View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Explore Bristol Research

Al-Salihi, S. A. A., Scott, T. A., Bailey, A. M., & Foster, G. D. (2017). Improved vectors for Agrobacterium mediated genetic manipulation of Hypholoma spp. and other homobasidiomycetes. *Journal of Microbiological Methods*, *142*, 4-9. https://doi.org/10.1016/j.mimet.2017.08.014

Peer reviewed version

License (if available):
CC BY-NC-ND

Link to published version (if available):
10.1016/j.mimet.2017.08.014

Link to publication record in Explore Bristol Research
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via ELSEVIER at http://www.sciencedirect.com/science/article/pii/S0167701217302221?via%3Dihub#ac0005 . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: http://www.bristol.ac.uk/pure/about/ebr-terms

Revised

Improved vectors for *Agrobacterium* mediated genetic manipulation of *Hypholoma spp.* and other homobasidiomycetes

Suhad A.A. Al-Salihi, Thomas A. Scott, Andy M. Bailey and Gary D. Foster*

Molecular Plant Pathology and Fungal Biology Group, University of Bristol, School of Biological Sciences, 24 Tyndall Avenue Bristol, BS8 1TQ, UK

*Author for correspondence gary.foster@bristol.ac.uk

Abstract

The basidiomycete fungi *Hypholoma fasciculare* and *H. sublateritium* are both prolific producers of sesquiterpenes and triterpenes, some of which have relevant pharmaceutical properties. Although *H. sublateritium* has been transformed in the past, the low reported efficiencies highlighted the need for establishing an effective simple transformation system for these valuable species. We have optimized *Agrobacterium tumefaciens*-mediated transformation through testing various parameters in these two *Hypholoma* species, showing that a mixture of homogenized mycelia and *Agrobacterium* (strain LBA4404) co-cultivated for 84hr at 25°C is optimal for efficient transformation in these basidiomycetes. This study also reveals the requirements for transgene expression, with the first report of GFP expression in these *Hypholoma*, the need for an intron for such transgene expression, and further demonstrates the functionality of the expression vector by its use in *Clitopilus passeckerianus*. This development of transformation system and expression constructs, can facilitate further genetic investigation such as gene functionality in these fungi.

Key words: Agaricales; *Agrobacterium*; *Hypholoma* promoter; GFP.

1. Introduction

With the recent advances in genome sequencing, and the increase of resistance of many human pathogenic bacteria to current antimicrobials, basidiomycete fungi have regained attention as an interesting source of novel biologically active compounds (**de Mattos *et al.,* 2016**) To explore this in detail and exploit this fully, requires establishment of genetic manipulation techniques to either deliver transgenes, or to silence/knockout specific genes and this has often been problematic for basidiomycete fungi **(Bailey *et al*., 2016).**

Hypholoma is a genus within the Strophariaceae family usually typified by the production of bright yellow or red mushrooms on rotting timber in temperate woodlands. In the UK, *H. fasciculare* is known as the "Sulphur Tuft" due to its abundant small sulphur-yellow fruiting bodies**.** Many *Hypholoma spp.* have been characterized as prolific producers of secondary metabolites and the vast majority of these compounds are terpenoids. Although numerous sesquiterpenoids and triterpenoids have been isolated from *Hypholoma* (**de Bernardi *et al.,* 1981; 1977; Ito *et al.,* 1967; Kleinwächter *et al.,* 1999; Shiono *et al.,* 2004; Shiono *et al.,* 2005),** to date, no corresponding gene cluster or related biosynthetic pathways have been characterized from these species. Since the first application of *Agrobacterium tumefaciens*-mediated transformation (ATMT) for fungi by **de Groot *et al.,* (1998),** increasing numbers of basidiomycetes and ascomycetes have been successfully transformed using this approach (**Michielse *et al.,* 2005; Mikosch *et al.,* 2001**). Although, ATMT has been used to transform *H. sublateritium* (**Godio *et al*., 2004**), this was with very low efficiency and did not address transgene expression.

44 Several basidiomycetes including *Coprinopsis cinerea, Agaricus bisporus, Clitopilus passeckerianus,* and *Armillaria mellea* (**Burns *et al.,* 2005; Kilaru *et al.,* 2009; Ford *et al.,* 2016**) have been shown to require an intron for successful GFP expression, however, this was not the case in *Pisolithus tinctorius (***Rodríguez *et al.,* 2005**) or *Hebeloma cylindrosporum (***Müller *et al.,* 2006***)* and can only be determined by experiment. Here we report the further development of ATMT for *Hypholoma spp.* showing that GFP transgene expression requires an intron, and that the use of native promoters enhances such gene expression. The parameters for ATMT were also investigated and optimised to provide a resilient efficient transformation system for *H. fasciculare* and *H. sublateritium*, and that these vectors were also functional in *Clitopilus passeckerianus.*

