Alignment and phylogeny of TPS conserved regions with important functions

The multiple sequence alignment of CaTPS revealed great variation between seven characterized and five putative Colletotrichum spp. fungal sesquiterpene synthases. However, between these sesquiterpene synthases, several conserved regions are shared or are shown to have high level of similarity to each other. This includes the two domains DDxxD/E (motif i) and NSE/DTE (NDxxS/TxxxE/D) (motif ii) and the H-α1 loop connected to the latter motif. These domains are essential for the catalytic activity of the cyclization mechanism (Agger et al., 2009; López-Gallego et al., 2010). The two aristolochene synthases functionally characterized from the filamentous fungi *P. roqueforti* (PrACS) and *A. terreus* (AtACS), together with putative aristolochene synthases of Colletotrichum origin, share identical conserved metal binding motifs i with CaTPS containing glutamic acid (E) in this aspartate rich region, while motif i varies between the remaining sesquiterpene...
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Terpenoid profile of yeast expressing CaTPS and the purified enzyme

CaTPS was successfully expressed in the FPP-overproducing yeast strain EPY300. The GC-MS chromatogram revealed that CaTPS catalyses the biosynthesis of seven sesquiterpene compounds (Fig. 2, Table S2). This includes the two major peaks representing an unidentified sesquiterpenoid (peak 1: calculated RI = 1428) and \( \beta \)-caryophyllene (peak 3) while five minor peaks represent unidentified sesquiterpenoids (peaks 2, 4, 5 and 7) and \( \alpha \)-humulene (peak 6). The unidentified sesquiterpenoid 2 showed similarity (<89%) to several sesquiterpenoids but could not be identified. As previously reported, EPY300 is a native producer of nerol (Fig. S4) (Pickel et al., 2012).

Purification of CaTPS and \textit{in vitro} enzyme kinetics

The CaTPS gene encodes a 35.8 kDa protein as expected; SDS-PAGE analysis revealed a strongly expressed protein of that size (Fig. S3). The optimal expression time was 17 h, and enzyme purity was estimated to be >90%. Initial enzyme assays (5 and 10 µg enzymes) without tritium labelled FPP were analysed by GC-MS. This demonstrated the purified enzyme’s ability to catalyse the formation of the unidentified products sesquiterpenoid 2 and \( \beta \)-caryophyllene when CaTPS is expressed in EPY300 (Fig. 2). For kinetics assays, data were processed using SigmaPlot 12 in order to obtain kinetic constants \( (K_m = 9.7 \pm 3.9 \text{ µM}; V_{max} = 0.179 \text{ µM s}^{-1}; K_{cat} = 0.628 \text{ s}^{-1}; K_{cat}/K_m = 0.0646 \text{ s}^{-1} \text{µM}^{-1}), \) and furthermore, analysis revealed that CaTPS exhibits substrate inhibition in the given experimental setup (Fig. 3). Typical \( K_m \) values of sesquiterpene synthases for FPP are in the range 0.4–10 mM (Asadollahi et al., 2010). Thus, CaTPS appears to have a normal affinity for FPP. Typical turnover numbers fall in the range 0.003–0.3 s\(^{-1}\) for sesquiterpene synthases (Asadollahi et al., 2010; Cane, 1990); here \( K_{cat} \) of CaTPS is slightly higher, but a \( K_{cat}/K_m \) of 0.0646 s\(^{-1}\) µM\(^{-1}\) is in the normal range of terpene synthases.

Terpenoid profile of \textit{Col. acutatum} in different culture media

The identification of terpenoids was achieved by comparing the mass spectra with the NIST and Wiley libraries and with standards of \( \alpha \)-humulene and \( \beta \)-caryophyllene. Spectra were considered coincident if the similarity index was higher than 90% and Kovats index differed by less than 10 retention index units from values in the literature. Unidentified sesquiterpenoids from \textit{in vitro} cultures, not fitting the Kovats index from the literature but having typical mass spectra of sesquiterpenes, were named sesquiterpenoids 1 and 2 and so on, starting the numbering in strawberry medium followed by PDB. In total, 24 sesquiterpenoids were produced by the fungus; 5 (including \( \alpha \)-humulene) of these are biosynthesized when \textit{Col. acutatum} is grown on PDB or strawberry medium, whereas the rest only occur in one of the two media (Table 1). In the strawberry medium, 3 known and 12 unidentified fungus-specific sesquiterpenoids were found while, in PDB, 6 known and 8 unidentified were detected. The majority of these sesquiterpenoids (e.g. sesquiterpenoid 2 and \( \alpha \)-humulene) were found in higher concentration when \textit{Col. acutatum} was grown on strawberry medium as compared to PDB, while the rest were found in similar amounts (e.g. \( \beta \)-caryophyllene).
CaTPS activity depends on the growth medium

CaTPS was found to catalyse the cyclization of seven sesquiterpene compounds in yeast. However, only three of these (i.e. sesquiterpenoid 2, β-caryophyllene and α-humulene) were detected from purified recombinant enzyme after adding FPP. The same three sesquiterpenoid products were detected in strawberry medium with the presence of Coll. acutatum, while only α-humulene was detected in PDB medium during growth of Coll. acutatum (Table 1).

