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# 1 Yeast-based heterologous production of the Colletochlorin family of fungal 2 secondary metabolites

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## 13 Abstract

14 Transcriptomic studies have revealed that fungal pathogens of plants activate the expression of  
15 numerous biosynthetic gene clusters (BGC) exclusively when in presence of a living host plant. The  
16 identification and structural elucidation of the corresponding secondary metabolites remain  
17 challenging. Here we adapted a polycistronic vector for efficient, seamless and cost-effective cloning  
18 of biosynthetic genes using *in vivo* assembly (also called transformation-assisted recombination)  
19 directly in *Escherichia coli* followed by heterologous expression in *Saccharomyces cerevisiae*. Two  
20 vectors were generated with different auto-inducible yeast promoters and selection markers. The  
21 effectiveness of these vectors was validated with fluorescent proteins. As a proof-of-principle, we  
22 applied our approach to the Colletochlorin family of molecules. These polyketide secondary  
23 metabolites were known from the phytopathogenic fungus *Colletotrichum higginsianum* but had  
24 never been linked to their biosynthetic genes. Considering the requirement for an halogenase, and  
25 by applying comparative genomics, we identified a BGC putatively involved in the biosynthesis of  
26 Colletochlorins in *C. higginsianum*. Following the expression of those genes in *S. cerevisiae*, we could  
27 identify the presence of the precursor Orsellinic acid, Colletochlorins and their non-chlorinated  
28 counterparts, the Colletorins. In conclusion, the polycistronic vectors described herein were adapted  
29 for the host *S. cerevisiae* and allowed to link the Colletochlorin compound family to their  
30 corresponding biosynthetic genes. This system will now enable the production and purification of  
31 infection-specific secondary metabolites of fungal phytopathogens. More widely, this system could  
32 be applied to any fungal BGC of interest.

## 33 Keywords:

34 secondary metabolism, heterologous expression, polycistronic vector, *Colletotrichum higginsianum*,  
35 *Saccharomyces cerevisiae*.

## 36 Abbreviations

37 BGC: biosynthetic gene cluster; CDS: coding sequence; DAPI: 4',6-diamidino-2-phenylindole; DMAPP:  
38 dimethylallyl pyrophosphate; ELSD: evaporative light scattering detector; GPP: geranyl  
39 pyrophosphate; HPLC: high-performance liquid chromatography; IVA: *in vivo* assembly; LC: liquid  
40 chromatography; MS: mass spectrometry; NLS: nuclear localization signal; NRPS: non-ribosomal  
41 peptide synthetase; OA: Orsellinic acid; OSMAC: one strain many compounds; PCR: polymerase chain  
42 reaction; PDA: potato dextrose agar; PKS: polyketide synthase; SM: secondary metabolite; TAR:  
43 transformation-assisted recombination; TEV: *Tobacco etch* virus; UV: ultraviolet; YNB: yeast nitrogen  
44 broth; YPD: yeast extract peptone dextrose.

## 45 **1. Introduction**

46 Fungi are rich sources of structurally diverse, small molecule natural products, as illustrated by the  
47 fact that more than 60% (20,304) of the 33,372 microbial natural products currently catalogued in  
48 the Natural Products Atlas were isolated from fungi (<https://www.npatlas.org>, May 2023)  
49 (van Santen et al., 2022). Well-known for their uses in medicine or agriculture, fungal natural  
50 products play important roles in the adaptation of fungi to their ecological niches, for example as  
51 toxins to compete with other microorganisms, as protection against environmental stresses, or in the  
52 case of pathogens, as effectors to facilitate the infection of plant or animal hosts (Collemare et al.,  
53 2019; Oberlie et al., 2018).

54 Fungal genes involved in the biosynthesis of natural products are typically located side-by-side in the  
55 genome as so-called biosynthetic genes clusters (BGCs). These clusters usually contain genes  
56 encoding one or two key enzymes that generate the backbone of the molecule and a varying number  
57 of genes encoding accessory enzymes that decorate the initial molecule. Genes coding for  
58 transporters or transcription factors can also form part of the BGC (Keller, 2019). This colocalization  
59 in the genome facilitates the identification of BGCs, and several bioinformatic tools (e.g. SMURF,  
60 antiSmash, MIBiG, CusProSe) have been developed to mine the ever-increasing number of  
61 sequenced fungal genomes released in public databases (Blin et al., 2021; Khaldi et al., 2010; Oliveira  
62 et al., 2023; Terlouw et al., 2023). Analysis with these tools has shown that a single fungal genome  
63 can contain more than 80 non-redundant BGCs (Han et al., 2016; Inglis et al., 2013; Liang et al., 2018;  
64 Valero-Jiménez et al., 2020). However, for the vast majority of these predicted BGCs, the chemical  
65 products are currently unknown.

66 *Colletotrichum higginsianum* is a plant-pathogenic ascomycete fungus that causes disease on various  
67 cultivated *Brassicaceae* as well as the model plant *Arabidopsis thaliana* (O'Connell et al., 2004).  
68 Resequencing the genome of *C. higginsianum* revealed the presence of 77 non-redundant BGCs, of  
69 which only 14 (18%) can be linked to known chemical products based on their similarity to BGCs

70 characterized in other fungi (Dallery et al., 2017; O'Connell et al., 2012). Transcriptional analysis  
71 showed that 19 *C. higginsianum* BGCs are induced at particular stages of plant infection. Among  
72 these, 14 are specifically expressed during the initial biotrophic phase of penetration and growth  
73 inside living plant cells, two are upregulated during later necrotrophic growth in dead tissues, while  
74 three are expressed at both infection stages (Dallery et al., 2017; O'Connell et al., 2012). These  
75 clusters are poorly expressed, or not at all, by axenic cultures of *C. higginsianum*, which has hindered  
76 isolation of the corresponding fungal metabolites in sufficient amounts for determination of their  
77 chemical structures and analysis of their biological activities.

78 Various strategies can be used to activate the expression of cryptic fungal BGCs in axenic cultures.  
79 For example, variation of the culture conditions (media composition, static/liquid cultures), as in the  
80 'One strain many compounds' (OSMAC) technique (Bode et al., 2002; Hewage et al., 2014) and co-  
81 cultivation with other microbes (Yu et al., 2021). Other approaches include over-expressing global or  
82 cluster-specific transcriptional activators (Chiang et al., 2009; von Bargen et al., 2013), and opening  
83 up chromatin structure by the chemical or genetic manipulation of epigenetic regulator proteins,  
84 such as CCLA, KMT6 and LAEA (Lyu et al., 2020; Pimentel-Elardo et al., 2015). In *C. higginsianum*, we  
85 previously deleted the CCLA subunit of the COMPASS protein complex, which mediates mono-, di-  
86 and trimethylation of lysine 4 in histone H3. Cultures of the resulting mutant over-produced a  
87 number of terpenoid compounds belonging to the Higginsianin, Colletorin/Colletochlorin and  
88 Sclerosporide families (Dallery et al., 2019).

89 Although these genome-wide strategies have been successfully used to isolate new compounds from  
90 some fungi, they are untargeted and do not activate all silent BGCs. Heterologous expression  
91 provides a way to activate specific BGCs of interest and facilitates the large-scale production of  
92 metabolites in axenic cultures. In this approach, an entire BGC is cloned into a heterologous microbial  
93 host that can be easily cultivated (Ahmed et al., 2020; Bond and Tang, 2019; Chiang et al., 2013;  
94 Gomez-Escribano and Bibb, 2011; Pfeifer et al., 2001; Zhang et al., 2018). For heterologous

95 production of fungal secondary metabolites, the most frequently used hosts have been *Escherichia*  
96 *coli* (Kealey et al., 1998; Pfeifer Blaine et al., 2003), the yeasts *Saccharomyces cerevisiae* and *Pichia*  
97 *pastoris* (Cochrane et al., 2016; Gao et al., 2013; Kealey et al., 1998; Xue et al., 2017) and some  
98 filamentous fungi, including species of *Aspergillus*, *Trichoderma* and *Penicillium* (Heneghan et al.,  
99 2010; Nielsen et al., 2013; Pohl et al., 2020; Shenouda et al., 2022). *S. cerevisiae* has the advantage of  
100 being easily genetically manipulated, grows rapidly in liquid culture and can support the protein  
101 folding and post-translational modifications occurring in filamentous fungi. Yeast also produces very  
102 few endogenous secondary metabolites, which facilitates the purification and isolation of  
103 heterologous compounds (Bond et al., 2016; Ishiuchi et al., 2012; Yu et al., 2013; Zhao et al., 2020).

104 To synchronously activate all the genes in a biosynthetic pathway during heterologous expression it  
105 is often necessary to laboriously change the native promoter and terminator of each gene (Harvey et  
106 al., 2018; Pahirulzaman et al., 2012). To avoid this, Hoefgen et al. (2018) designed an expression  
107 vector that allows the concerted expression of multiple genes as a single polycistron, where all the  
108 genes are placed under the control of a single promoter, allowing their co-expression. The self-  
109 splicing porcine teschovirus P2A DNA sequence is inserted at the 3' end of each gene of the  
110 polycistron, which induces bond skipping by the ribosome during translation, thereby releasing the  
111 individual proteins (Kim et al., 2011). The vector also incorporates a split fluorescent reporter gene so  
112 that correct translation of the polycistronic transcript can be monitored by microscopy. The vector  
113 was successfully used to transfer the psilocybin biosynthetic pathway into *A. nidulans* (Hoefgen et al.,  
114 2018).

115 In the present study, we have adapted the polycistronic vector of Hoefgen et al. (2018) for the  
116 heterologous expression of fungal BGCs in baker's yeast. To validate this expression system, we  
117 chose the Colletochlorins and their non-chlorinated counterparts, the Colletorins, a well-  
118 characterized family of prenylated polyketide compounds produced by *C. higginsianum* (Dallery et  
119 al., 2019). Using genome mining and comparative genomics, we identified a candidate BGC that

120 could be involved in this family of molecules. Four genes, encoding a polyketide synthase, a non-  
121 ribosomal peptide synthase-like enzyme, a putative prenyltransferase and an halogenase, were  
122 expressed in *S. cerevisiae*. The resulting yeast culture supernatants were found to contain the  
123 expected products and intermediates of this biosynthetic pathway, namely Orsellinic acid, Colletorins  
124 B and D, Colletochlorin B and D and Colletorin D acid.

## 125 **2. Materials and Methods**

### 126 **2.1. Biological material and growth conditions**

127 A summary of the *Saccharomyces cerevisiae* strains used in this study is given in Supplementary File  
128 1. *In vivo* assembly and propagation of plasmids was performed using the *E. coli* TOP10 strain.  
129 Bacteria were maintained and propagated in LB broth, supplemented with antibiotics and agar as  
130 required. The yeast strains were cultivated at 28°C in the selective medium YNB (cat. no. Y0626,  
131 Sigma), supplemented with drop-out without leucine (cat. no. Y1376, Sigma), without uracil (cat. no.  
132 Y1501, Sigma) or without both leucine and uracil (cat. no. Y1771, Sigma), and supplemented with 2%  
133 glucose and 2% agar. For the heterologous production of metabolites, the yeast strains were  
134 cultivated in YPD liquid medium (yeast extract 10 g·L<sup>-1</sup>, peptone 20 g·L<sup>-1</sup> and glucose 20 g·L<sup>-1</sup>) for 72 h  
135 at 30°C with agitation on a rotary shaker at 250 rpm.