54 **2. Experimental strategies**

55 **2.1. Microbial isolates and culture conditions**

56 *H. sublateritium* FD-334 SS-4 was a kind gift from Dr. David Hibbett, Clark University USA, and *H. fasciculare* was kindly provided by Dr. Alice Banks, University of Bristol, UK. The identity of all species was confirmed by sequence verification of two housekeeping genes *β-tubulin* and *gpd* and the ITS region. Isolates were maintained on PDA (potato dextrose broth 24 g/L, agar 15 g/L) at 25°C in the dark. *Saccharomyces cerevisiae* YPH499, Y10000 and *Escherichia coli* DH5α were used for yeast recombination and plasmid maintenance. *Agrobacterium tumefaciens* strains AgL1, LBA1126 and LBA4404 were routinely maintained on LBA (NaCl 10 g/L, tryptone 10 g/L, yeast extract 5 g/L, agar 15 g/L,) supplemented with appropriate antibiotics (75 µg/ml carbenicillin or 20 µg/ml rifampicin).

65 For drug sensitivity determination, plates of YMG (yeast extract 4 g/L, malt extract 10 g/L, glucose 4 g/L, agar 15 g/L), MEA (malt extract 3 g/L, agar 15 g/L) or PDA, supplemented with hygromycin, glufosinate or carboxin were used. For ATMT, co-cultivation plates (IM per liter: 50 ml stock A [K_2HPO_4 34.84 g/L, KH_2PO_4 27.22 g/L, NaCl 2.93 g/L], 50 ml stock B [$MgSO_4.7H_2O$ 9.86 g/L, $(NH_4)_2SO_4$ 10.58 g/L], 10 ml 70mM $CaCl_2$, 1 ml 9 mM $FeSO_4$, 10 ml 1M D-glucose, 40 ml 1M MES, 10 ml 50 %glycerol, 15 g agar) were used.

71 **2.3. *Agrobacterium* transformation**

72 Mycelia of *Hypholoma* were prepared using the method of **Kilaru *et al.,* (2009)**. Arthrospore suspensions were prepared by flooding a 30 day-old agar culture with 10 ml sterile water and scraping the surface with an inoculating loop to liberate the spores. These were quantified using a haemocytometer and adjusted to a final concentration 1×10^6/ml. *A. tumefaciens*-mediated transformation was carried out following **Kilaru *et al.,* (2009),** and modified according to experimental outcomes. Plasmids used were the pCAMBIA based vector pBGgHg (**Chen *et al.,* 2000**), or vectors made as outlined below, using the yeast-adapted pCAMBIA0380YA backbone.

80 Mixtures of *Agrobacterium* and fungal material were spread onto 9 cm cellophane discs overlaid on co-cultivation agar. After an appropriate period of time, these were aseptically transferred onto selective agar containing 10 or 40 µg/ml of hygromycin for *H. sublateritium* and *H. fasciculare* respectively along with 100 µg/ml ticarcillin-clavanate, and incubated until colonies appeared.

85 *C. passeckerianus* was transformed by protoplast PEG/$CaCl_2$ method as outlined by **Kilaru *et al.* (2009),** using intact plasmid DNA.

87 Hygromycin resistant colonies were subcultured three times under selective conditions by transfer of small blocks of mycelia from the growing edge of the colony before analysis.

89 **2.4. PCR confirmation of the *hph* gene in *Hypholoma spp.* transformants**

90 To prepare genomic DNA for analysis, 100 ml of PDB (potato dextrose broth 24 g/L)
91 was inoculated with a 6mm diameter plug from an agar culture of the fungus. The culture was
92 incubated at 25°C in the dark for 10 days with shaking at 200 rpm. Filtered mycelia were then
93 freeze dried for 48 hr and DNA extracted following the method of **Liu *et al.,* (2000**). 0.5 µg
94 DNA was used as template in PCR to assess the presence of the *hph* gene, with DNA quality
95 confirmed by amplification of the ITS region, using primers Hygro-1 and Hygro-2, or ITS1
96 and ITS4 respectively (table S1). PCR reactions were carried out using 2X Dream Taq DNA
97 polymerase (Thermo Scientific) following the cycling program: initial denaturation at 95 °C
98 for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55
99 °C for 30 seconds, extension at 72 °C for 60 seconds and final extension at 72 °C for 10
100 minutes.

101 **2.5. GFP vector construction**

102 The *in silico* design of the two vectors pCAM-*hph*-Hs*gpd*GFP and pCAM-*hph*-
103 Hs*gpd*iGFP was performed using Clone-manager (Scientific and Educational Software). The
104 yeast-based recombination method of **Gietz and Woods (2002)** was adapted for the *in vivo*
105 construction. Both vectors were identical in terms of the pCAMBIA0380YA backbone,
106 hygromycin selection cassette and GFP expression cassette, differing only in the presence of
107 intron sequences in the *H. sublateritium gpd* promoter driving GFP expression. DNA segments
108 were amplified from either pBGgHg (**Chen *et al.,* 2000**) or from the *H. sublateritium* genome
109 with primer extensions to provide overlaps with the cut ends of the backbone (figures S1 and
110 S2). Vector sequences were verified using the GATC BIOTECH sequencing service, then
111 transferred to *Agrobacterium tumefaciens* LBA4404.

