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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⁻¹, peptone 20 g·L⁻¹ and glucose 20 g·L⁻¹) 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 μ 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*⁺ 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 μ origin, *KanR*⁺ 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) β -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 μL 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 H₂O

264 to CH₃CN, both containing 0.1% formic acid, for 50 min at 0.7 mL·min⁻¹. 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⁻¹
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⁻¹, for 5 μL injected. The mobile phases used
274 for the chromatographic separation were: (A) 0.1% formic acid in H₂O; 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⁻¹; 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⁻ 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⁻ metabolomic data used for molecular network analyses were searched
307 against the available MS² 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).

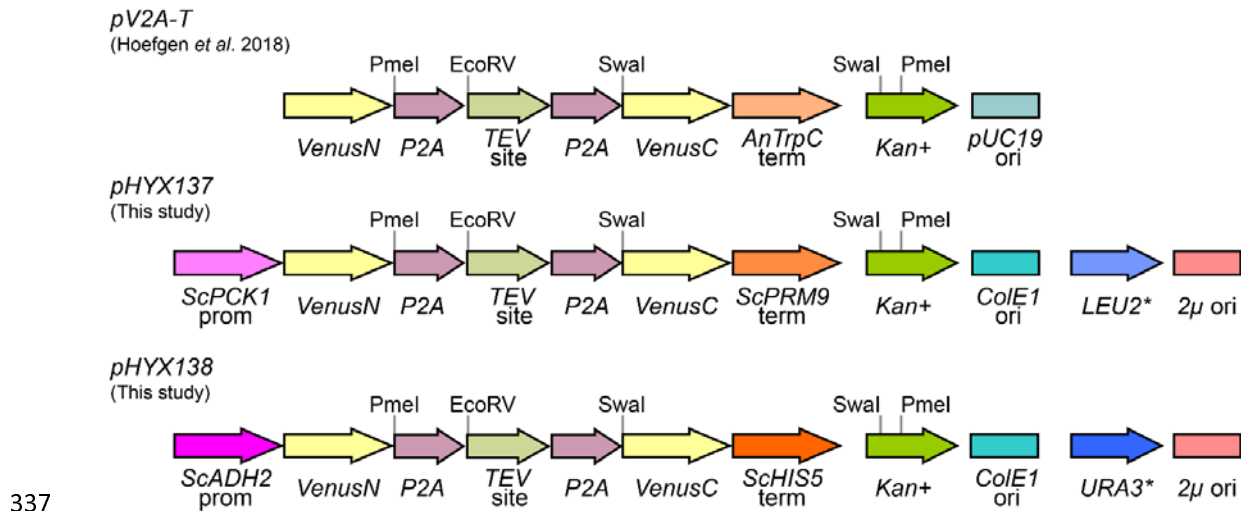


Figure 1: Features of the *pV2A-T* plasmid described by Hoefgen et al. (2018) and of the two plasmids, *pHYX137* and *pHYX138*, adapted for *S. cerevisiae* expression, described in this study. Complete maps are shown in Supplementary File 2.