### 136 **2.2. Yeast expression vector construction**

137 All vectors developed in the present study were built by *in vivo* assembly (IVA) of DNA fragments in *E.*  
138 *coli*. The PCR primers used to amplify the fragments were designed to provide a minimum of 22 bp of  
139 overlapping sequence between adjacent fragments for homologous recombination in the bacteria.  
140 The amount of fragment used for IVA varied according to the fragment size: 200-300 bp: 1.5 pmol,  
141 300-600 bp: 1.0 pmol, 600-1000 bp: 0.5 pmol, 1000-3000 bp: 0.2 pmol, 3000-5000 bp: 0.1 pmol, >  
142 5000 bp: 0.05 pmol. Residual plasmid matrix in the PCR reactions was removed by digestion at 37°C  
143 for 2 h with 10U of DpnI enzyme before proceeding further. All the cloning fragments were obtained  
144 by PCR amplification using Q5 polymerase according to the manufacturer's instructions (cat. no.

145 M0491L, New England Biolabs). Diagnostic PCR was performed using GoTaq polymerase (cat. no.  
146 M7805, Promega).

147 The plasmid pHYX104 was constructed by amplifying by PCR the polycistronic fragment (*VenusN-P2A-*  
148 *TEV-P2A-VenusC*) from pV2A-T (Hoefgen et al., 2018) using primers P953 and P954, the *URA3* marker  
149 gene, 2 $\mu$  origin, *AmpR* and ColE1 origin from pNAB-OGG (Schumacher, 2012) with primers P949 and  
150 P950, the *ScADH2* promoter with primers P951 and P952 and the *ScHIS5* terminator with primers  
151 P955 and P956. The four fragments were assembled by IVA in *E. coli*. The resulting plasmid pHYX104  
152 was amplified by PCR with primers P963 and P964 to remove the *AmpR* gene and replace it with the  
153 modified *KanR*<sup>+</sup> gene amplified with primers P965 and P966 from pV2A-T to take advantage of the  
154 Swal/PmeI restriction sites. The resulting plasmid pHYX105 was further modified with an EcoRV-free  
155 version of the *URA3* gene obtained by gene synthesis to give rise to pHYX138.

156 The plasmid pHYX106 was constructed by amplifying the polycistronic fragment (*VenusN-P2A-TEV-*  
157 *P2A-VenusC*) from pV2A-T with primers P953 and P954, the *LEU2* marker gene from M4755 (Voth et  
158 al., 2003) with primers P971 and P972, the 2 $\mu$  origin, *KanR*<sup>+</sup> and ColE1 origin from pHYX105 with  
159 primers P967 and P950, the *ScPCK1* promoter with primers P975 and P976 and the *ScPRM9*  
160 terminator with primers P977 and P978. The five fragments were assembled by IVA in *E. coli*. The  
161 resulting pHYX106 was further modified by PCR with primers P1204 and P1205 to insert a single  
162 nucleotide mutation to remove the EcoRV restriction site present in the *LEU2* marker gene, giving  
163 rise to the plasmid pHYX137 (Supplementary File 2).

164 The plasmid pHYX163 was constructed by digesting the plasmid pHYX153 with Swal and the pHYX154  
165 with PmeI following the strategy described by Hoefgen et al. (2018). The digestion was performed  
166 overnight (nearly 16h). After heat inactivation, the digested fragments (0.1 pmol of each fragment of  
167 interest) were assembled by IVA in *E. coli*. The resulting plasmid pHYX163 was digested by PmeI and  
168 assembled with the plasmid pHYX152 previously digested with Swal, to obtain the pHYX164 plasmid.

169 The plasmid pHYX172 was derived from plasmid pCfB2312, and was constructed by amplifying

170 pCfB2312 with primers P1312 and P1313, and the prenyltransferase (codon-adapted for yeast) from  
171 pHYX154 with primers P1310 and P1311. The two fragments were gel-purified and assembled by IVA  
172 in *E. coli*. All primers used are listed in Supplementary File 3. The following plasmids were deposited  
173 with Addgene: pHYX137 (#202814), pHYX138 (#202815), pHYX143 (#202816) and pHYX173  
174 (#202817).

### 175 **2.3. Modifications of the yeast chassis strain**

176 The yeast strain *S. cerevisiae* BJ5464-NpgA was modified by inserting the *Botrytis cinerea* NADPH  
177 cytochrome P450 reductase gene *BcCPR1* (Bcin12g03180) at the yeast locus XI-3 as described by  
178 Mikkelsen et al. (2012). Competent yeast cells were prepared according to Knop et al. (1999). The  
179 CRISPR-Cas9 transformation was performed as described by Jessop-Fabre et al. (2016) using the  
180 plasmids pCfB2312, pCfB3045 and XI-3-bccpr1 yielding the strain BJNBC (Supplementary File 1).

### 181 **2.4. Comparative analyses of the biosynthetic gene cluster 16**

182 The protein sequences of the genes belonging to the BGC16 of *C. higginsianum* IMI 349063  
183 (CH63R\_05468 to CH63R\_05483) as predicted in Dallery et al. (2017) were used to query the NCBI nr  
184 database using cblaster v1.3.9 (Gilchrist et al., 2021) with the `search` module and default  
185 parameters but limiting the investigations to fungi with `-eq "txid4751[orgn]"` for the  
186 Colletochlorin part of BGC16, or limiting the investigations to *Colletotrichum* spp. with the parameter  
187 `-eq "txid5455[orgn]"` for the entire BGC16. The BGCs identified were retrieved with the module  
188 `extract_clusters` and used as input for `clinker` software v0.0.23 using default parameters  
189 (Gilchrist and Chooj, 2021). Manual editing of the cartoons was performed to represent the contig  
190 ends where appropriate.

### 191 **2.5. Cloning of reporter genes and biosynthetic genes**

192 The gene predictions of CH63R\_05468 to CH63R\_05471 were manually inspected for correctness  
193 using RNA-Seq datasets previously published (O'Connell et al., 2012). The CDS of CH63R\_05468  
194 appeared to be composed of six exons instead of only five in the initial prediction. Each gene was



195 then synthesized without stop codons and with optimization of the codons for *S. cerevisiae* and  
196 exclusion of common restriction enzyme sites using the GenSmart™ Codon Optimization tool  
197 (Genscript Biotech B.V., Netherlands). The optimized sequences can be found in Supplementary File  
198 4. Each coding sequence was individually cloned into the EcoRV linearized pHYX137 and  
199 subsequently assembled together following the strategy described by Hoefgen et al. (2018) using  
200 SwaI and PmeI restriction enzymes followed by IVA in *E. coli*. The gene coding mScarlet-I was  
201 amplified from the plasmid Double UP mNeogreen to mScarlet (Addgene #125134) by PCR using  
202 primers P1214 and P1215 and cloned into the EcoRV linearized pHYX137 by IVA in *E. coli*. The gene  
203 coding Tobacco Etch Virus (TEV) protease was synthesized (Genscript Biotech B.V., Netherlands),  
204 then amplified by PCR using primers P1177 and P1178 and cloned into the EcoRV linearized pHYX137  
205 by IVA in *E. coli*. All coding sequences were verified by Sanger sequencing after their initial cloning  
206 and their presence was further verified by PCR after subsequent combination of vectors for  
207 multigene expression. A summary of the plasmids constructed in this study is presented in  
208 Supplementary File 5.

## 209 **2.6. Yeast transformation**

210 Established protocols were used for the transformation of plasmids into yeast strain BJNBC and the  
211 preparation of frozen competent yeast cells (Knop et al., 1999). To obtain the strain BJNBC015, yeast  
212 transformation was performed in two steps. First, the plasmids pHYX164 and pHYX172 were  
213 integrated, yielding the strain BJNBC014, and then the plasmid pHYX173 was integrated into strain  
214 BJNBC014, giving the strain BJNBC015.

## 215 **2.7. Microscopy**

216 For confocal microscope observations, yeast strains were cultivated in YPD media at 28°C for 2 days.  
217 For staining nuclei, samples were fixed with 4% formaldehyde in PBS for 30 min, spun down and  
218 rinsed once in PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min and rinsed three times in  
219 PBS. Samples were then incubated in 15  $\mu\text{g}\cdot\text{mL}^{-1}$  of DAPI (4',6-diamidino-2-phenylindole) in PBS for  
220 30 min, washed for 5 min and mounted on microscope slides prior to observation. Samples were

221 imaged by sequential scanning using a Leica TCS SPE laser scanning microscope (Leica Microsystems)  
222 equipped with an APO 40× (1.15 NA) oil immersion objective. Venus, mScarlet-I and DAPI were  
223 excited using the 488 nm, 532 nm and 405 nm laser lines, respectively.

## 224 **2.8. Protein extraction**

225 For total protein extraction, yeast strains were cultivated in the appropriate YNB medium until OD  
226 reached 0.4. The cultures were then centrifuged ( $3,000 \times g$ ) for 10 min and the pellets resuspended  
227 in fresh YNB medium supplemented with 2% (w/v) glucose and 3% (v/v) ethanol. Yeast cells were  
228 pelleted by centrifugation at  $3,000 \times g$  for 5 min at 4°C and immediately resuspended in lysis buffer  
229 (8 M urea, 5% [w/v] SDS, 40 mM Tris-HCl pH 6.8, 0.1 mM EDTA,  $0.4 \text{ mg}\cdot\text{mL}^{-1}$  bromophenol blue)  
230 supplemented with 1% (v/v)  $\beta$ -mercaptoethanol, 1× protease inhibitor cocktail (cat.no. P8215,  
231 Sigma-Aldrich),  $5 \text{ }\mu\text{g}\cdot\text{mL}^{-1}$  leupeptin (cat.no. L2884, Sigma-Aldrich) and 1 mM PMSF (cat. no. P7626,  
232 Sigma-Aldrich). PMSF was renewed every 7 min until the samples were frozen at -80°C or loaded on a  
233 gel. Immediately after resuspension, glass beads were added up to the meniscus and the mixture  
234 incubated at 70°C for 10 min. Cells were disrupted using a vortex mixer for 1 min and debris were  
235 pelleted by centrifugation at  $18,000 \times g$  for 5 min at 4°C. After the supernatants were recovered on  
236 ice, 75  $\mu\text{L}$  of lysis buffer was added to the pellets, boiled at 100°C for 5 min, centrifuged again and  
237 the supernatants were finally combined with those from the first centrifugation.