112 **2.6. Microscopy analysis**

113 GFP was assessed by epifluorescence microscopy with a 20x objective via Leica
114 DM750 microscope. Images were captured using a Leica ICC50 HD camera software (Leica
115 application Suite V 4.4). Small blocks of agar culture from 10 day old plates were excised from
116 PDA, placed on slides and examined for any discernible green fluorescence.

117 **3. Results**

118 **3.1. Selectable marker choice**

119 Due to the lack of reliable auxotrophic systems, and the dikaryotic nature of both
120 *Hypholoma*, antibiotic resistance was used for ATMT in this work. A preliminary evaluation
121 of hygromycin, glufosinate and carboxin showed hygromycin to inhibit fungal growth more
122 effectively than the other antibiotics, therefore experiments proceed with a range of
123 hygromycin concentrations (0-100 µg/ml). There were differences in the level of susceptibility
124 between the two species, and between the media used (figures S3 and S4). For example, *H.*
125 *fasciculare* failed to grow on PDA and YMG plates supplemented with 20 µg/ml of
126 hygromycin, while the same degree of growth inhibition was only obtained on MEA plates
127 with 30 µg/ml hygromycin. *H. sublateritium* was generally more sensitive than *H. fasciculare*
128 irrespective of the media used. Based on these results, plates of PDA supplemented with 10
129 and 40 µg/ml of hygromycin were used for transformants selection for *H. sublateritium* and *H.*
130 *fasciculare* respectively.

131

132

3.2. *Agrobacterium* transformation

Various studies in Basidiomycota have indicated the parameters that need to be optimized to achieve efficient transformation using ATMT (**e.g. Godio *et al.,* 2004; Michielse *et al.,* 2005**). Sections below report the parameters tested in this work. These included the strain of *Agrobacterium* employed, the requirement for addition of acetosyringone, nature of the fungal material employed, as well as cultivation times and temperatures.

3.2.1. *Agrobacterium* strain and recipient cell combinations

Two different fungal tissues (arthrospores and homogenized mycelia) and three strains of *Agrobacterium* (AgL1, LBA1126 and LBA4404) all carrying the pBGgHg vector were tested concurrently in both *Hypholoma* species. As shown in figure 1, transformants were successfully obtained for both species, although *H. fasciculare* consistently yielded more hygromycin resistant transformants than did *H. sublateritium*. Strains LBA4404 and AgL1 were broadly comparable in their productivity of transformants, whilst LBA1126 produced significantly fewer transformants. A similar transformation efficiency was observed when either spores or homogenized mycelia were used as fungal recipient tissue in both species. Based on these observations, the combination of *Agrobacterium* LBA4404 and homogenized mycelia was chosen as co-cultivation combination for further optimization in both *Hypholoma* species.