DISCUSSION

Fungi emit a broad spectrum of volatile organic compounds including sesquiterpenoids, which have diverse ecological functions such as phytotoxic and antimicrobial activities (Kramer & Abraham, 2011; Minerdi et al., 2009). Very little is known about the biochemistry and molecular biology of Coll. acutatum and its infection process of strawberry (Brown et al., 2008). As shown here, we have characterized the sesquiterpene synthase CaTPS and identified volatile sesquiterpenoids released by the strawberry phytopathogen Coll. acutatum strain SA 0-1. Three sesquiterpenoids (sesquiterpenoid 2, β-caryophyllene and α-humulene) secreted by the fungus during in vitro growth were biosynthesized by CaTPS. β-Caryophyllene has been shown to have phytotoxic activity towards different plants (Wang et al., 2010), exhibiting fungal stimulatory activity (Fantaye et al., 2015), and inhibitory properties towards bacteria and fungi (Dahham et al., 2015; Muroi & Kubo, 1993). β-Caryophyllene also has activity as an insect attractant (Köllner et al., 2008) and provides synergistic effects to activities of α-humulene and more complex terpenoids (Legault & Pichette, 2007). α-Humulene, produced in small amounts by SA 0-1, has also been shown to play a minor role in the induction of infection symptoms in plants (Stokłosa et al., 2012) but is known to have antifungal activities (Minerdi et al., 2009). The high expression of the CaTPS during strawberry infection as seen in our qPCR along with the in vitro production of β-caryophyllene, sesquiterpenoid 2 and α-humulene by CaTPS indicate that CaTPS may play a role in fruit infection.

The volatile sesquiterpenoids are often precursors that are biosynthesized into more complex molecules with toxic

Table 1. Sesquiterpenoids produced by Coll. acutatum

Sesquiterpenoids produced by Coll. acutatum grown in either strawberry or PDB medium. Amounts of sesquiterpenoids are shown as area of the given peak relative to the internal standard (×1000). RT=retention time.

<table>
<thead>
<tr>
<th>Sesquiterpenoid</th>
<th>RT</th>
<th>Coll. acutatum growth on strawberry medium</th>
<th>Coll. acutatum growth on PDB medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7 days 12 days 17 days 22 days</td>
<td>14 days</td>
</tr>
<tr>
<td>Sesquiterpenoid 1</td>
<td>19.3</td>
<td>0.6</td>
<td>0.6</td>
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<tr>
<td>Sesquiterpenoid 2</td>
<td>21.2</td>
<td>6.0</td>
<td>17.7</td>
</tr>
<tr>
<td>Sesquiterpenoid 3</td>
<td>21.6</td>
<td>4.6</td>
<td>4.2</td>
</tr>
<tr>
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<td>4.1</td>
<td>4.5</td>
</tr>
<tr>
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<td>21.9</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
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<td>0.7</td>
</tr>
<tr>
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<td>0.9</td>
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<tr>
<td>Sesquiterpenoid 8</td>
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<td>0.4</td>
<td>1.0</td>
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<tr>
<td>α-Cedrene</td>
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<td>7.4</td>
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<tr>
<td>β-Caryophyllene</td>
<td>23.0</td>
<td>3.1</td>
<td>4.8</td>
</tr>
<tr>
<td>β-Cedrene</td>
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<tr>
<td>α-Copaene</td>
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<tr>
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<td>δ-Cadinene</td>
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<tr>
<td>Z-Calemene</td>
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<td></td>
</tr>
<tr>
<td>Sesquiterpenoid 17</td>
<td>32.5</td>
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</table>
activities (Jeleń, 2002; Pinedo et al., 2008; Proctor & Hohn, 1993). The volatile α-humulene and β-caryophyllene are often found as precursors or intermediates to non-volatile sesquiterpenoids (Kramer & Abraham, 2011). Thus, these and sesquiterpenoid 2 could be precursors to more complex terpenoids found in Coll. acutatum. The biosynthesis of sesquiterpenoid mycotoxins (e.g. PR toxin) can be accompanied by the release of volatile precursors (e.g. aristolochene) and intermediates produced during in vitro growth of fungus (Jeleń, 2002; Proctor & Hohn, 1993), as observed for products emitted by Coll. acutatum in strawberry medium. However, the first cyclization product generated by the fungal sesquiterpenoid synthase (i.e. presilphifolan-8-β-ol) might not be secreted to the ambient environment during the biosynthesis of complex phytotoxins (Pinedo et al., 2008). This might be what is observed when Coll. acutatum is grown on PDB medium, where CaTPS is expressed, but two of the main products are not found in the headspace analysis of the fungus.