## 238        **2.9. Immunoblot assay**

239        Proteins were separated by SDS-PAGE on 4-15% gradient Mini-Protean TGX Stain-free gels (cat. no.  
240        4568083, Bio-Rad), transferred onto PVDF membranes (cat. no. 1704273, Bio-Rad) and subsequently  
241        blocked with 5% (w/v) BSA in TBST buffer. The membranes were incubated for 1 h at RT with a  
242        mouse anti-2A primary antibody (cat. no. MABS2005, Merck) diluted 1:2000 in 1% (w/v) BSA in TBST.  
243        The membranes were then rinsed 15 min in TBST then 3 x 5 min in TBST before incubation for 1 h at  
244        ambient temperature with HRP-coupled goat anti-mouse secondary antibody diluted 1:5000 (cat.no.  
245        ab6728, Abcam). The membranes were then rinsed with TBST as above before chemiluminescence  
246        detection using the Clarity Western ECL substrate kit (cat. no. 1705060, Bio-Rad). Gels and blots were  
247        recorded with a ChemiDoc Imaging System (Bio-Rad).

## 248        **2.10.        General chemistry procedures**

249        The cultivation of yeast strains harboring each plasmid and isolation of the chemical compounds  
250        were as described previously by Harvey et al. (2018). After preculturing the yeast strains in selective  
251        medium supplemented with 2% (w/v) glucose (2 days at 28°C with shaking at 250 rpm), the  
252        preculture (10 mL) was inoculated into 1 L of YPD medium in a 2 L Erlenmeyer flask (total 4 x 1L) and  
253        incubated for 72 h at 30°C with shaking at 250 rpm. The YPD culture was then centrifuged aseptically  
254        (5000 x g, 5 min) and the supernatant was incubated overnight with sterile XAD-16N resin (Dow  
255        Chemicals) for solid phase extraction (Dallery et al., 2019). The resin was collected by filtration and  
256        extracted for 2 h in ethyl acetate (100 mL) followed by 2 h in methanol (100 mL). Lyophilized cell  
257        pellets were resuspended in acetone (3 x 30 mL), sonicated for 3 x 15 min, centrifuged at 5000 x g, 5  
258        min between each acetone addition, followed by extraction with methanol (3 x 30 mL). Ethyl acetate  
259        extracts were dried over anhydrous sodium sulphate. Similar extracts were pooled, evaporated  
260        under reduced pressure and resuspended in HPLC grade methanol. The crude extracts were then  
261        analyzed on an Alliance 2695 HPLC instrument equipped with a 2998 photodiode array, a 2420  
262        evaporative light scattering and an Acquity QDa mass detector (Waters Corporation). The HPLC  
263        column used was a 3.5 µm C-18 column (Sunfire 150 x 4.6 mm) operating a linear gradient from H<sub>2</sub>O

264 to CH<sub>3</sub>CN, both containing 0.1% formic acid, for 50 min at 0.7 mL·min<sup>-1</sup>. Thin layer chromatography  
265 plates (Si gel 60 F 254) were purchased from Merck. Purified standards of Orsellinic acid, Colletorin D,  
266 Colletorin D acid, Colletochlorin D, Colletochlorin B and Colletochlorin A were dissolved at 1 mg·mL<sup>-1</sup>  
267 in methanol. All standards were purified as previously described (Dallery et al., 2019) except  
268 Orsellinic acid that was purchased from ThermoFisher Scientific (cat.no. 453290010).

## 269 **2.11. Untargeted analysis of different colletochlorin derivatives**

270 Untargeted analysis was performed using a UHPLC system (Ultimate 3000 Thermo) coupled to  
271 quadrupole time of flight mass spectrometer (Q-ToF Impact II Bruker Daltonics).

272 Separation was performed on an EC 100/2 Nucleoshell Phenyl-Hexyl column (200×100 mm, 2.7 μm;  
273 Macherey-Nagel) at 40°C, with a flow rate of 0.4 mL·min<sup>-1</sup>, for 5 μL injected. The mobile phases used  
274 for the chromatographic separation were: (A) 0.1% formic acid in H<sub>2</sub>O; and (B) 0.1% formic acid in  
275 acetonitrile. Elution was as follows: 5% phase B for 2 min, the gradient elution increased linearly to  
276 50% phase B in 13 min, followed by a further linear increase to 100% phase B in 10 min, then 100%  
277 phase B for 3 min and the final gradient linear elution decreased to 5% phase B for 7 min.

278 Data-dependent acquisition methods were used for mass spectrometer data in negative ESI mode  
279 using the following parameters: capillary voltage, 4.5 kV; nebulizer gas flow, 2.1 bar; dry gas flow,  
280 6 L·min<sup>-1</sup>; drying gas in the heated electrospray source temperature, 200°C. Samples were analysed  
281 at 8 Hz with a mass range of 100–1500 m/z. Stepping acquisition parameters were created to  
282 improve the fragmentation profile with a collision RF from 200 to 700 Vpp, a transfer time from 20  
283 to 70 μsec, and collision energy from 20 to 40 eV. Each cycle included a MS fullscan and 5 MS/MS  
284 CID on the 5 main ions of the previous MS spectrum.

## 285 **2.12. Data processing of untargeted metabolomic data**

286 The .d data files (Bruker Daltonics) were converted to .mzXML format using the MSConvert software  
287 (ProteoWizard package 3.0; Chambers et al., 2012). mzXML data processing, mass detection,  
288 chromatogram building, deconvolution, samples alignment and data export were performed using  
289 MZmine-2.37 software (<http://mzmine.github.io/>) for negative data files. The ADAP chromatogram

290 builder (Myers et al., 2017) method was used with a minimum group size of scan 9, a group intensity  
291 threshold of 1000, a minimum highest intensity of 1000 and  $m/z$  tolerance of 10 ppm.  
292 Deconvolution was performed with the ADAP wavelets algorithm using the following setting: S/N  
293 threshold 10, peak duration range 0.01–2 min RT wavelet range 0.02–0.2 min, MS2 scan were  
294 paired using a  $m/z$  tolerance range of 0.05 Da and RT tolerance of 0.5 min. Then, isotopic peak  
295 grouper algorithm was used with a  $m/z$  tolerance of 10 ppm and RT tolerance of 0.1 min. All the  
296 peaks were filtered using feature list row filter keeping only peaks with MS2 scan. The alignment of  
297 samples was performed using the join aligner with an  $m/z$  tolerance of 10 ppm, a weight for  $m/z$  and  
298 RT at 1, a retention time tolerance of 0.2 min. Metabolites accumulation was normalized according  
299 to the weight of dried extract for the relative quantification. Molecular networks were generated  
300 with MetGem software (Olivon et al., 2018; <https://metgem.github.io>) using the .mgf and .csv files  
301 obtained with MZmine2 analysis. The molecular network of ESI<sup>-</sup> datasets was generated using cosine  
302 score thresholds of 0.60.

### 303 **2.13. Metabolite annotation of untargeted metabolomic data**

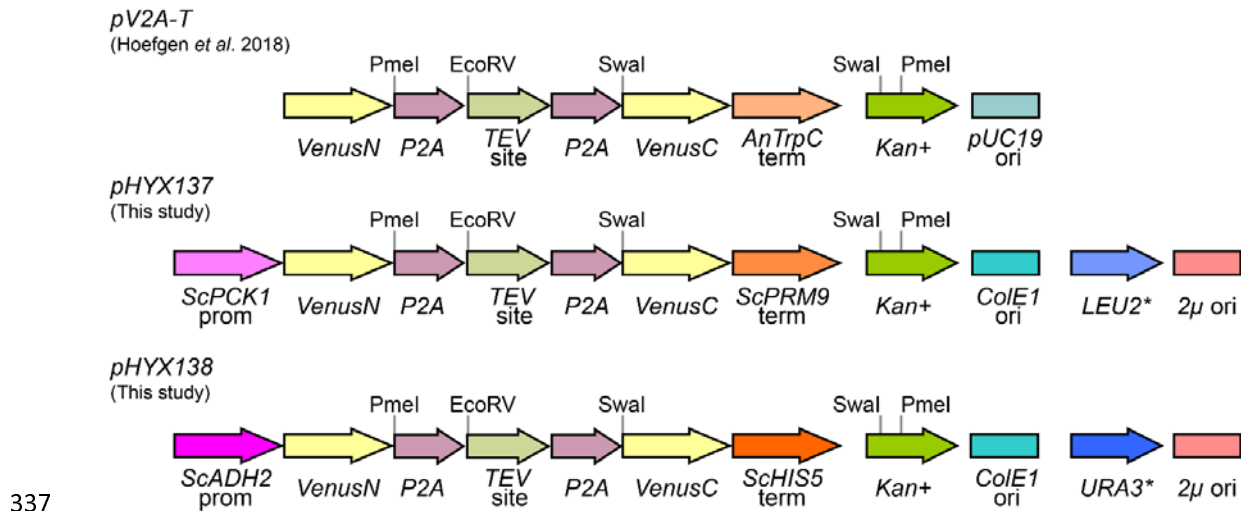
304 Metabolite annotation was performed in three consecutive steps. First, the obtained RT and  $m/z$   
305 data of each feature were compared with our library containing the 6 standards based on their RT  
306 and  $m/z$ . Second, the ESI<sup>-</sup> metabolomic data used for molecular network analyses were searched  
307 against the available MS<sup>2</sup> spectral libraries (Massbank NA, GNPS Public Spectral Library, NIST14  
308 Tandem, NIH Natural Product and MS-Dial), with absolute  $m/z$  tolerance of 0.02, 4 minimum  
309 matched peaks and minimal cosine score of 0.60. Third, not-annotated metabolites that belong to  
310 molecular network clusters containing annotated metabolites from steps 1 and 2 were assigned to  
311 the same chemical family and annotation was carried out on the basis of MS/MS spectrum  
312 comparisons.

### 313 **3. Results**

#### 314 **3.1. Heterologous expression vectors and modification of the yeast recipient** 315 **strain**

316 In their study, Hoefgen et al. (2018) described the polycistronic plasmid pV2A-T designed to express  
317 multiple secondary metabolism (SM) genes under the control of a single promoter. In this  
318 polycistronic system each gene is separated by a TEV-P2A sequence. The P2A sequence encodes a  
319 self-cleaving peptide releasing the upstream protein with a 33 amino acids tail in C-term and the  
320 downstream protein with a proline in N-term. The TEV peptide is recognized and cut by the TEV-  
321 protease enzyme, reducing the C-term tail to 6 amino acids. Each polycistron contained the *VenusN*  
322 and *VenusC* genes on the first and last position of the coding sequence, respectively. Because both  
323 genes contain a nuclear localization signal (NLS), when the polycistronic transcript is translated, the  
324 *VenusN* and *VenusC* proteins accumulate in the yeast nucleus, where they self-assemble to produce  
325 a yellow fluorescent protein. The presence of this fluorescence in the nucleus is thus an indicator of  
326 the production of the polycistronic proteins. By digestion with the *EcoRV* restriction enzyme,  
327 biosynthetic genes from the cluster of interest can be introduced between the *VenusN* and *VenusC*  
328 genes. Polycistronic plasmids containing the desired genes can then be fused after digestion by *SwaI*  
329 or *PmeI* (Hoefgen et al., 2018).