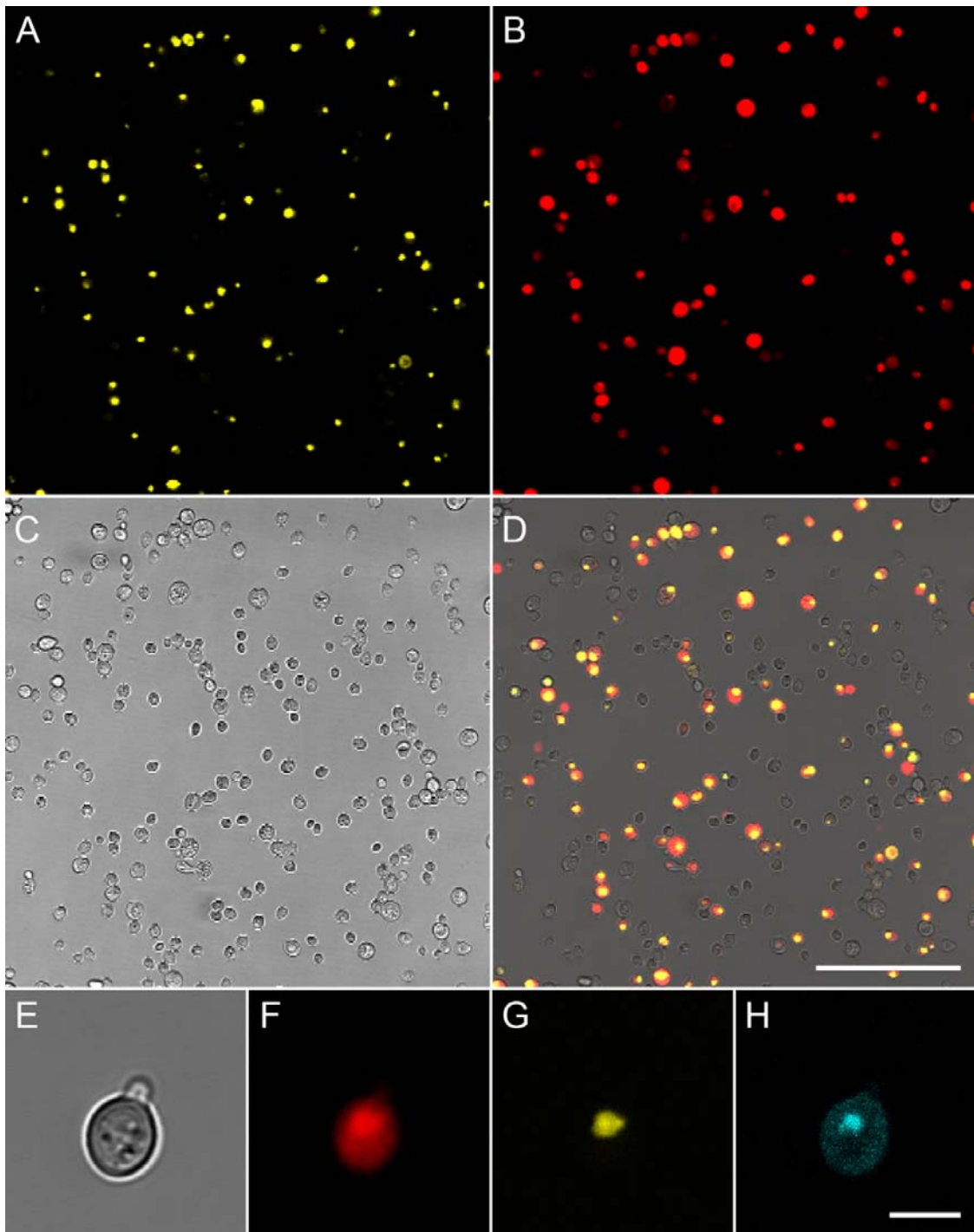
In addition to the polycistronic gene, the plasmids *pHYX137* and *pHYX138* possess a yeast 2μ origin of replication and the *pUC19* origin of replication was also replaced by the *ColE1* origin. The nutritional selection genes, either *LEU2* or *URA3*, were included and silent mutations were introduced 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.

