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Revised

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

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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.

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. sublateralitium*, and that these vectors were also functional in *Clitopilus passeckerianus*.

2. Experimental strategies

2.1. Microbial isolates and culture conditions

H. sublateralitium 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).

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 [K₂HPO₄ 34.84 g/L, KH₂PO₄ 27.22 g/L, NaCl 2.93 g/L], 50 ml stock B [MgSO₄·7H₂O 9.86 g/L, (NH₄)₂SO₄ 10.58 g/L], 10 ml 70mM CaCl₂, 1 ml 9 mM FeSO₄, 10 ml 1M D-glucose, 40 ml 1M MES, 10 ml 50 % glycerol, 15 g agar) were used.

2.3. *Agrobacterium* transformation

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 1×10⁶/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.

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. sublateralitium* and *H. fasciculare* respectively along with 100 µg/ml ticarcillin-clavulanate, and incubated until colonies appeared.

C. passeckerianus was transformed by protoplast PEG/CaCl₂ method as outlined by Kilaru *et al.* (2009), using intact plasmid DNA.

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.

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

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

2.5. GFP vector construction

The *in silico* design of the two vectors pCAM-*hph*-Hs*gpd*GFP and pCAM-*hph*-Hs*gpd*iGFP was performed using Clone-manager (Scientific and Educational Software). The yeast-based recombination method of **Gietz and Woods (2002)** was adapted for the *in vivo* construction. Both vectors were identical in terms of the pCambia0380YA backbone, hygromycin selection cassette and GFP expression cassette, differing only in the presence of intron sequences in the *H. sublateritium gpd* promoter driving GFP expression. DNA segments were amplified from either pBGgHg (**Chen et al., 2000**) or from the *H. sublateritium* genome with primer extensions to provide overlaps with the cut ends of the backbone (figures S1 and S2). Vector sequences were verified using the GATC BIOTECH sequencing service, then transferred to *Agrobacterium tumefaciens* LBA4404.

2.6. Microscopy analysis

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

3. Results

3.1. Selectable marker choice

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

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. sublateralitium*. 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. sublateralitium* (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.

Different concentrations of acetosyringone (from 0-400 μ M) were added to *Agrobacterium* pre-cultures to evaluate the levels needed for efficient induction of the T-DNA transfer pathway. No transformants were produced in the absence of this phytochemical, whilst 100-200 μ M gave the highest numbers (figure S5). To determine the optimal OD₆₀₀ of *Agrobacterium* induction culture (AIC), a series of densities (0.2, 0.3, 0.4 and 0.5) were tested. *Agrobacterium* culture density of OD₆₀₀ = 0.3 and 0.4 were the optimum in producing hygromycin resistant colonies for *H. fasciculare*, and *H. sublateritium* respectively (figure S6).

3.2.3. Co-cultivation conditions

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

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.

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

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

3.4. GFP visualization in *Hypholoma* species

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

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

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*-HsgpdGFP 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 clavarinic 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. fasciculare* 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.*,

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

Like many other researchers we found that the presence of acetosyringone in the *Agrobacterium* pre-culture was essential for successful transformation, however this is not always the case as observed in studies on the ectomycorrhizal *Hebeloma cylindrosporum* or the plant pathogenic *Colletotrichum trifolii* (Michielse *et al.*, 2005). We also found that the increase of *Agrobacterium* culture OD₆₀₀, co-cultivation temperature and period, could increase the number of transformants. In our co-cultivation evaluation, we found that the highest number of resistant colonies was obtained after 84hr for both *H. fasciculare* and *H. sublateralitium* however we did not determine whether these longer cocultivation times led to an increase in multiple integration events. It is more common to use shorter co-cultivation times, typically three days or less, prior to applying the selection, (Godio *et al.*, 2004) however with slow growing fungi such as *Hypholoma*, the longer incubation allowed reliable transformation without deleterious effects on selection of transformants. However, there is clearly an optimum for each parameter that can be utilised (Michielse *et al.*, 2005).

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

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

Conflicts of interest

No conflict of interest has been declared by the authors.

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