330 Here, we generated the plasmids pHYX137 and pHYX138, both of which can be used in *S. cerevisiae*  
331 (Figure 1; Supplementary File 2). Both vectors harbour auto-inducible promoters from yeast, namely  
332 *pPCK1* or *pADH2*, respectively, which are repressed in the presence of glucose and activated after the  
333 diauxic shift during ethanol-anaerobic fermentation (Harvey et al., 2018). This allows to disconnect  
334 biomass accumulation from SM production, which is a valuable feature when the SM are toxic. Both  
335 promoters are poorly induced in selective medium, in contrast to rich medium (Lee and DaSilva,  
336 2005).



**Figure 1:** Features of the *pV2A-T* plasmid described by Hoefgen et al. (2018) and of the two plasmids, *pHYX137* and *pHYX138*, adapted for *S. cerevisiae* expression, described in this study. Complete maps are shown in Supplementary File 2.

In addition to the polycistronic gene, the plasmids *pHYX137* and *pHYX138* possess a yeast  $2\mu$  origin of replication and the *pUC19* origin of replication was also replaced by the *ColE1* origin. The nutritional selection genes, either *LEU2* or *URA3*, were included and silent mutations were introduced to remove the *EcoRV* restriction sites. This allows to linearize the *pHYX137* or *pHYX138* with *EcoRV* and to clone individually the coding sequences by *in vivo* assembly (IVA) in *E. coli*, which is a fast, cost-effective and simple method. Likewise, the coding sequences are successively assembled in a single polycistron by digesting the plasmids with either *Swal* or *PmeI* and direct transformation of *E. coli* with the unpurified plasmid fragments for IVA.

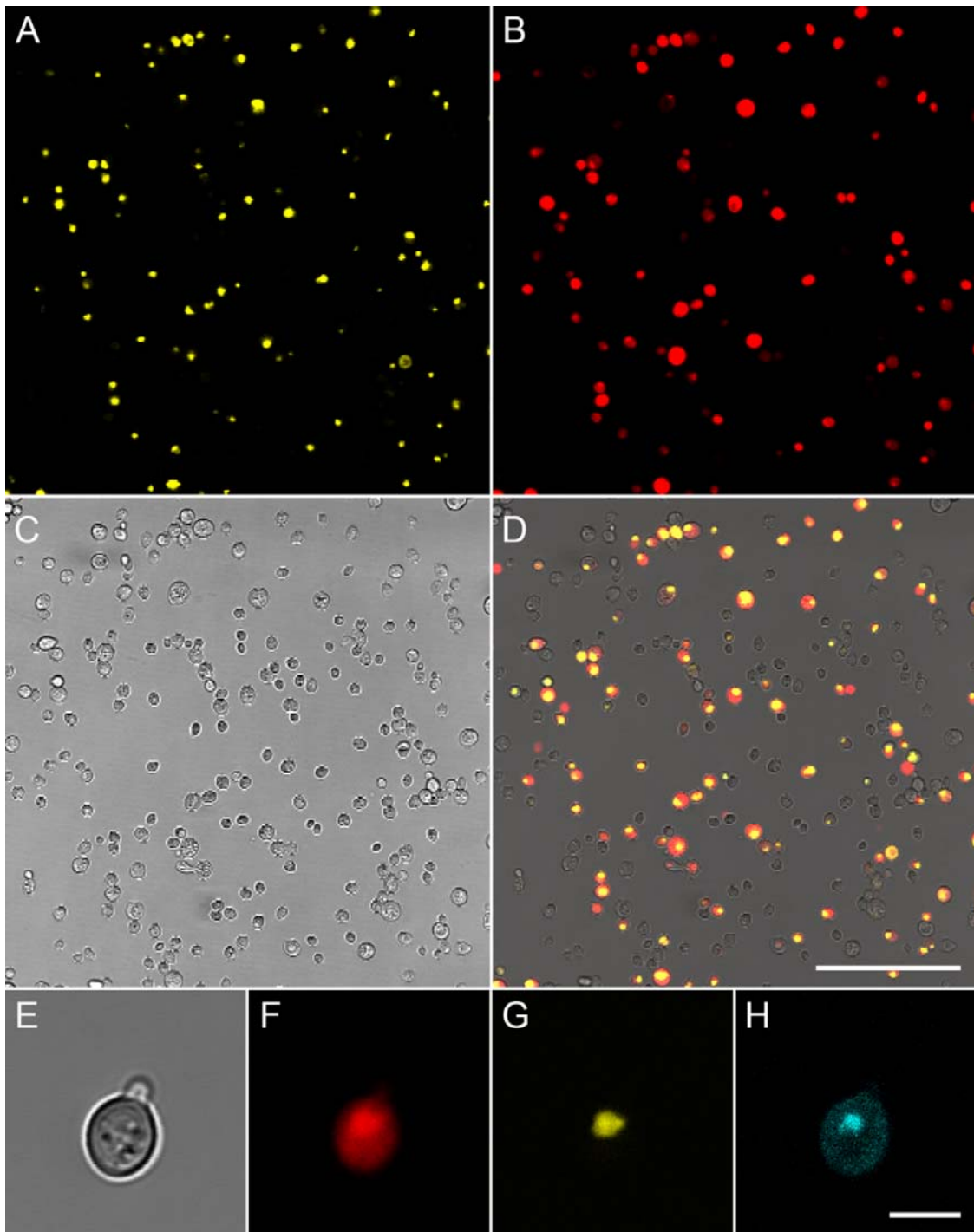
To enhance the production of heterologous polyketides in the yeast *S. cerevisiae*, the strain BJ5464-*npga* was previously modified by the deletion of two vacuolar proteases, *PEP4* and *PRB1* (Lee et al., 2009) and the introduction of the *npga* gene (Bond et al., 2016) involved in activating the ACP domain of PKS enzymes. Here, we used the strain BJNBC, a derivative of BJ5464-*npga* that we generated in the frame of a project involving BGCs with numerous cytochrome P450 enzymes. The modification involved the integration of an NADPH-cytochrome reductase from a filamentous fungus.

355 This NADPH-cytochrome reductase was successfully used to enhance the heterologous production of  
356 *B. cinerea* abscisic acid in *S. cerevisiae* (Otto et al., 2019).

### 357 **3.2. Fluorescence-based validation of the yeast heterologous expression** 358 **system**

359 To verify the proper transcription of the polycistronic gene, and correct translation and separation of  
360 the individual proteins, a gene coding for the mScarlet-I red fluorescent protein was introduced  
361 between the coding sequences of the *VenusN* and *VenusC* genes. The *mScarlet-I* gene was first  
362 introduced into plasmid pHYX137, giving the plasmid pHYX143, which was then transformed into the  
363 BJNBC yeast strain. The transformed yeast was cultivated 24 h in YNB medium supplemented with  
364 2% (w/v) glucose without leucine. Epi-fluorescence microscopy revealed that the yellow fluorescence  
365 of Venus was present in the yeast nucleus, where it colocalized with the blue fluorescent DNA stain  
366 DAPI, whereas the red fluorescence of mScarlet-I, which lacked an NLS, was distributed through both  
367 the cytoplasm and nucleus (Figure 2). These observations confirm that proteins encoded by the  
368 polycistronic gene had been well-transcribed and separately translated in yeast, and that Venus was  
369 correctly assembled in the yeast nucleus from the two complementary non-fluorescent fragments.



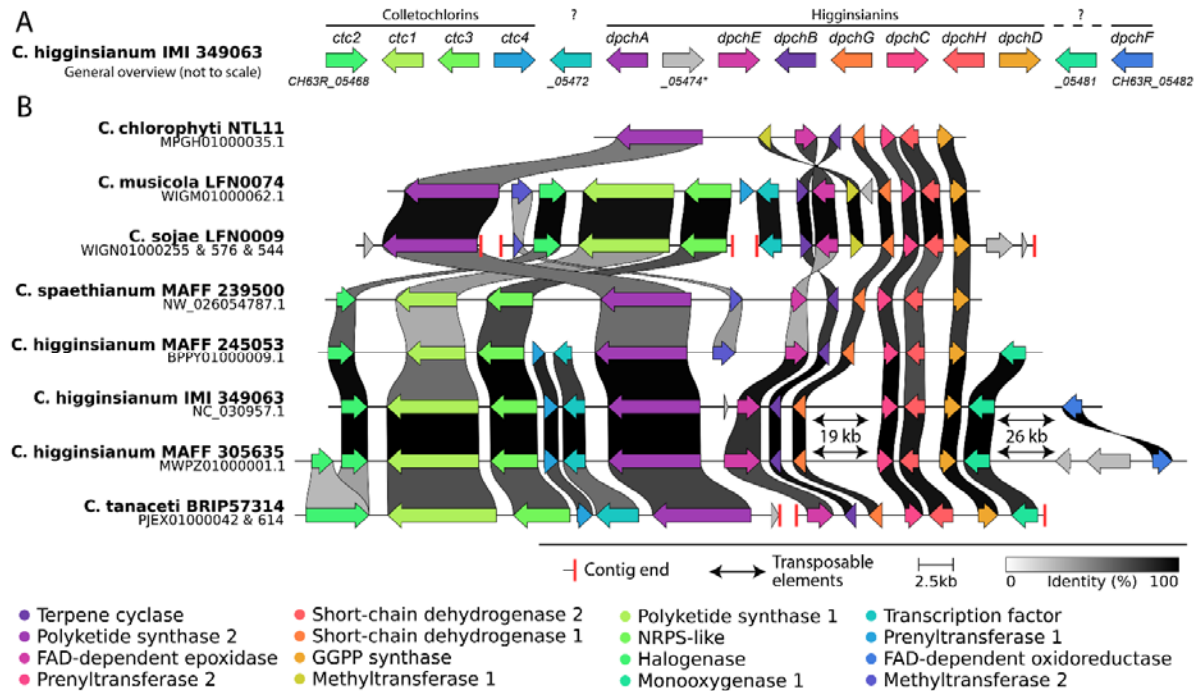


370

371 **Figure 2:** Confocal microscopy of the yeast strain BJNBC-003 expressing Venus-NLS and mScarlet-I fluorescent  
372 proteins and stained with DAPI to detect DNA. A-D, Bar= 50  $\mu$ m. E-H, from left to right, bright-field image  
373 showing the yeast cells, red fluorescence corresponding to mScarlet-I, yellow fluorescence corresponding to  
374 Venus and, blue fluorescence corresponding to DAPI. The mScarlet-I signal is distributed throughout the cell  
375 whereas the Venus signal is co-localized with DNA in the nucleus. Bar = 5  $\mu$ m.