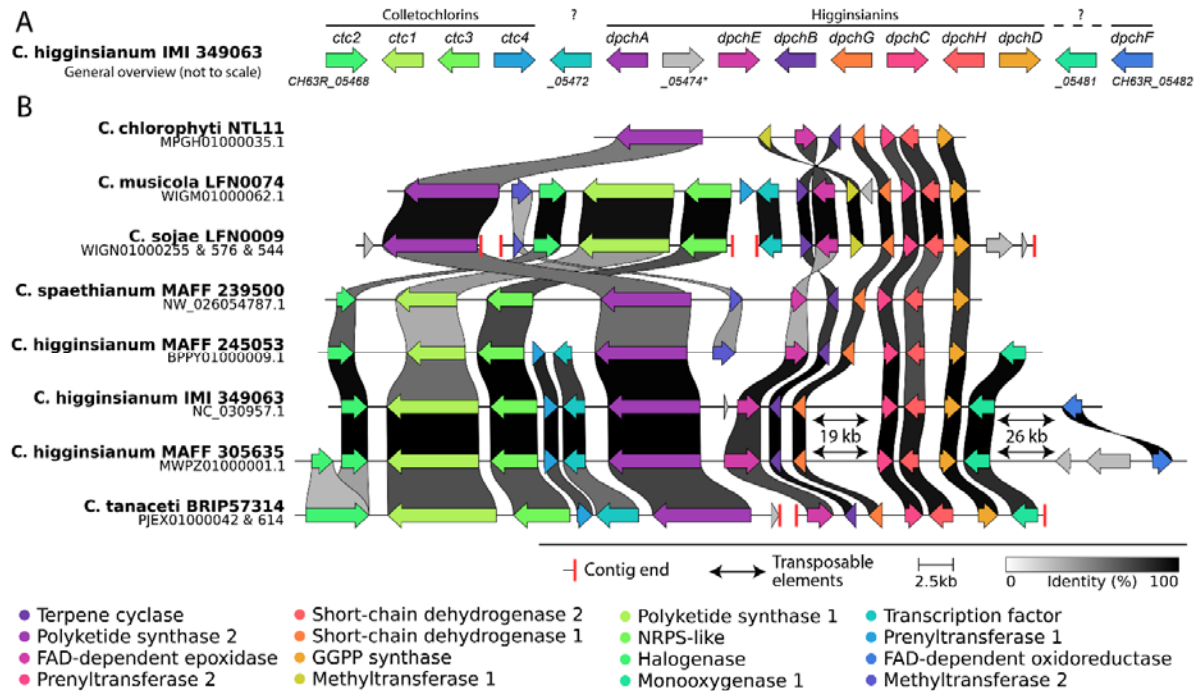


370

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 μ 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 μ 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).