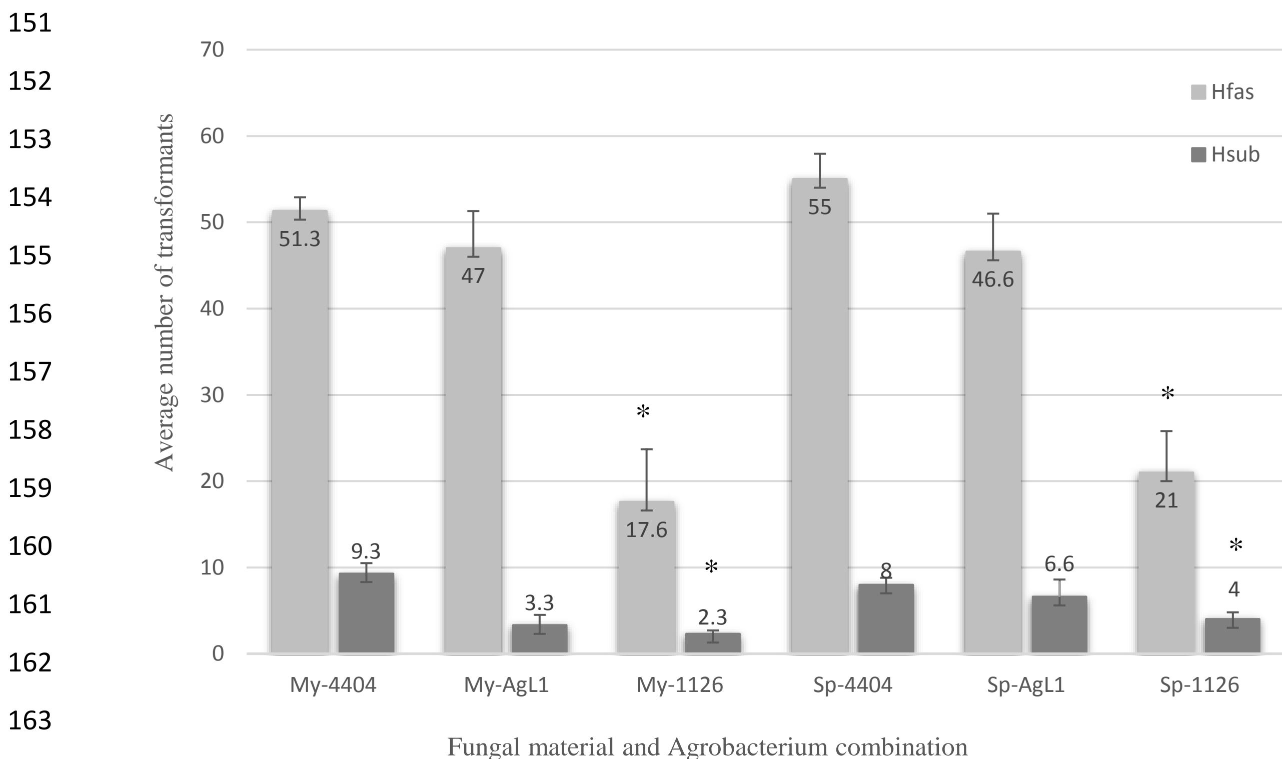


Figure 1: Average number of transformants obtained using different combinations of *Agrobacterium* strain and fungal material for both *H. fasciculare* (Hfas) and *H. sublateritium* (Hsub). My-4404 = homogenized mycelia + LBA4404 *Agrobacterium*, My-AgL1 = homogenized mycelia + AgL1 *Agrobacterium*, My-1126 = homogenized mycelia + LBA1126 *Agrobacterium*, Sp-4404 = arthrospores + LBA4404 *Agrobacterium*, Sp-AgL1 = arthrospores + AgL1 *Agrobacterium* and Sp-1126 = arthrospores + LBA1126 *Agrobacterium*. * indicates significant difference compared to other Agrobacterium strains, determined by factorial ANOVA and confirmed by Tukey honest significant difference (HSD) post-hoc test.

170 Different concentrations of acetosyringone (from 0-400 μM) were added to
171 *Agrobacterium* pre-cultures to evaluate the levels needed for efficient induction of the T-DNA
172 transfer pathway. No transformants were produced in the absence of this phytochemical, whilst
173 100-200 μM gave the highest numbers (figure S5). To determine the optimal OD_{600} of
174 *Agrobacterium* induction culture (AIC), a series of densities (0.2, 0.3, 0.4 and 0.5) were tested.
175 *Agrobacterium* culture density of OD_{600} = 0.3 and 0.4 were the optimum in producing
176 hygromycin resistant colonies for *H. fasciculare*, and *H. sublateritium* respectively (figure S6).

177 **3.2.3. Co-cultivation conditions**

178 *Hypholoma spp.* are reasonably thermotolerant, therefore co-cultivation plates were
179 incubated at 20, 25, 28 and 30°C for 72hr. Under these conditions, 25°C yielded the highest
180 number of hygromycin resistant colonies (figure 2A). A range of co-cultivation times was also
181 assessed, with incubations for 48, 60, 84 or 108 hr. A co-cultivation time of 84hr was the
182 optimal for both species (figure 2B).