### 376 **3.3. Selection of a biosynthetic pathway to test the expression system**

377 Previously, we isolated several members of the Colletochlorin family of secondary metabolites from  
378 *C. higginsianum* and we proposed an hypothetical biosynthetic pathway based on the isolated  
379 molecules and the plausible expected enzymatic activities (Dallery et al., 2019). In order to identify  
380 the biosynthetic gene cluster (BGC) responsible for producing Colletochlorins, we looked for putative  
381 halogenases (InterPro signature IPR006905) in the *C. higginsianum* IMI 349063 genome. The only  
382 BGC with an IPR006905 signature was BGC16, comprising genes CH63R\_05468 to CH63R\_05483 in  
383 the original prediction (Dallery et al., 2017). Recently, Tsukada et al. (2020) reported the  
384 heterologous production of Higginsianins as well as other decalin-containing diterpenoid pyrones by  
385 expressing 8 of the 16 genes in BGC16. None of the Higginsianins are chlorinated and only one PKS  
386 (*ChPKS11*) was required for the biosynthesis of Higginsianins despite the presence of a second PKS  
387 (*ChPKS10*) in the cluster, suggesting the BGC16 comprises two BGCs side-by-side or intertwined. To  
388 test this hypothesis, we examined the conservation of BGC16 in 57 genome-sequenced  
389 *Colletotrichum* spp. (NCBI taxid 5455) using the cblaster tool (Figure 3). Interestingly, the BGC16 was  
390 found in six species belonging to four different species complexes, namely *C. higginsianum* and  
391 *C. tanacetii* (Destructivum complex), *C. musicola* and *C. sojiae* (Orchidearum complex), *C. spaethianum*  
392 (Spaethianum complex) and *C. chlorophyti*. In *C. tanacetii* and *C. sojiae*, the BGC16 homologous genes  
393 were found respectively on two and three different contigs with each part being located at contig  
394 ends, suggesting problems of genome assembly rather than locations on different chromosomes.  
395 Interestingly, in *C. chlorophyti* only the genes required for making Higginsianin-like molecules were  
396 retrieved. Homologues of the genes CH63R\_05468 to CH63R\_05472 were absent from the *C.*  
397 *chlorophyti* NTL11 genome and were hypothesized to be involved in the biosynthesis of  
398 Colletochlorins (Figure 3).

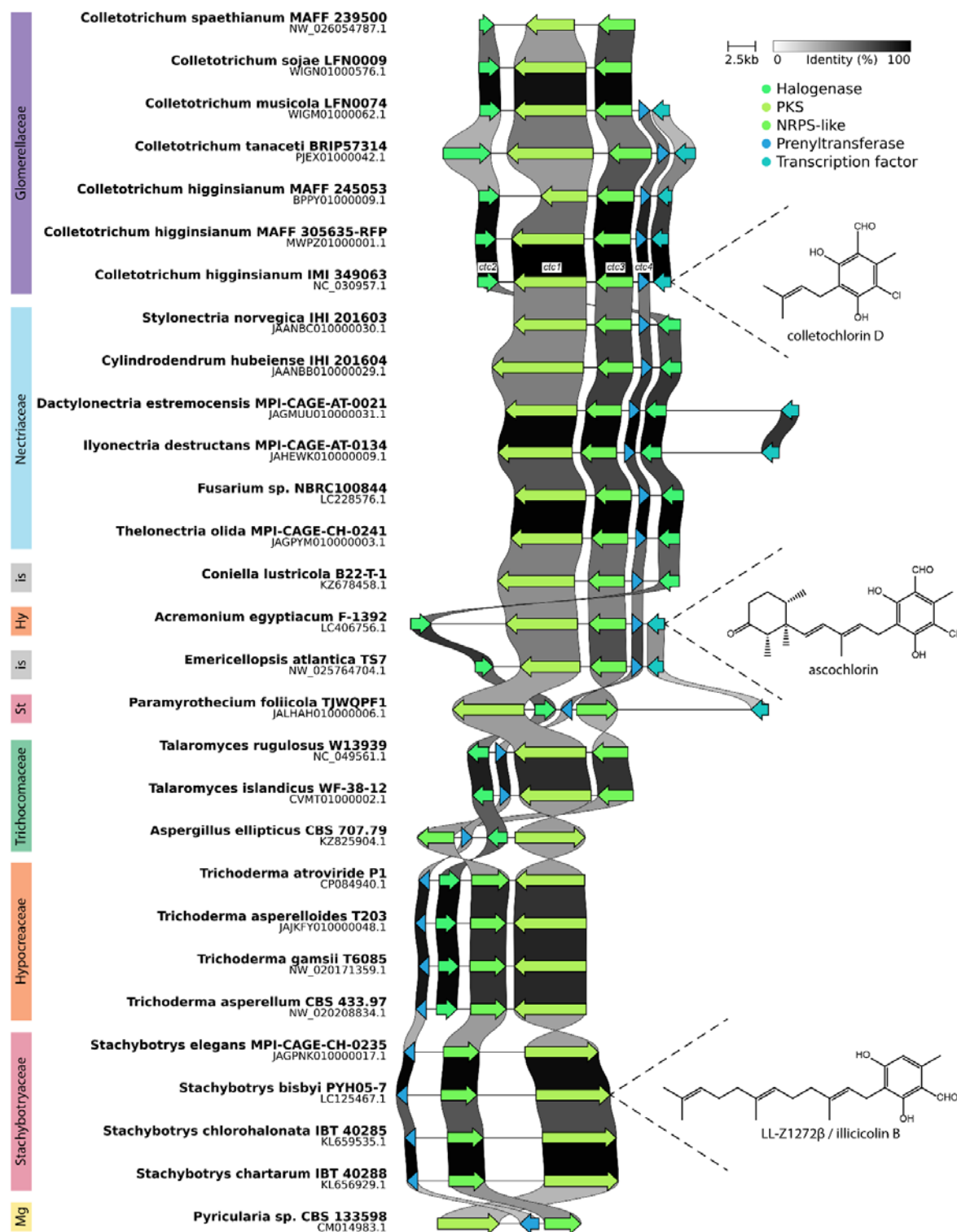


399

400 **Figure 3: General overview of the biosynthetic gene cluster BGC16 (A) and its conservation and microsynteny in**  
 401 **genome-sequenced *Colletotrichum* spp. (B).** The BGC is actually composed of two BGCs side-by-side or intertwined, one  
 402 for biosynthesis of Colletochlorins and the other for biosynthesis of Higginsianins. The Higginsianin genes (*dpch*) were  
 403 characterized by Tsukada et al. (2020). The gene *CH63R\_05474* is a pseudogene whereas the gene *CH63R\_05472*  
 404 encodes a predicted transcription factor. When present, regions composed of repeated transposable elements are shown  
 405 as double-headed arrows together with their length. Vertical red bars denote contig ends. Fungal BGCs are often  
 406 misassembled during genome sequencing and split over several contigs due to difficult-to-assemble long stretches of  
 407 repeats. The intensity of grey/black shading represents the percentage of amino acid identity.

408 Using the cblaster tool, we investigated the conservation of genes putatively responsible for  
 409 Colletochlorins biosynthesis (*CH63R\_05468* to *CH63R\_05472*) in other fungi. Clustered gene  
 410 homologues were found mostly in Sordariomycetes belonging to the *Glomerellaceae*, *Nectriaceae*,  
 411 *Hypocreaceae* and *Stachybotryaceae* families (Figure 4). Only three Eurotiomycetes had homologous  
 412 BGCs (*Aspergillus ellipticus* and two *Talaromyces* spp.). None of the four homologous clusters found  
 413 in *Stachybotrys* species contained an halogenase-encoding gene. Consistently, *Stachybotrys bisbyi*  
 414 cultures produced only non-chlorinated prenylated derivatives of Orsellinic acid, notably LL-Z1272 $\beta$ ,  
 415 also called Illicicolin B (Li et al., 2016). Among the retrieved homologues, we also found the BGC in

416 *Acremonium egyptiacum* responsible for biosynthesis of Ascochlorin, another prenylated yet  
417 chlorinated derivative of Orsellinic acid (Figure 4). Based on these findings and knowledge of the  
418 experimentally-determined biosynthetic route for LL-Z1272 $\beta$  and Ascochlorin, as well as the  
419 predicted pathway for Colletochlorins, we selected genes CH63R\_05468 (*ctc2*, halogenase),  
420 CH63R\_05469 (*ctc1*, also known as *ChPKS10*), CH63R\_05470 (*ctc3*, also known as *ChNRPS-like04*) and  
421 CH63R\_05471 (*ctc4*, prenyltransferase) for heterologous expression in *S. cerevisiae*.  
422

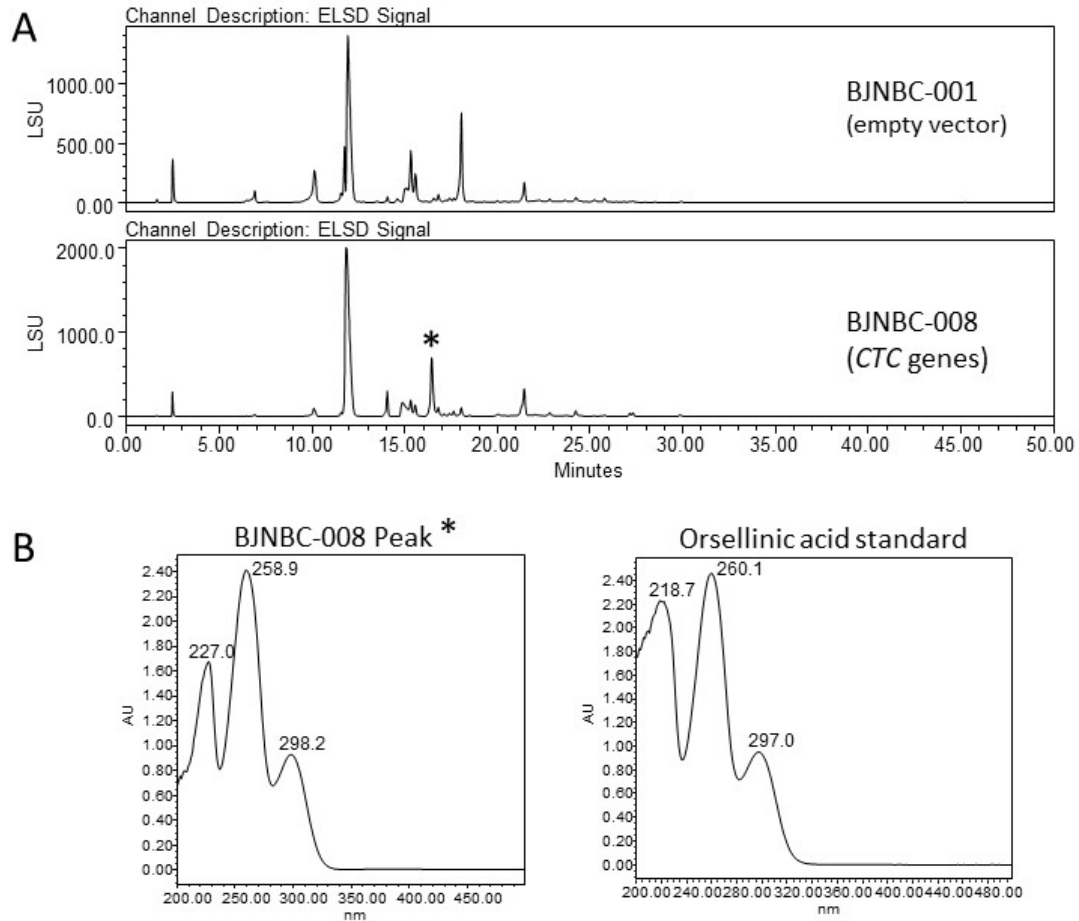


424 **Figure 4: Conservation and microsynteny of the Colletochlorin (ctc) biosynthetic genes cluster in fungi.** In  
 425 *Colletotrichum higginsianum* IMI 349063, this BGC was initially predicted to be a single BGC16 with the *dpch* BGC  
 426 responsible for *Higginsianins* biosynthesis, located side-by-side. Genes are color-coded according to their putative or  
 427 experimentally-confirmed functions. The intensity of grey/black shading represents the percentage of amino acid