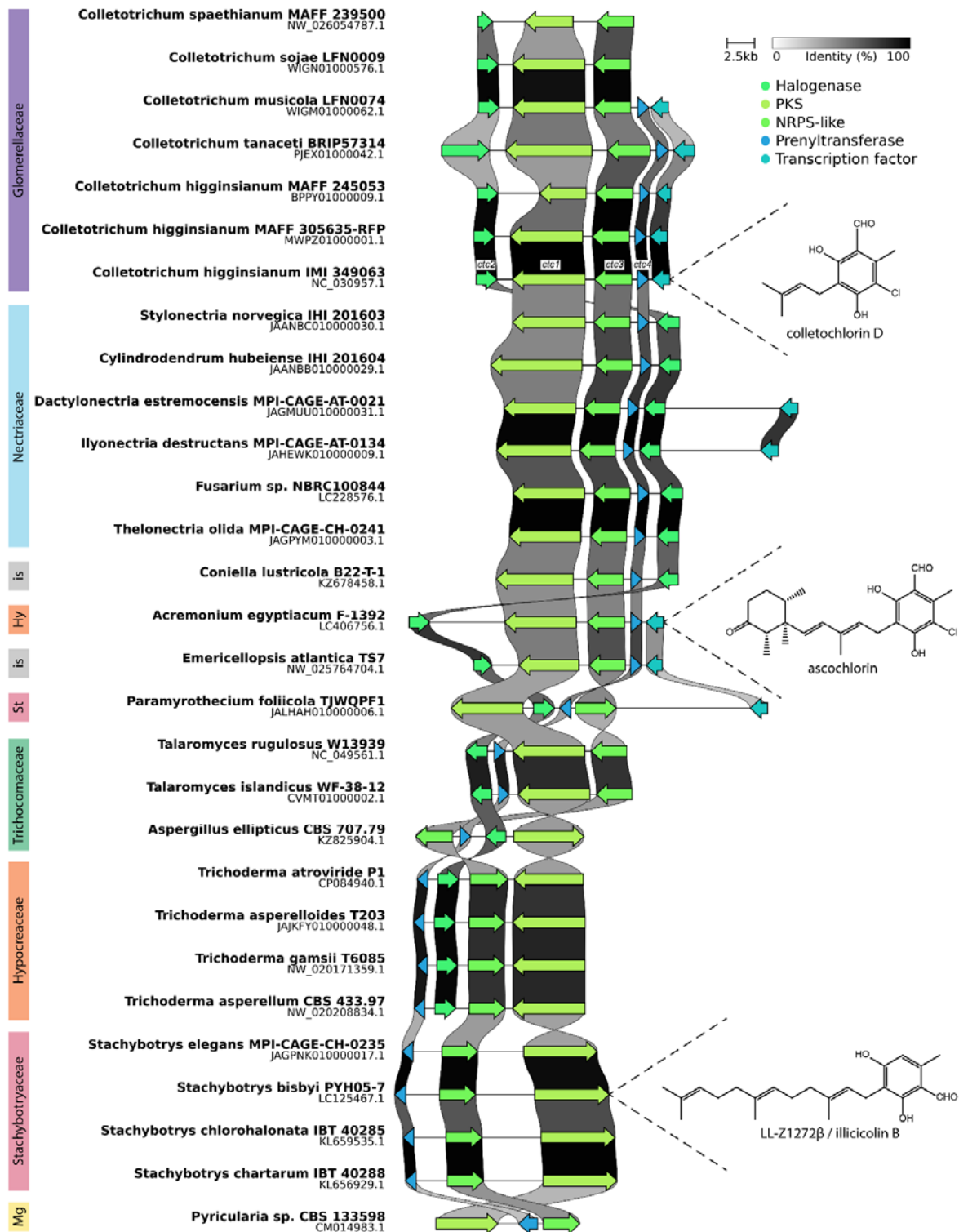


399

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 β ,
 415 also called Illicicolin B (Li et al., 2016). Among the retrieved homologues, we also found the BGC in