183

184

185

186

187

188

189

190

191

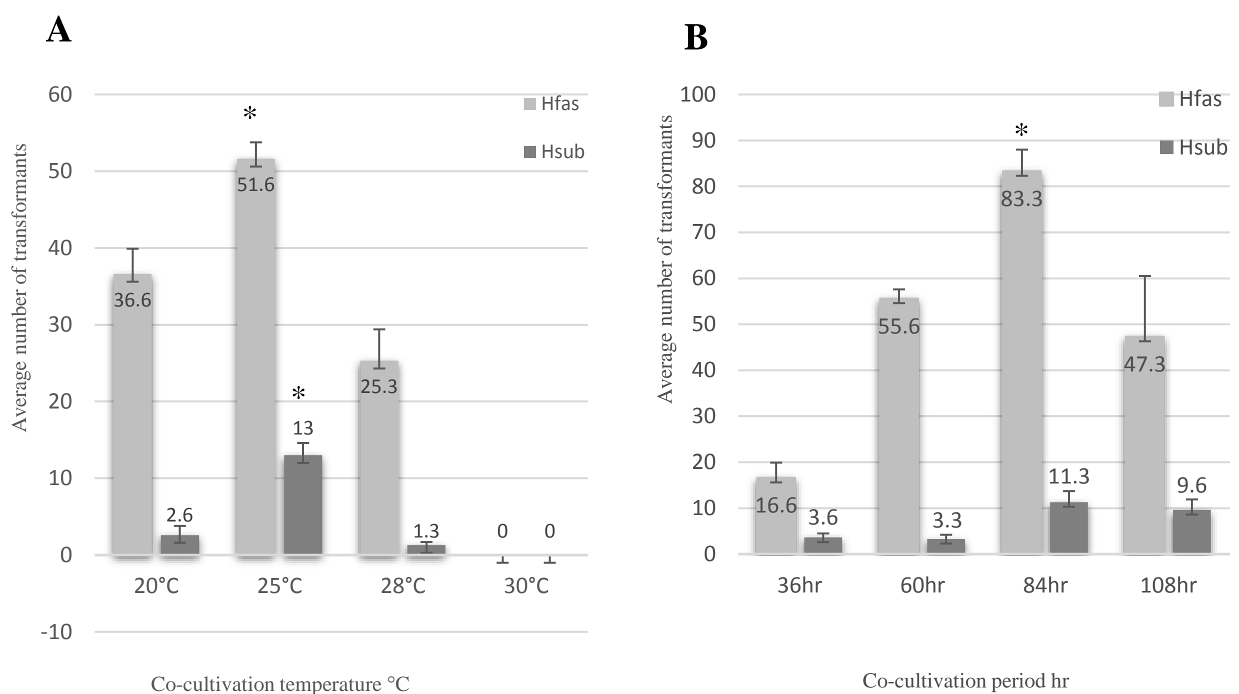
192

193

194

195

196



197

198

199

Figure 2: Average number of transformants obtained using A: different co-cultivation temperatures and B: co-cultivation period, for both *H. fasciculare* (Hfas) and *H. sublateritium* (Hsub). * indicates significant difference compared to other co-cultivation conditions, determined by factorial ANOVA and confirmed by Dunnett's post-hoc test where P<0.05.

200

201

202

203

204

205

206 **3.3. Detection of *hph* in putative *Hypholoma* transformants**

207 10 randomly selected putative transformants for each species were subcultured three
208 times on selective agar (PDA + hygromycin). PCR amplification of the ITS region from all
209 strains confirmed the quality of the gDNA was suitable for analysis, and subsequent PCR
210 amplification with primers Hygro-1 & -2 of a 1kb amplicon in all transformants was the size
211 predicted for the *hph* gene, indicative of successful transformation in all cases (figures S7, S8
212 and S9).

213

214 **3.4. GFP visualization in *Hypholoma* species**

215 The ten *hph*-positive transformants of each *Hypholoma* species obtained using pBGgHg
216 were screened for GFP fluorescence by microscopy, however none gave discernible green
217 fluorescence compared to the wild-type fungus. This has been reported in other species **(Godio**
218 ***et al.*, 2004; Burns *et al.*, 2005; Ford *et al.*, 2016),** where either the promoter needed to be
219 altered, or an intron included within the GFP cDNA region in order to get GFP expression.
220 Therefore two new expression vectors were constructed, both using the *H. sublateritium gpd*
221 promoter to drive GFP expression, and without or with an intron directly upstream of the start
222 codon of the GFP coding region; pCAM-*hph*-Hs*gpd*GFP (intronless vector) and pCAM-*hph*-
223 Hs*gpd*iGFP (intron containing vector) respectively.

224 The T-DNA regions were mobilised into mycelia of *H. fasciculare* and *H. sublateritium*
225 using *A. tumefaciens* LBA4404 and hygromycin resistant colonies were selected. To avoid any
226 bias, all transformants were purified (irrespective of colony size) and were then analysed by
227 microscopy for green fluorescence. When transformants were obtained using the intronless
228 construct, no expression of GFP protein was observed in any of the 49 *H. fasciculare* and 32
229 *H. sublateritium* transformants. When those obtained with the intron-containing vector were
230 assessed, 43 out of 52 *H. fasciculare,* transformants gave detectable green fluorescence.
231 Similarly, 20 out of 28 *H. sublateritium* transformants showed visible GFP expression (figure
232 3). It was observed that the strength of the fluorescence differed between transformants.