428 *identity. Experimentally-verified BGCs are shown with one of their molecular products. Hy, Hypocreaceae; is, incertae*  
429 *sedis; Mg, Magnaporthaceae; St, Stachybotryaceae.*

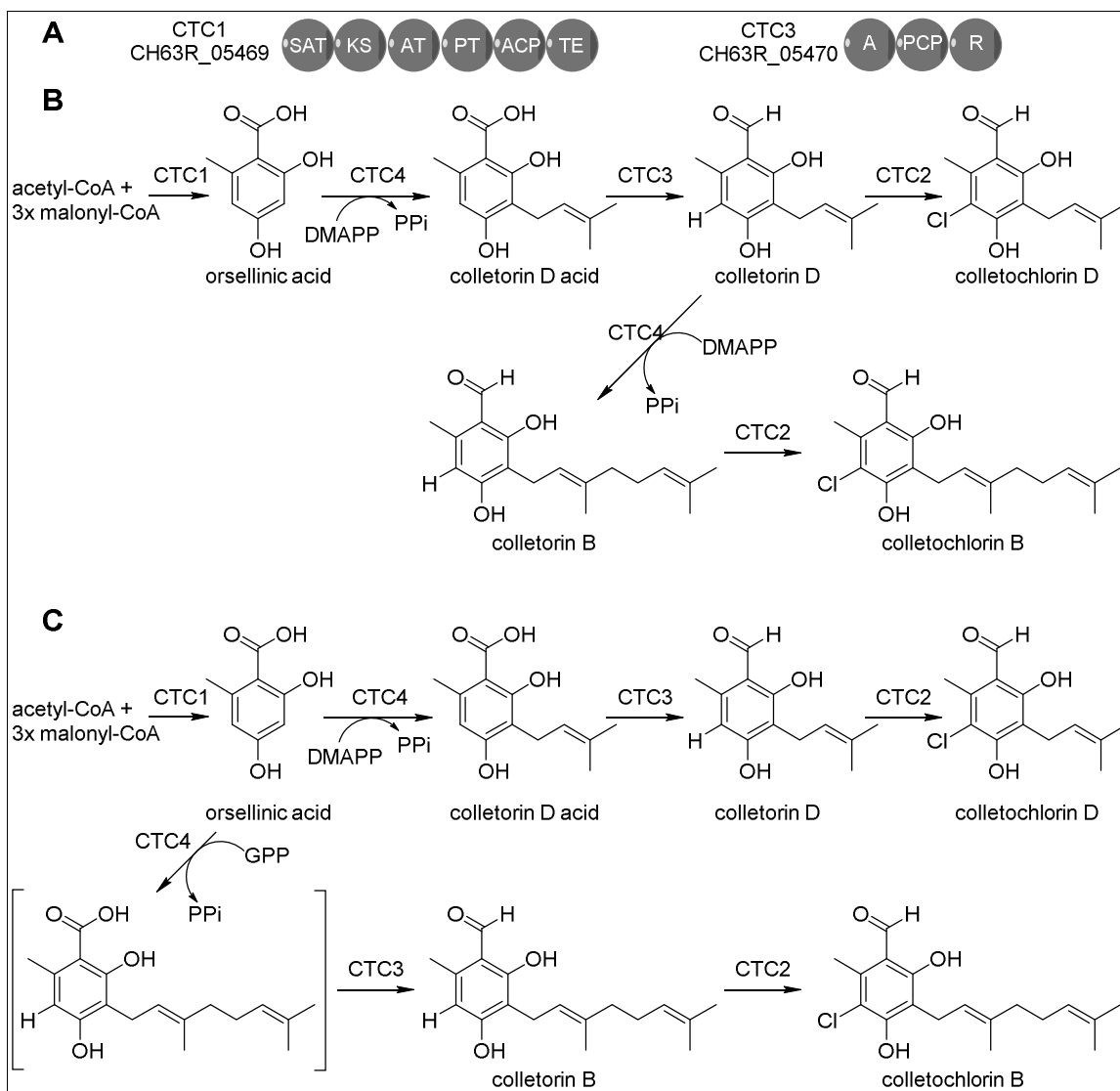
#### 430 **3.4. Heterologous production of Orsellinic acid in yeast**

431 After careful examination of the gene models and intron borders using available RNA-Seq data, the  
432 coding sequences (CDS) of *ctc1* to *ctc4* were codon-optimized for *S. cerevisiae* and *de novo*-  
433 synthesized. Each CDS was then cloned individually into the plasmid pHYX137. A polycistron  
434 containing the four *ctc* CDS under control of the *PCK1* promoter of *S. cerevisiae* was constructed by  
435 successive digestions with either *Swa*I or *Pme*I followed by *in vivo* assembly (Transformation-Assisted  
436 Recombination, TAR) directly in *E. coli*. This plasmid was named pHYX164 and was transformed into  
437 the adapted yeast strain BJNBC, giving BJNBC-008. All plasmids and strains used in this study are  
438 described in Supplementary File 1 and 5.



439

440 Figure 5: Monitoring the production of Colletochlorin biosynthetic intermediates using HPLC-PDA-ELSD-MS. (A)  
441 ELSD chromatogram of crude extracts of strains BJNBC-001 (empty vector) and BJNBC-008 (*ctc1* to *ctc4* genes  
442 expressed from a polycistron). Only Orsellinic acid could be detected among the known intermediates of the  
443 Colletochlorin family. (B) UV spectra of the differential peak identified in BJNBC-008 (asterisk) and of the  
444 Orsellinic acid standard.



445  
446

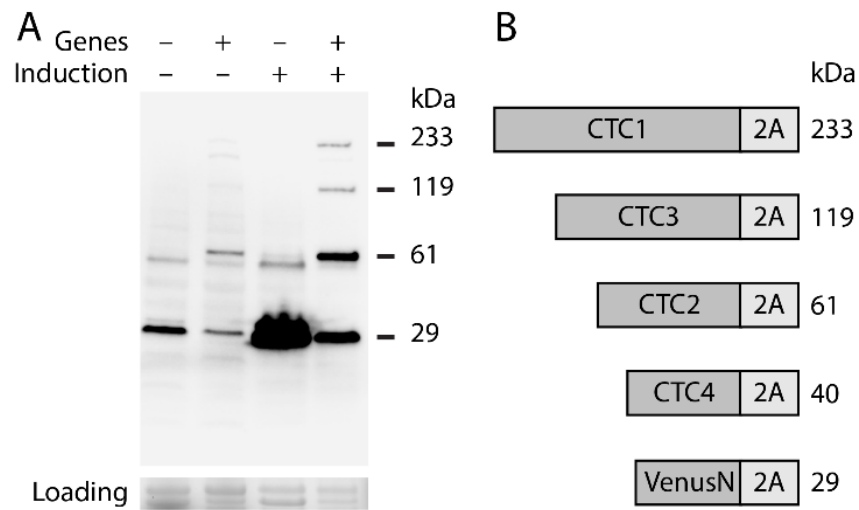
447 *Figure 6: Proposed biosynthetic pathways of the Colletochlorins. (A) Domain structure of CTC1 and CTC3*  
 448 *proteins. (B) Hypothetical scenario 1 where the CTC4 prenyltransferase accepts only DMAPP*  
 449 *(dimethylallylpyrophosphate) as isoprene donor. (C) Hypothetical scenario 2 where the CTC4 prenyltransferase*  
 450 *accepts both DMAPP and GPP (geranylpyrophosphate) as isoprene donors. Domains: ACP, acyl-carrier protein;*  
 451 *AT, acyl transferase; KS, ketosynthase; PT, product template; SAT, starter-unit acyltransferase; TE, thioesterase;*  
 452 *A, adenylation; PCP, peptidyl-carrier protein; R, reduction.*

453

454 The yeast strains BJNBC-001 (containing the empty polycistronic plasmid pHYX137) and BJNBC-008  
 455 (harboring the Colletochlorin gene cluster in pHYX164) were cultured for three days and then



456 metabolites were extracted and analysed by HPLC. Only one molecule was detected in BJNBC-008  
457 that was not present in BJNBC-001. This molecule had a retention time of 16.5 min, a molecular  
458 weight of 168 and a UV spectrum with maxima at 227, 259 and 298 (Figure 5). These same  
459 characteristics were also shown by an orsellinic acid (OA) standard, indicating that the molecule  
460 detected in culture extracts is OA (Figure 5). The presence of OA, the first molecule in the proposed  
461 biosynthetic pathway, but none of the other expected molecules, suggests that the polyketide  
462 synthase was functional but not the prenyltransferase (Figure 6).



463  
464 *Figure 7: Immunodetection of the CTC proteins and VenusN. (A) Immunoblot of whole-cell protein extracts from*  
465 *the strains BJNBC001 (empty vector) and BJNBC008 (ctc genes) at t0 (optical density of 0.4; repressive medium*  
466 *replaced by inductive medium) and t24 after induction. The proteins were detected with an anti-2A antibody.*  
467 *Note that VenusN is present also in the empty vector. Equal loading was assessed using TGX Stain-Free gels. (B)*  
468 *Schematic representation of the expected proteins with their size.*

469 All the enzymes expressed from this polycistron retain a P2A tag at their carboxyl terminus. To verify  
470 that all the enzymes in the pathway were present in the transgenic yeast BJNBC-008, we used  
471 immunoblotting with antibodies raised against the P2A peptide (Hoefgen et al., 2018). Protein  
472 samples were collected at two time-points, the first (t0) corresponded to when the culture reached  
473 an OD<sub>600</sub> of 0.4 and the culture medium was changed to a new one containing 2% glucose and 3%  
474 ethanol. At that point, the polycistronic gene under control of the *PCK1* promoter was expected to be

475 repressed, as *PCK1* is repressed in glucose-containing media. The second time-point (t24)  
476 corresponds to 24 h after t0. At t24, all the glucose was supposed to be consumed by the yeast and  
477 ethanol fermentation had started (Lee and DaSilva, 2005), thus activating the polycistronic gene  
478 expression.