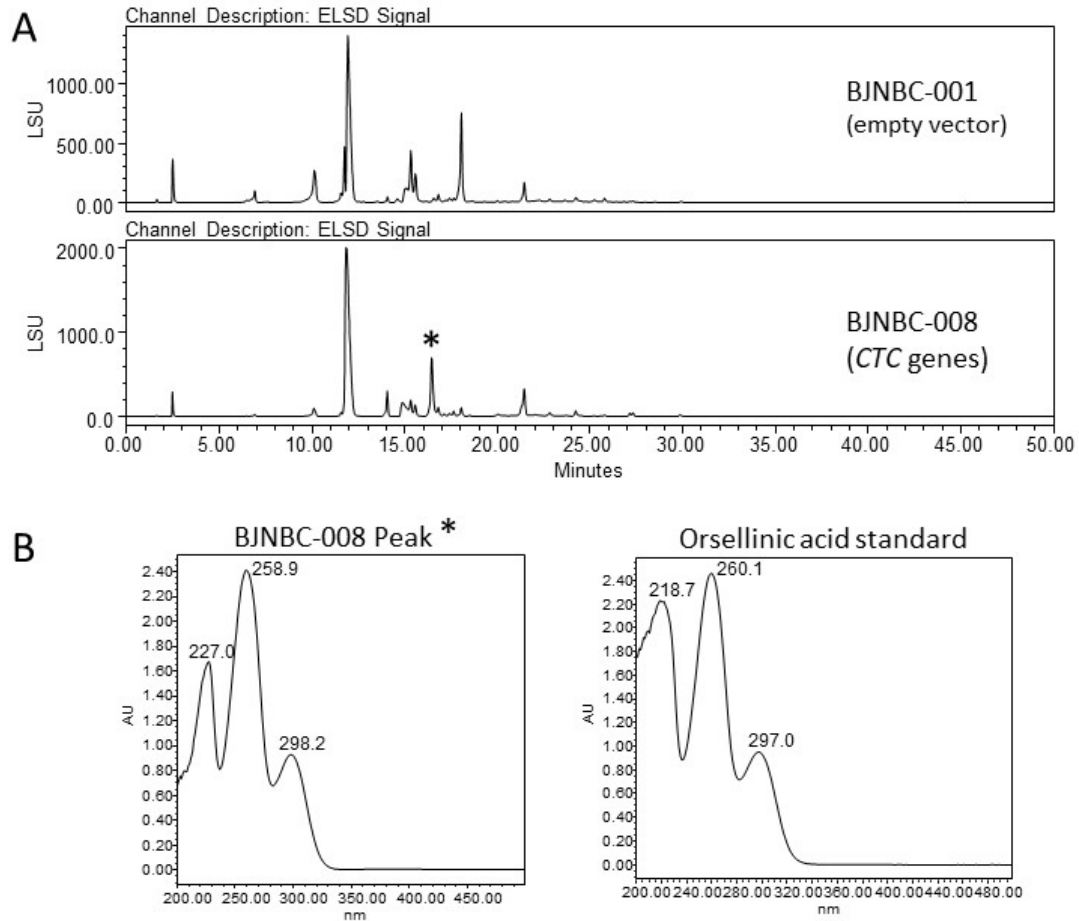
416 *Acremonium egyptiacum* responsible for biosynthesis of Ascochlorin, another prenylated yet
417 chlorinated derivative of Orsellinic acid (Figure 4). Based on these findings and knowledge of the
418 experimentally-determined biosynthetic route for LL-Z1272 β and Ascochlorin, as well as the
419 predicted 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