The biosynthesis of terpenoids in Coll. acutatum during in vitro growth generates different terpenoid profiles depending on the medium used. The strawberry medium profile mimics that of unripe fruits, the natural host of Coll. acutatum. This indicates that an external stimulus affects the fungus and induces biosynthesis of higher diversity of sesquiterpenoids. Coll. acutatum generates a more diverse terpenoid profile in media compared to when CaTPS is expressed in yeast (i.e. 7). Thus, the terpenoid profile of Coll. acutatum must be related to the activity of several sesquiterpene synthases and their gene clusters. These additional TPSs remain to be discovered. This is further supported as several sesquiterpene synthases are reported within a member of the Coll. acutatum species complex based on genome sequencing analysis, though none of these is characterized (Baroncelli, 2012).

α-Humulene and β-caryophyllene are found in essential oils of a variety of plants but are neither common products of strawberry plants nor detected in uninoculated strawberry or PDB medium (see supplementary data). Thus, together with sesquiterpenoid 2, these sesquiterpenoids are potential chemical biomarkers for Coll. acutatum infection of strawberries. However, this requires further clarification of the activities of such TPSs and possible metabolite profiling of infected fruits.

NR-BLAST of CaTPS showed similarity to putative and functionally characterized aristolochene synthases. These enzymes share similar protein sequences of the two metal binding active site motifs i (Fig. 1a) and ii (Fig. 1b). Therefore, these enzymes were expected to catalyse analogous or related cyclization reactions. Surprisingly, the in vitro and in vivo cultures of Coll. acutatum did not contain the products related to aristolochene biosynthesis (Cane & Kang, 2000; Felicetti & Cane, 2004). Biosynthesis of aristolochene by aristolochene synthases proceeds via germacrene through C1-C10 bond formation (Yu et al., 2007) and the precursor is normally further biosynthesized into various mycotoxins by different fungi (Proctor & Hohn, 1993). CaTPS does not catalyse this reaction; instead, the biosynthesis of α-humulene and β-caryophyllene proceeds though trans-humulyl cation 1,11 ring closure (Fig. 4). Many fungi use these humulene and caryophyllene skeletons to biosynthesize commercially valuable products with bicyclic and tricyclic sesquiterpene structures (Abraham et al., 1990; Brock et al., 2013; Kramer & Abraham, 2011). Therefore, we may expect to find sesquiterpene synthases in such species that have a similar motif ii and H-α loop to CaTPS, whereas motif i is more important for the coordination of Mg2+ in the active site of the enzyme and allows for greater flexibility (López-Gallego et al., 2010). CaTPS and the F. fujikuroi sesquiterpene synthase (Ffs34) produce some of the same products; however, the amino acid sequence of CaTPS shares more similarity with characterized and putative aristolochene synthases (Fig. 1a, b) but forms a closely related sister clade to these (Fig. 1c). These small changes of the active sites of a TPS clearly have a significant impact on the product formation as also shown for plant TPSs (Gao et al., 2012; O’Maille et al., 2008). Interestingly, the H-α1 loop which is part of the lid of the active site in microbial sesquiterpene synthases is diverse among the characterized enzymes (López-Gallego et al., 2010), but it still hinders the quenching of the formation of the CaTPS.

Fig. 4. A proposed reaction mechanism for the formation of the major products of CaTPS. It represents the cyclization pathways based on previous studies on cyclization reactions catalysed by other related sesquiterpene synthases reported elsewhere in the literature (Brock et al., 2013; Kramer & Abraham, 2011).
carbocation by water and allows the enzyme to perform the proton abstraction. Some of the genomes within members of the Colletotrichum species are identified with two different putative aristolochene synthases that are located in each of the mentioned clades. This suggests that those closely related to CaTPS may have similar product profile and have an incorrect gene annotation as aristolochene synthase.

In summary, we performed, we believe, the first functional characterization of a sesquiterpene synthase (CaTPS) of Colletotrichum species responsible for the formation of multiple sesquiterpenoids. We successfully used yeast as a platform for production of CaTPS for both in vivo and in vitro experiments. A broad spectrum of terpenoids is formed by Col. acutatum in PDB and strawberry fruit medium and in the latter, the major products of CaTPS are detected. CaTPS is highly active during strawberry fruit infection, suggesting that it could be involved in the pathogenic process. CaTPS is most closely related to aristolochene synthases, but we show that it is a multiple product enzyme with β-caryophyllene being one of the major ones. In the future, it would be interesting to investigate the remaining TPSs from Col. acutatum and identify all the products of CaTPS.

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