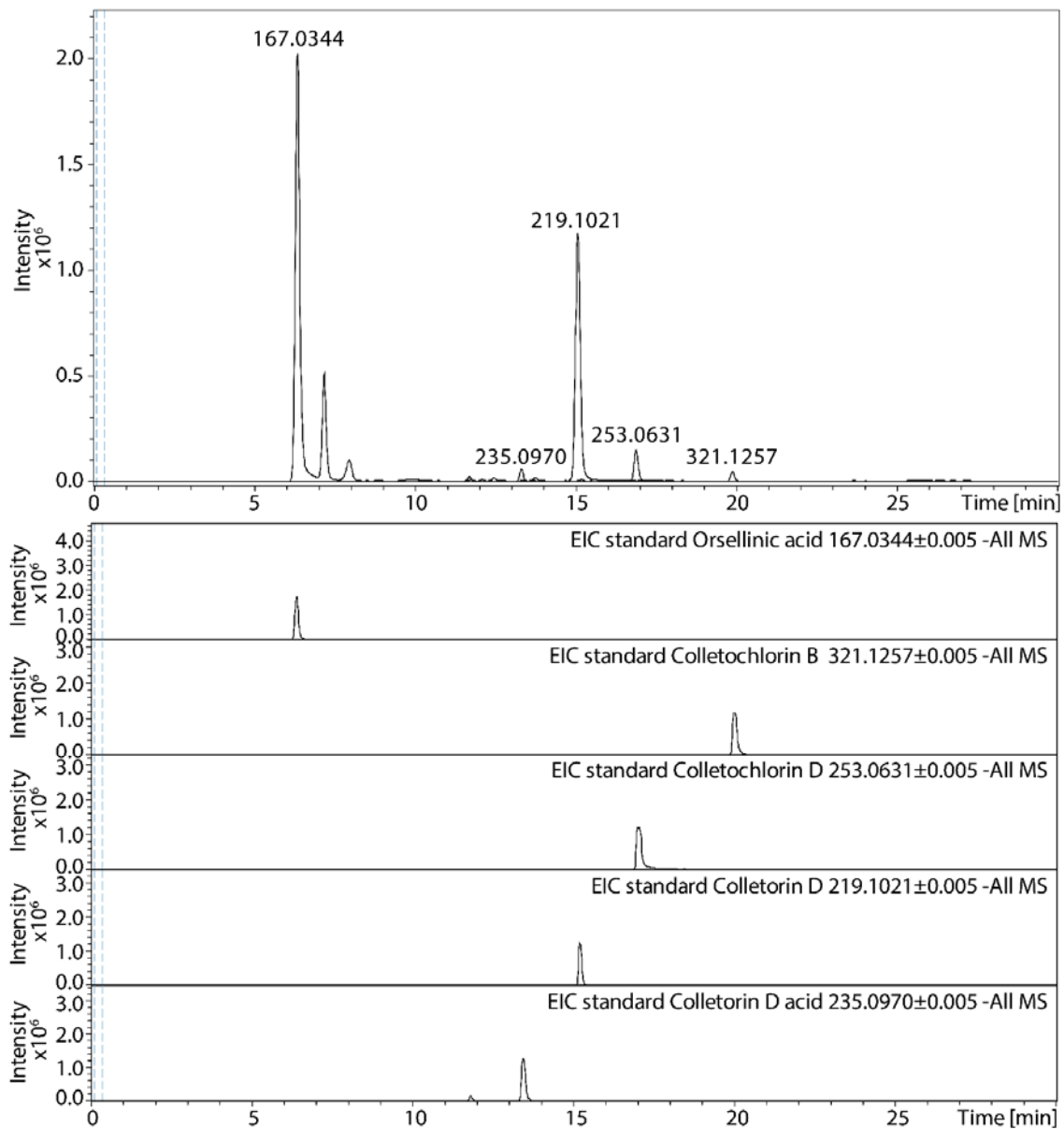
479 Before induction (t0), a band at 29kDa corresponding to the VenusN protein was detected in both  
480 BJNBC-001 and BJNBC-008. The detection of VenusN in non-induced conditions (t0) showed that the  
481 polycistron was expressed at a very low level in these conditions. After induction (t24h), only the  
482 VenusN protein was found in BJNBC-001, whereas in the BJNBC-008 yeast, four bands were detected  
483 at 29, 61, 119 and 233 kDa, corresponding to VenusN, CTC2, CTC3 and CTC1, respectively (Figure 7).  
484 However, the prenyltransferase CTC4 (expected Mr = 40 kDa) was not detectable. This apparent  
485 absence of the prenyltransferase could explain why only the first molecule in the pathway, Orsellinic  
486 acid, was obtained from BJNBC-008 cultures.

### 487 **3.5. Heterologous production of the Colletochlorin metabolites in yeast**

488 To overcome the absence of the CTC4 protein in the BJNBC-008 protein extract, a new plasmid  
489 pHYX172 was made containing only the prenyltransferase gene under the control of the strong and  
490 constitutive *TEF1* promoter. Another plasmid (pHYX173) containing the gene encoding the TEV  
491 protease was also introduced into the yeast strain BJNBC, which allows cleavage of the C-terminal  
492 P2A-tail from the polycistronic enzymes. Two new yeast strains were generated: BJNBC-015  
493 containing all three plasmids pHYX164 (polycistron with Colletochlorin genes), pHYX173 (polycistron  
494 with TEV protease gene) and pHYX172 (*ctc4* alone), and as a control, the strain BJNBC-017 containing  
495 the pHYX137 and pHYX138 empty vectors. Metabolites were extracted from 3-day-old cultures and  
496 then analysed by LC-QToF-MS.

497 The expected molecular ions corresponding to Colletorins and Colletochlorins were readily found in  
498 samples from BJNBC-015 and with retention times and masses similar to those for the purified  
499 standards Orsellinic acid (RT, 6.40;  $m/z$  167.0344 [M-H]<sup>-</sup>), Colletorin D acid (RT, 13.51;  $m/z$  235.0970

500 [M-H]<sup>-</sup>), Colletorin D (RT, 15.22; *m/z* 219.1021 [M-H]<sup>-</sup>), Colletochlorin B (RT, 20.02; *m/z* 321.1257 [M-  
501 H]<sup>-</sup>) and Colletochlorin D (RT, 17.06; *m/z* 235.0631 [M-H]<sup>-</sup>) (Figure 8).



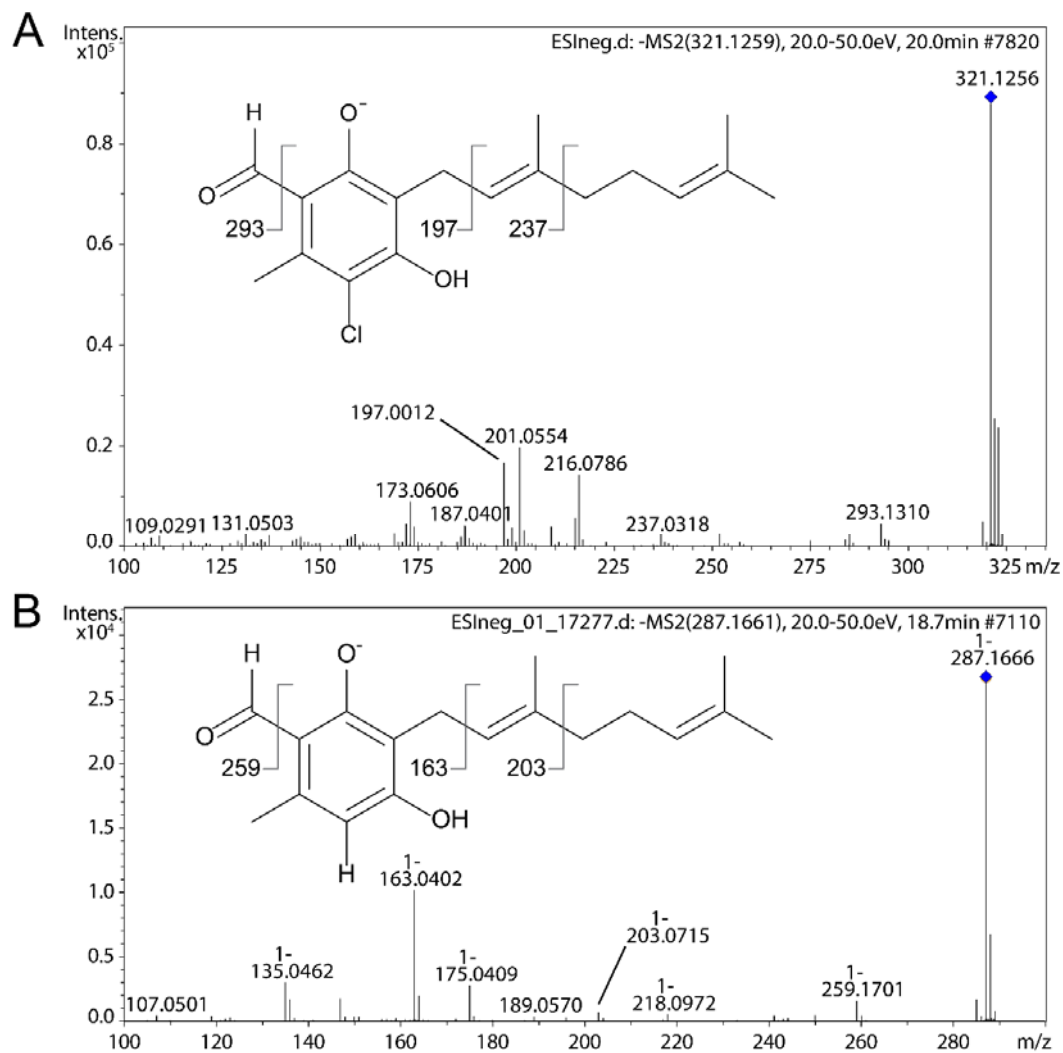
502

503 *Figure 8: Combined extracted ion chromatograms (EIC) of the culture supernatant of the strain BJNBC015*  
504 *expressing all four ctc genes in a polycistron and an additional copy of ctc4 (prenyltransferase) with its own*  
505 *promoter. EIC of standards of the different biosynthetic intermediates in the Colletochlorin pathway are also*  
506 *represented.*

507 Next, we performed a non-targeted analysis, comparing the BJNBC015 extract with the control  
508 BJNBC-017 extract. This confirmed the detection of all the standard molecules previously described

509 in the BJNBC-015 extract. In addition, another molecule (RT, 18.71;  $m/z$  287.1666 [M-H]<sup>-</sup>) was  
510 detected. The molecular mass of this molecule corresponds to that of Colletorin B, the non-  
511 chlorinated form of Colletochlorin B. In order to validate this hypothesis, we carried out a  
512 comparative analysis of the fragmentation pattern of Colletochlorin B and Colletorin B (Figure 9;  
513 Supplementary File 6). Taken together, the results show that Orsellinic acid, Colletorin D acid,  
514 Colletorin B and D, and Colletochlorin B and D were detected in the yeast harbouring genes *ctc1* to  
515 *ctc4* of *C. higginsianum* BGC16, and validated that the proposed gene cluster does indeed encode the  
516 Colletochlorin biosynthetic pathway.