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

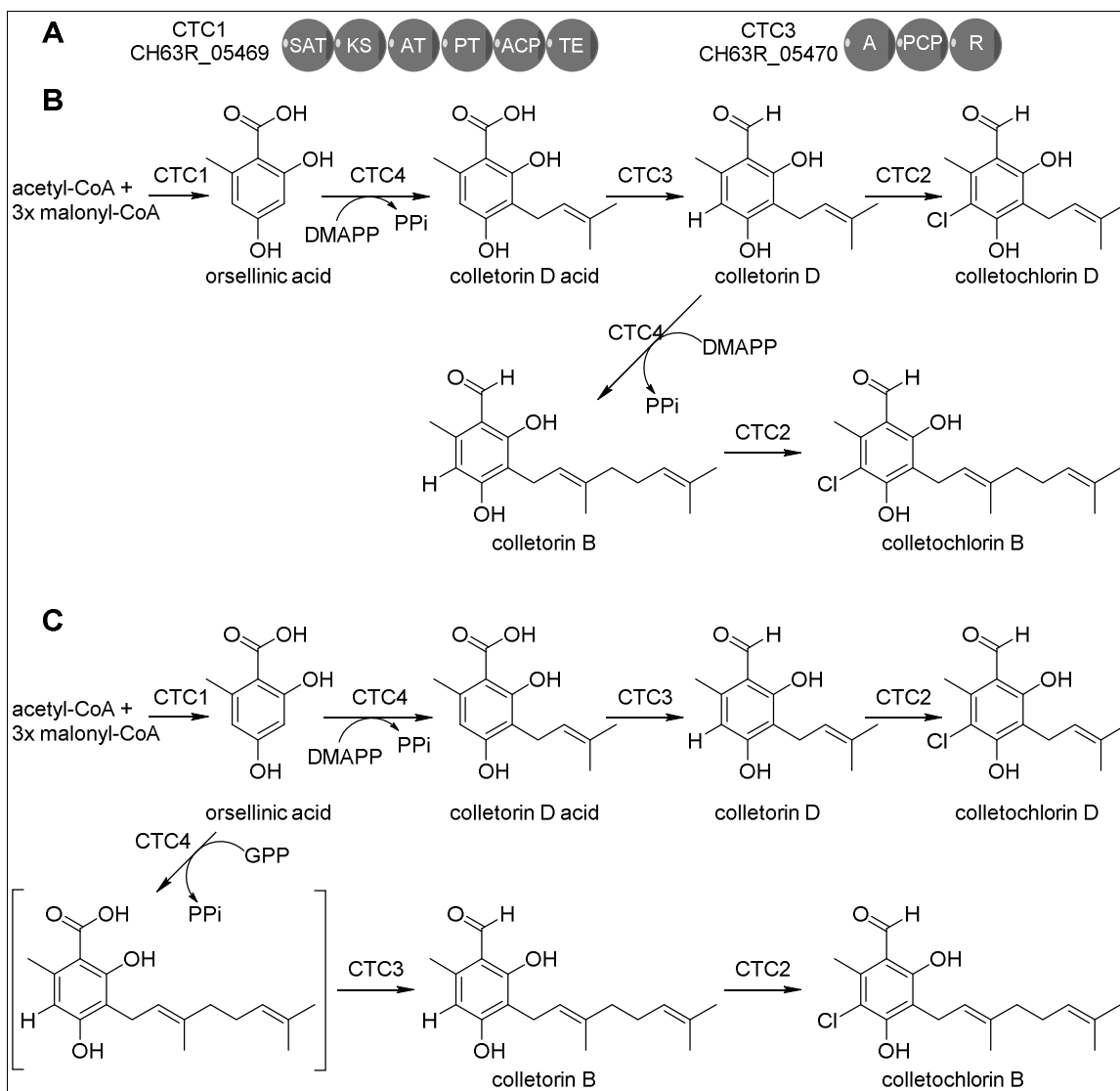
430 **3.4. Heterologous production of Orsellinic acid in yeast**

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.*



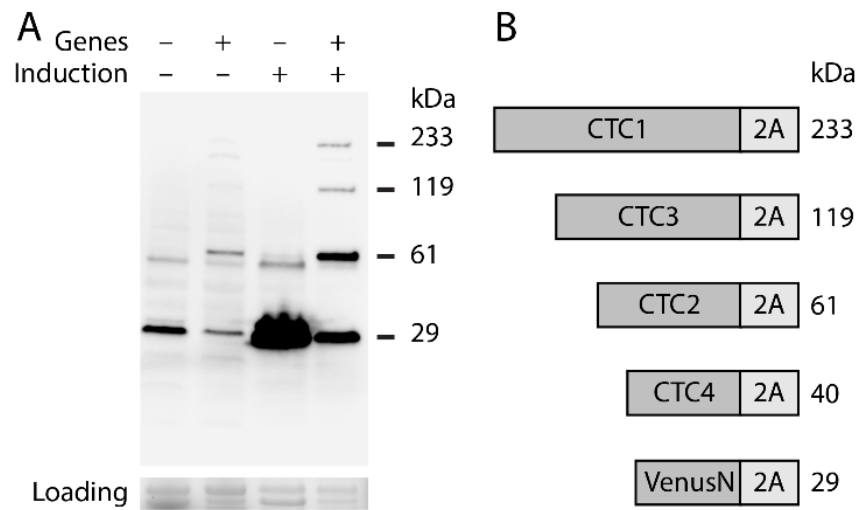
445
446

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,*
 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 OD₆₀₀ 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.