233

234

235

236

237

238

239

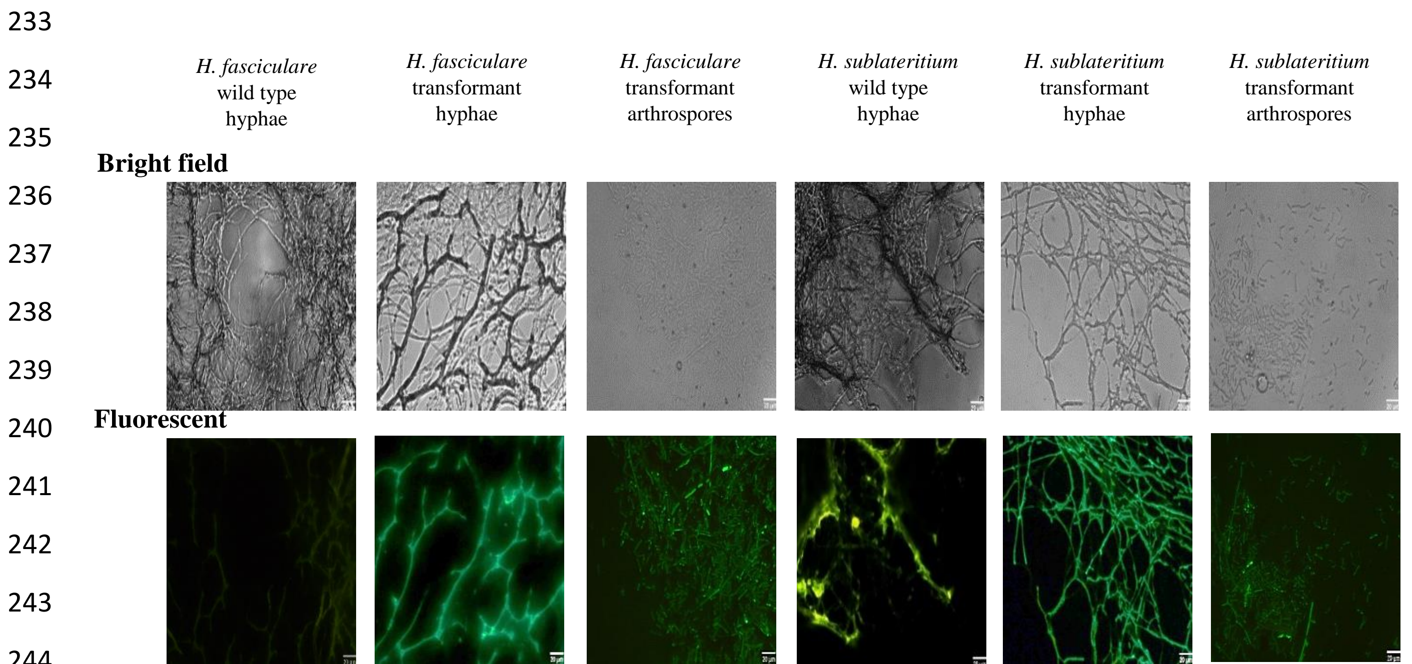
240

241

242

243

244



245 Figure 3: Microscopic images showing limited yellow autofluorescence in the wild type, and bright green fluorescence in selected GFP transformants of both *Hypholoma*. Scale bars 20µm.

To prove the wider functionality of *H. sublateritium gpd* promoter beyond the Strophariaceae family, the intron-containing construct pCAM-*hph*-Hs*gpd*iGFP was transferred to protoplasts of the Entolomataceae species *Clitopilus passeckerianus*, and this transformation also yielded transformants with bright green fluorescence indicative of GFP expression (figure 4).

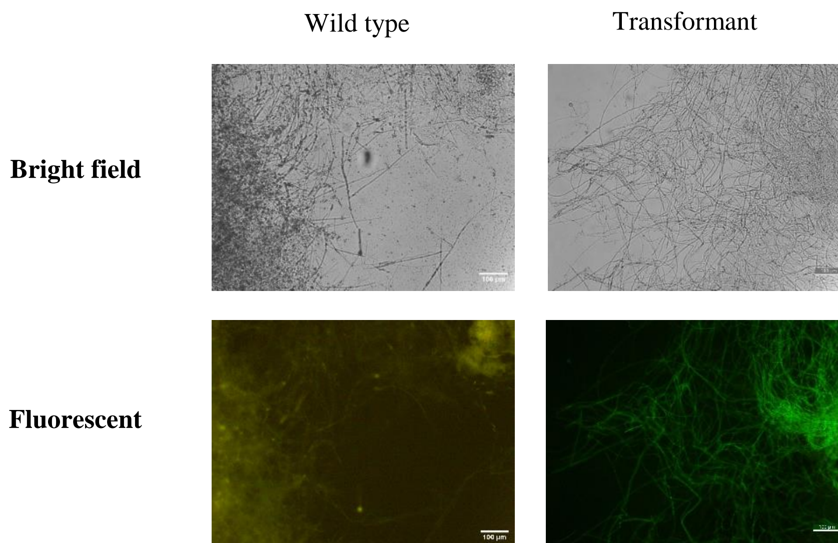


Figure 4: Microscopic images showing limited yellow autofluorescence in wild type *Clitopilus passeckerianus*, and bright green fluorescence in a selected GFP transformant. Scale bars 100µm.

4. Discussion

One of the most distinctive properties of basidiomycetes is their capability to produce biologically active substances. *Hypholoma* sp. are prolific producers of terpenoid like compounds, such as the antitumor clavaric acid and the antibacterial neamatolin, however very limited information is available relating to methods for genome manipulation for these fungi. We aimed in this work to optimise ATMT for *H. fasciculare* and *H. sublateritium,* to establish efficient methods for gene delivery, which could be then used in the potential manipulation of targeted genes. In order to assess the feasibility of transgene expression it is common practise to utilise the reporter genes GFP or DsRed and like some others, we found an intron was a prerequisite for efficient GFP expression.

In general, *H. fascicluare* was more amenable to transformation than *H. sublateritium*, although in each species a comparable transformation frequency was obtained with either arthrospores or homogenized mycelia. Given that spore production by both *Hypholoma* required at least 30 days incubation, while homogenised mycelia could be generated within 10 days, homogenized mycelia were selected for further optimization, as the objective was to establish a simple, fast and efficient transformation system. Previous reports of ATMT in fungi, has highlighted bespoke conditions for each species for efficient transformation. It was reported that *Suillus grevillei* was transformed most efficiently using mycelia **(Murata *et al.,***

283 **2006)**, whereas *Hypsizygus marmoreus* was more readily transformed using protoplasts as
284 recipient tissue (**Jing *et al.,* 2014**).