517



518

519 *Figure 9: LC-MS/MS fragmentation pattern of, A, a Colletochlorin B standard and, B, a molecule annotated as*  
520 *Colletorin B from the BJNBC-015 strain. Detailed fragmentation patterns are presented in Supplementary File 6.*

## 521 **4. Discussion**

522 Fungi are a huge and underestimated reservoir of bioactive natural products. While genetic and  
523 chemical manipulations of fungi are common strategies to activate biosynthetic pathways in  
524 laboratory conditions, they require extensive trial-and-error. In order to facilitate the discovery of  
525 new fungal specialized metabolites, we developed the system described in this study to provide an  
526 easily-applicable tool for the heterologous expression of entire secondary metabolite gene clusters in  
527 engineered *S. cerevisiae*. The expression system should facilitate the discovery of new fungal  
528 specialized metabolites, especially those produced by silent BGCs, or BGCs that are only expressed at  
529 low levels *in vitro* or uniquely during interactions with the host plant. Apart from the possible  
530 discovery of high-value natural products including new medicines or biopesticides, this will provide a  
531 better understanding of the ecological role of these molecules, including their contribution to the  
532 pathogenesis of plant pathogens. The host organism, *Saccharomyces cerevisiae*, is a GRAS (generally  
533 recognized as safe) organism and is easily cultured on a large scale, while the polycistronic plasmid  
534 allows for simultaneous enzyme production and avoids multiple cloning and transformation steps  
535 with many vectors for introducing each gene with its own promoter in the engineered recipient  
536 strain. In addition, the fluorescent reporter protein VENUS provides a simple way to check the  
537 transcription and translation of the polycistronic gene. The production of toxic metabolites may also  
538 be possible given the use of the inducible promoters *pADH2* and *pPCK1*, which are activated after the  
539 diauxic shift (Harvey et al., 2018), allowing yeast biomass to increase before potentially toxic  
540 metabolites start to be produced.

541 The expression system was firstly validated in yeast by introducing the *mScarlet* reporter gene into  
542 the adapted polycistronic plasmid. We then introduced the genes coding the key and tailoring  
543 enzymes (CTC1 to CTC4) of *C. higginsianum* BGC16 and showed that the resulting yeast cultures

544 heterologously produced Orsellinic acid, Colletorin D acid, Colletochlorin B and D and Colletorin B.  
545 The chemical structure of these metabolites validates the correct production and enzymatic activity  
546 of the PKS, prenyltransferase, NRPS-like and halogenase enzymes in the heterologous system.  
547 Moreover, it demonstrates that *ctc1* to *ctc4* encode all the enzymes necessary for the biosynthesis of  
548 Colletochlorins B and D, Colletorin B and D, Colletorin D acid and Orsellinic acid.

549 In the first experiment, one bottleneck was the proper functioning of the prenyltransferase CTC4.  
550 This enzyme was a predicted UbiA-like membrane-bound prenyltransferase, possessing the typical  
551 NDXXDXXXD motif and potentially seven transmembrane domains according to TMHMM (Chang et  
552 al., 2021; Krogh et al., 2001). The main hypothesis explaining a non-functional prenyltransferase is  
553 the absence of insertion of the protein into the membrane or misfolding followed by rapid  
554 degradation of the protein. Although not detected by the signal peptide predictor SignalP (Petersen  
555 et al., 2011), this enzyme is predicted to have a plasma-membrane addressing signal by WoLF PSORT  
556 (Horton, 2007). Generally, proteins destined to be transported into the membrane are synthesized  
557 with a targeting-sequence, usually at the N-terminus and possibly at the C-terminus (Schatz and  
558 Dobberstein, 1996). The porcine tescho virus P2A self-cleaving peptide (P2A) has the peculiarity to  
559 add 21 amino acids at the C-terminus of the upstream protein and a proline at the N-terminus of the  
560 downstream protein (Kim et al., 2011). The presence of these extra residues likely caused the loss of  
561 function observed for the prenyltransferase CTC4. An example of non-recognition of an N-terminal  
562 signal peptide due to the presence of the P2A-proline was reported previously for an N-myristoylated  
563 protein (Hadpech et al., 2018).

564 Another hypothesis could be that the prenyltransferase is correctly translocated into the membrane  
565 but the catalytic site of the enzyme is non-active due to presence of the P2A tail, as was reported by  
566 Mattern et al. (2017) for an O-acetyltransferase. However, this explanation is less probable because  
567 in that case the prenyltransferase would have been detected by western blot. Whatever the  
568 explanation, proper functioning of the prenyltransferase appears to have been prevented by the P2A

569 peptide, because Colletochlorins were successfully produced when the prenyltransferase was  
570 expressed from a separate plasmid with a conventional construction involving a strong promoter and  
571 terminator, in addition to the polycistronic plasmid containing the other *ctc* genes. In future  
572 experiments, we recommend to check for the presence of predicted signal peptide and  
573 transmembrane domains in the enzymes of each pathway of interest and to clone genes encoding  
574 this type of enzyme in a separate plasmid. Alternatively, the TEV protease enzyme may be used to  
575 cut the P2A C-terminal tail to avoid interference with the enzyme activity.

576 The use of a polycistronic 2A sequence for the production of heterologous specialized metabolites in  
577 yeast was previously used by Beekwilder et al. (2014). They successfully produced  $\beta$ -carotene with a  
578 polycistronic plasmid containing three genes separated by the *Thosea asigna* virus 2A sequence  
579 (T2A). Jiao et al. (2018) tried to improve the Beekwilder *et al.* experimental procedure by studying  
580 the order of the genes introduced in the polycistronic plasmid. They concluded that the first gene  
581 was more highly expressed than the two following ones. It may thus be better to put the gene  
582 encoding the most rate-limiting enzyme at the beginning of the polycistron. Liu et al. (2017) also  
583 found that gene expression level progressively decreases with distance from the N-terminus of the  
584 polycistron. One limitation of the polycistronic plasmid may therefore be the number of genes  
585 introduced. To overcome this issue, we designed two polycistronic plasmids pHYX137 and pHYX138  
586 with different markers of prototrophy, namely *LEU2* and *URA3*, respectively. Distributing the genes  
587 of the BGC between these two polycistronic plasmids may allow a more homogeneous expression  
588 level for BGCs containing numerous genes.

589 The successful heterologous production of Colletochlorins demonstrated that four genes of the  
590 BGC16 of *C. higginsianum* are sufficient for the production of these molecules. Eight other genes in  
591 the BGC16 were previously assigned to Higginsianins biosynthesis (Tsukada et al., 2020), while the  
592 gene CH63R\_05474 is a methyltransferase relict that underwent pseudogenization, CH63R\_05472 is  
593 a putative transcription factor and CH63R\_05481 has no characterized function. Two biosynthetic

594 pathways for the production of Colletochlorins were proposed (Figure 6). Various lines of evidence  
595 suggest that the second pathway is the most probable. Li et al. (2016) described the cluster involved  
596 in LL-Z1272 $\beta$  (Illicicolin B) synthesis, which contains three genes coding for a PKS *StbA*, a  
597 prenyltransferase *StbC* and an NRPS-like *StbB*. These authors showed that the PKS is involved in the  
598 formation of Orsellinic acid, which is then converted into Grifolic acid by the prenyltransferase. The  
599 NRPS-like *StbB* is only able to convert the prenylated form of Orsellinic acid (i.e. Grifolic acid) into LL-  
600 Z1272 $\beta$  and does not accept Orsellinic acid as a substrate. In the literature, NRPS-like enzymes that  
601 require prenylated substrates have been rarely described. Comparison of the Adenylation domains of  
602 the NRPS-like accepting Orsellinic acid (ATEG\_03630) or only prenylated-orsellinic acid (*StbB*) as  
603 substrate showed differences in their protein sequence. At position 334, ATEG\_03630 possesses a  
604 leucine and *StbB* a glycine, while at position 358, essential for ATEG\_03630 substrate specificity  
605 (Wang and Zhao, 2014), ATEG\_03630 has a histidine and *StbB* a phenylalanine. The *C. higginsianum*  
606 NRPS-like enzyme CTC3 has the same amino acids involved in substrate specificity as *StbB*, suggesting  
607 that it may have a similar substrate specificity towards prenylated Orsellinic acid. Finally, the  
608 proposed ability of prenyltransferase CTC4 to accept both DMAPP and GPP moieties as substrates is  
609 known to occur in other aromatic prenyltransferases (Chen et al., 2017; Cheng and Li, 2014; Kalén et  
610 al., 1990; Suzuki et al., 1994; Swiezewska et al., 1993). Further experiments are now needed to  
611 confirm the Colletochlorin biosynthetic pathway, notably by purifying the prenyltransferase and  
612 NRPS-like enzymes for assessing their substrate specificity.

613 The Colletochlorins were previously isolated from a *C. higginsianum* mutant with a partially deficient  
614 COMPASS complex (Dallery et al., 2019) and several of them were shown previously to be biologically  
615 active. For example, Colletorin B and Colletochlorin B displayed moderate herbicidal, antifungal and  
616 antibacterial activities towards *Chlorella fusca*, *Ustilago violacea*, *Fusarium oxysporum*, and *Bacillus*  
617 *megaterium* (Hussain et al., 2015), while Colletochlorin B had a significant antibacterial effect against  
618 *Bacillus subtilis* (minimum inhibitory concentration, 2  $\mu\text{g}\cdot\text{mL}^{-1}$ ) (Kemkuignou et al., 2022).



## 619 **5. Conclusions**

620 Our findings demonstrate the utility of this synthetic biology tool for the metabolic engineering of  
621 yeast to produce fungal metabolites from BGCs of interest in bulk liquid cultures. This is a  
622 prerequisite for subsequent structural characterization and bioactivity profiling of SM products from  
623 BGCs that are otherwise silent in their native organisms when cultured in laboratory conditions.

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## 639 **8. Contribution statement**

640 Conceptualization: JFD, RJO, MV; Methodology: JFD, AGK; Resources: JO, GM; Investigation: AGK,  
641 JFD, JCT, GLG, JV, KS; Formal analysis: AGK, JCT, GLG, JFD; Visualization: AGK, JFD, JCT, JV, KS; Writing  
642 – Original Draft: AGK, JFD, RJO, MV, JCT; Writing – Review & Editing: JFD, RJO, MV, AGK; Supervision:  
643 JFD, RJO, MV, JO, GM; Funding acquisition: JFD, MV, GM, JO; Project administration: JFD.

## 644 **9. Conflict of interest**

645 The authors declare no conflict of interest.

## 646 **10. Supplementary Files**

647 Supplementary File 1: List of strains used in this study.

648 Supplementary File 2: Maps of the plasmids pHYX137 and pHYX138.

649 Supplementary File 3: List of the primers used in this study.

650 Supplementary File 4: Sequences of the *CTC* genes codon-adapted for *Saccharomyces cerevisiae*.

651 Supplementary File 5: List of the plasmids used in this study.

652 Supplementary File 6: Fragmentation patterns of Colletochlorin B standard and Colletorin B.

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