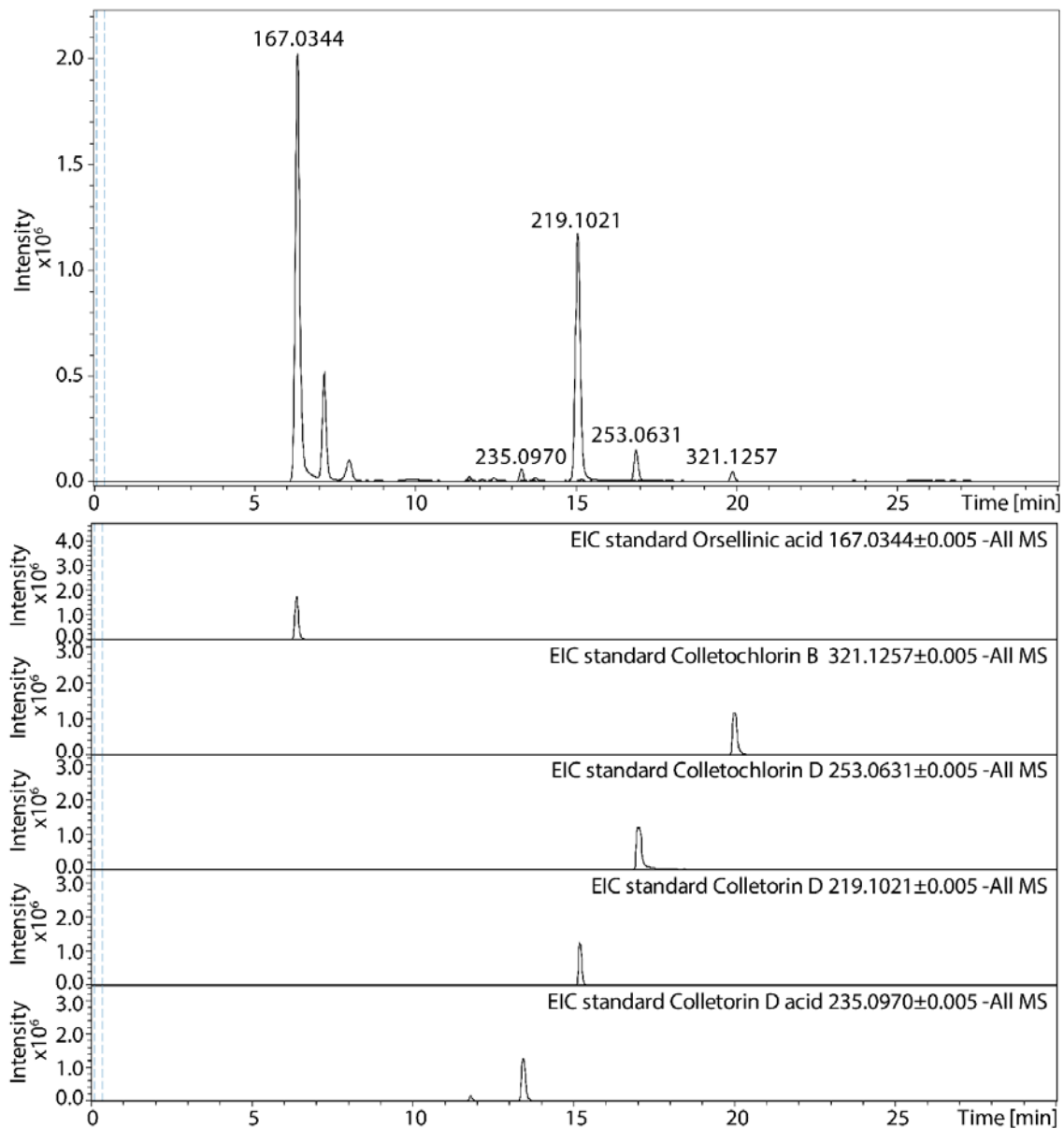
479 Before induction (t0), a band at 29kDa corresponding to the VenusN protein was detected in both
480 BJNBC-001 and BJNBC-008. The detection of VenusN in non-induced conditions (t0) showed that the
481 polycistron was expressed at a very low level in these conditions. After induction (t24h), only the
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]⁻), Colletorin D acid (RT, 13.51; m/z 235.0970