285 Like many other researchers we found that the presence of acetosyringone in the
286 *Agrobacterium* pre-culture was essential for successful transformation, however this is not
287 always the case as observed in studies on the ectomycorrhizal *Hebeloma cylindrosporum* or
288 the plant pathogenic *Colletotrichum trifolii* **(Michielse *et al.,* 2005).** We also found that the
289 increase of *Agrobacterium* culture OD_{600}, co-cultivation temperature and period, could increase
290 the number of transformants. In our co-cultivation evaluation, we found that the highest number
291 of resistant colonies was obtained after 84hr for both *H. fascicluare* and *H. sublateritium*
292 however we did not determine whether these longer cocultivation times led to an increase in
293 multiple integration events. It is more common to use shorter co-cultivation times, typically
294 three days or less, prior to applying the selection, (**Godio *et al.,* 2004**) however with slow
295 growing fungi such as *Hypholoma*, the longer incubation allowed reliable transformation
296 without deleterious effects on selection of transformants. However, there is clearly an optimum
297 for each parameter that can be utilised **(Michielse *et al.,* 2005).**

298 Two important factors have been shown to impact the efficiency of heterologous gene
299 expression in basidiomycota; a functional promoter and intron presence. Previous studies have
300 highlighted the varied functionality of some promoters when moved between species. For
301 example, the *Agaricus bisporus gpdII* promoter was functional in driving the expression of
302 *hph*, but not for GFP (**Burns *et al.,* 2005),** and this was also observed in this work. However,
303 this was not the case for *Hebeloma cylindrosporum*, where the same promoter was able to drive
304 the expression of both genes *hph* and GFP **(Muller *et al.,* 2006)**. Efficient transgene expression
305 (as defined by readily discernible GFP) was also shown to require a 5' intron in the transgene.
306 This confirms observations in some other species such as *A. bisporus*, *C. passeckerianus* and
307 *A. mellea*, (**Burns et al., 2005**; **Kilaru et al., 2009**; **Ford *et al.,* 2016**), although the reasons for
308 the intron requirement is not yet determined, and this needs to be assessed on a species by
309 species basis. Whilst the GFP fluorescence levels varied between transformants carrying the
310 same construct, this is likely to be due to differences in the chromatin context into which the
311 DNA element has inserted, and possibly also variations in copy number.

312 In conclusion, in this work, we have developed an efficient, simple ATMT system in
313 *H. fasciculare* and *H. sublateritium*, species that are known to produce a wide range of
314 biologically active secondary metabolites. Also, we demonstrate the use of the *H. sublateritium*
315 *gpd* promoter in driving transgene expression, and highlight the need for including its first
316 intron for successful expression of GFP in both *Hypholoma* species. We would predict that
317 careful optimisation of ATMT protocols and evaluation of a number of fungal tissues, coupled
318 with appropriately designed vectors, should pave the way to effective biotechnological
319 exploitation of basidiomycetes in the near future.

320

321

322

323

Conflicts of interest

No conflict of interest has been declared by the authors.

Acknowledgments

SAAA-S was funded by the Ministry of Higher Education and Scientific research (IRAQ). The authors thank Dr. David Hibbett, Clark University (USA) for providing the *H. sublateritium* isolate.

References

Bailey, A.M., Alberti, F., Kilaru, S., Collins, C.M., de Mattos-Shipley, K., Hartley, A.J., Hayes, P., Griffin, A., Lazarus, C.M., Cox, R.J. and Willis, C.L. (2016). Identification and manipulation of the pleuromutilin gene cluster from *Clitopilus passeckerianus* for increased rapid antibiotic production. *Scientific reports*, *6*, p.25202. doi:10.1038/srep25202.

Burns, C., Gregory, K.E., Kirby, M., Cheung, M.K., Riquelme, M., Elliott, T.J., Challen, M.P., Bailey, A. and Foster, G.D. (2005). Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. *Fungal Genetics and Biology*, *42*(3), pp.191-199. http://dx.doi.org/10.1016/j.fgb.2004.11.005.

Chen, X., Stone, M., Schlagnhaufer, C. and Romaine, C.P. (2000). A fruiting body tissue method for efficient Agrobacterium-mediated transformation of *Agaricus bisporus*. *Applied and Environmental Microbiology*, *66*(10), pp.4510-4513. doi: 0.1128/AEM.66.10.4510-4513.2000.

de Bernardi, M., Mellerio, G., Vidari, G., Vita-Finzi, P., Fronza, G., Kocòr, M. and St. Pyrek, J. (1981). Fungal metabolites. IX. Triterpenes from *Naematoloma sublateritium*. *Journal of Natural Products*, *44*(3), pp.351-356. doi: 10.1021/np50015a020.

de Groot, M.J., Bundock, P., Hooykaas, P.J. and Beijersbergen, A.G. (1998). *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology*, *16*. doi:10.1038/nbt0998-839.

de Mattos-Shipley, K.M.J., Ford, K.L., Alberti, F., Banks, A.M., Bailey, A.M. and Foster, G.D. (2016). The good, the bad and the tasty: The many roles of mushrooms. *Studies in Mycology*, *85*, pp.125-157. https://doi.org/10.1016/j.simyco.2016.11.002

Ford, K.L., Baumgartner, K., Henricot, B., Bailey, A. and Foster, G. (2016). A native promoter and inclusion of an intron is necessary for efficient expression of GFP or mRFP in *Armillaria mellea*. *Scientific Reports*, *6*(29226). doi:10.1038/srep29226.