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



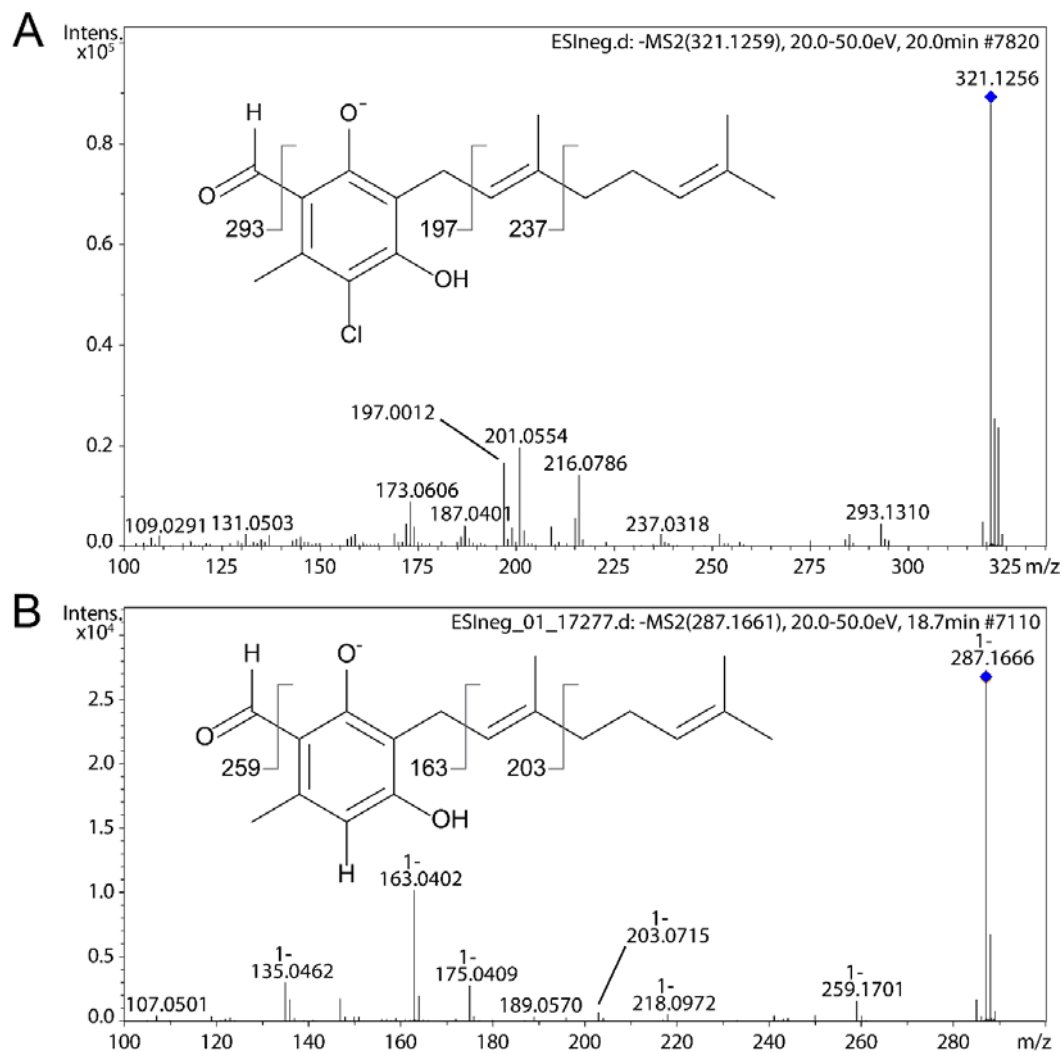
502

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]⁻) 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 β -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 β (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 β 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.

653 **11. References**

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