Gietz, R.D. and Woods, R.A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods in Enzymology*, *350*, pp.87-96. https://doi.org/10.1016/S0076-6879(02)50957-5

Godio, R.P., Fouces, R., Gudina, E.J. and Martin, J.F. (2004). *Agrobacterium tumefaciens*-mediated transformation of the antitumor clavaric acid-producing basidiomycete *Hypholoma sublateritium*. *Current Genetics*, *46*(5), pp.287-294. doi:10.1007/s00294-004-0533-5

Ito, Y., Kurita, H., Yamaguchi, T., Sato, M. and Okuda, T. (1967). Naematolin, a new biologically active substance produced by *Naematoloma fasciculare* (Fr.) Karst. Chemical and Pharmaceutical Bulletin 15, 2009–10. http://doi.org/10.1248/cpb.15.2009.

jing Zhang, J., Shi, L., Chen, H., qi Sun, Y., wen Zhao, M., Ren, A., jie Chen, M., Wang, H. and yong Feng, Z. (2014). An efficient *Agrobacterium*-mediated transformation method for the edible mushroom *Hypsizygus marmoreus*. *Microbiological research*, *169*(9), pp.741-748. https://doi.org/10.1016/j.micres.2014.01.004.

Kilaru, S., Collins, C.M., Hartley, A.J., Bailey, A.M. and Foster, G.D. (2009). Establishing molecular tools for genetic manipulation of the pleuromutilin-producing fungus *Clitopilus passeckerianus*. *Applied and Environmental Microbiology*, *75*(22), pp.7196-7204. doi:10.1128/AEM.01151-09.

Kleinwächter, P., Luhmann, U., Schlegel, B., Heinze, S., Härtl, A., Kiet, T.T. and Gräfe, U. (1999). New fasciculol-type triterpene compounds from *Hypholoma fasciculare*. *Journal of Basic Microbiology*, *39*(5-6), pp.345-349. doi: 10.1002/(SICI)1521-4028(199912)39:5/6<345::AID-JOBM345>3.0.CO;2-K.

Liu, D., Coloe, S., Baird, R. and Pedersen, J. (2000). Rapid mini-preparation of fungal DNA for PCR. *Journal of Clinical Microbiology*, *38*(1), pp.471-471.

Michielse, C.B., Hooykaas, P.J., van den Hondel, C.A. and Ram, A.F. (2005). *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Current Genetics*, *48*(1), pp.1-17. doi: 10.1007/s00294-005-0578-0.

Mikosch, T.S., Lavrijssen, B., Sonnenberg, A.S. and Van Griensven, L.J. (2001). Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. *Current Genetics*, *39*(1), pp.35-39. doi:10.1007/s002940000178.

Müller, T., Benjdia, M., Avolio, M., Voigt, B., Menzel, D., Pardo, A., Frommer, W.B. and Wipf, D. (2006). Functional expression of the green fluorescent protein in the ectomycorrhizal model fungus *Hebeloma cylindrosporum*. *Mycorrhiza*, *16*(6), pp.437-442. doi:10.1007/s00572-006-0060-y.

Murata, H., Sunagawa, M., Yamazaki, T., Shishido, K. and Igasaki, T. (2006). Expression of the autofluorescent protein, DsRed2, in the recombinants of the ectomycorrhizal basidiomycete, *Suillus grevillei*, generated by *Agrobacterium*-mediated transformation. *Mycorrhiza*, *16*(6), pp.407-412. doi:10.1007/s00572-006-0058-5.

Rodríguez-Tovar, A.V., Ruiz-Medrano, R., Herrera-Martínez, A., Barrera-Figueroa, B.E., Hidalgo-Lara, M.E., Reyes-Márquez, B.E., Cabrera-Ponce, J.L., Valdés, M. and Xoconostle-Cázares, B. (2005). Stable genetic transformation of the ectomycorrhizal fungus *Pisolithus tinctorius*. *Journal of microbiological methods*, *63*(1), pp.45-54. https://doi.org/10.1016/j.mimet.2005.02.016.

Shiono, Y., Akasaka, H., Hiramatsu, F., Sato, K., Murayama, T. and Ikeda, M. (2005). Three sesquiterpenoids, fascicularones E, F, and G produced by the fungus *Hypholoma fasciculare*. *Zeitschrift für Naturforschung B*, *60*(8), pp.880-884. https://doi.org/10.1515/znb-2005-0811.

Shiono, Y., Matsuzaka, R., Wakamatsu, H., Muneta, K., Murayama, T. and Ikeda, M. (2004). Fascicularones A and B from a mycelial culture of *Naematoloma fasciculare*. *Phytochemistry*, *65*(4),pp.491-496. http://dx.doi.org/10.1016/j.phytochem.2003.10.002.