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Development of an efficient genetic system in a gene cluster-rich endophytic fungus *Calcarisporium arbuscula* NRRL 3705

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ABSTRACT

Filamentous fungi are emerging as attractive producers of natural products with novel structures, diverse bioactivities and unprecedented enzymology. But their genetic systems are poorly developed, especially in some non-model endogenic fungi, which have hampered our genetic manipulation of their natural product development. *Calcarisporium arbuscula* NRRL 3705 is an endophytic filamentous fungus rich in biosynthetic gene clusters and primarily producing mycotoxin aurovertins. Here we optimized *Agrobacterium tumefaciens*-mediated transformation (ATMT)-based efficient DNA introduction into *C. arbuscula*. By complementation of the monooxygenase gene *aurC* in *ΔaurC* mutant as a model, we showed that a strong but down-regulated promoter *aurAp* and three strong constitutive promoter *gpdAp*, *tef1p* and *tubCp* could be used for gene overexpression. Meanwhile, red fluorescence protein (RFP) was expressed in this fungus under the control of *tubCp*, potentially paving the way for enzyme localization determination during natural product biosynthesis. Furthermore, we developed efficient and convenient gene disruption in *C. arbuscula* based on ATMT, as exemplified by deletion of *aurA* in *ΔaurC* mutant. Our efficiency of deletion ran at about 40%. These results suggest that ATMT-based transformation for gene ectopic expression or deletion is an efficient strategy for genetic manipulation of *C. arbuscula*, and can be readily adapted to other rare filamentous fungi, potentially to promote discovery and development of natural products.

1. Introduction

Filamentous fungi, producing a large number of secondary metabolites with novel structures and activities, are regarded as inexhaustible sources for the production of clinical drugs or lead compounds (Newman and Cragg, 2016), such as penicillin from *Penicillium chrysogenum* (Martín, 2017) and lovastatin from *Aspergillus terreus* (Pérez-Sánchez et al., 2017), etc. With the rapid expansion of fungi genome information (http://1000.fungalgenomes.org), a large number of biosynthetic gene clusters (BGCs) have been identified for the potential production of invaluable natural products (Ziemert et al., 2016), particularly in some rare fungi, these fungi being distinct from the traditional genera *Aspergillus*, *Penicillium* or *Trichiderma* (Alberti et al., 2017). However, problems with the efficient genetic manipulation systems, such as the overexpression and the deletion of certain genes in BGCs to understand the biosynthetic pathways, have impeded discovery and development of these natural products.

The most commonly used method of genetic manipulation of filamentous fungi is PEG-mediated protoplast transformation (Turgeon et al., 2010). Recently, the CRISPR/Cas9 system has been established in some model fungi, such as *Trichoderma reesei* (Nødvig et al., 2015), *Neurospora crassa* (Matsuura et al., 2015) and *Penicillium chrysogenum* (Pohl et al., 2016) etc. But cumbersome operation and unsatisfactory repetitiveness have limited the wide application of CRISPR/Cas9 system in filamentous fungi. *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been developed in filamentous fungi for two decades (Groot et al., 1998). It is based on the capacity of the transfer DNA (T-DNA) between the left border (LB) and the right border (RB) of tumor-inducing (Ti) plasmid, randomly integrating into the host cell genome (Citovsky et al., 2007). ATMT is more convenient and reproducible than the traditional protoplast transformation and can be applied in fungal cells independent of their growth stages (Groot et al., 1998). Moreover, to take full advantage of BGCs for natural product development, some effective genetic elements, such as strong promoters, have been extensively developed for activation, upregulation or hetero-expression of BGCs in fungi (Alberti et al., 2017). These include inducible promoters *glaAp*, *alcAp* (Chiang et al., 2009, Reilly et al., 2018), and constitutive promoters *gpdAp*, *tef1p*, etc. (Chen et al., 2010;

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D'Espaux et al., 2017). It has been reported that *alcAp* has been used for the overexpression of a transcription regulator in order to activate production of asperfuranone in *Aspergillus nidulans*. (Chiang et al., 2009). Additionally, *gpdAp* has been used for the over-production of monacolin K in *Monascus pilosus* (Chen et al., 2010).

Calcarisporium arbuscula NRRL 3705 is an endophytic fungus in fruit-bodies of mushrooms, producing aurovertin-type mycotoxins that are potent against F0F1-ATPase and breast cancers (Huang et al., 2008; Van Raaij et al., 1996). In addition, 68 BGCs were found in this fungus and most of the BGCs were silent. Previously, intriguing compounds with attractive structures and bioactivities were discovered after epigenetic activation (Mao et al., 2015a,b). And many valuable silent BGCs are worthy of further study. However, only protoplast-mediated gene knock-out has been developed in *C. arbuscula* until now, and the transformation and gene deletion efficiencies were low.

So far, more attention has been paid to model filamentous fungi, such as *Aspergillus, Penicillium, Neurospora, Trichoderma,* etc. Fungi in other genera, partially because of their inconspicuous living environments and lack of genetic tools, are rarely studied. For example, only 5 papers have reported characterization of the *Calcarisporium* genus (Barnett, 1958; Evans, 1971; Haller and Loeffler, 1969; Somrithipol and Jones, 2006; Watson, 1955). Here using *C. arbuscula* as a model of rare filamentous fungi, we aim to develop a genetic manipulation system, including the over-expression tools, fluorescent protein expression system, and efficient gene disruption based on ATMT. The methods will greatly benefit natural products research in *C. arbuscula,* and could be applicable in other BGC-rich filamentous fungi as well.

2. Methods and materials

2.1. Strains and culture conditions

Calcarisporium arbuscula NRRL 3705 (Mao et al., 2015a,b) was the fungal wild type strain, and the *ΔaurC* mutant, deficient of *aurC* (encoding a monooxygenase for aurovertin biosynthesis), was constructed previously (Mao et al., 2015a,b). *ΔaurCΔaurA* double mutant was constructed by further deletion of *aurA* (encoding aurovertin polyketide synthase) in the *ΔaurC* mutant. *C. arbuscula* strains were cultured at 25°C on potato dextrose agar (PDA) (Sigma) for sporulation, and in potato dextrose broth (PDB) (Sigma) for protein preparation with flask shaking at 150 rpm or secondary metabolite production after stationary culture. *Escherichia coli* strain DH5α was used for routine sub-cloning and cultured in the lysogenic broth (LB) medium at 37°C. *Agrobacterium tumefaciens* EHA105 (Michielse et al., 2008) was used for introduction of DNA into filamentous fungi and cultured at 28°C in LB medium supplemented with 50 μg/ml streptomycin.

For antibiotic sensitivity assays, spores of *C. arbuscula* were spread on PDA plates with 0, 25, 50, 100, 150, 200 μg/ml hygromycin B, geneticin (G418) or phleomycin, respectively, incubated at 25°C for 7 days and photographed.

2.2. Plasmid construction

All the plasmids and primers used in this study were listed in Table S1 and S2, respectively, and scheme-diagram of construction was shown in Fig. S1-S3. *C. arbuscula* genomic DNA was prepared as described (Mao et al., 2015a,b). pFGL815N (Yang and Naqvi, 2014) is a T-DNA for *Agrobacterium tumefaciens*-mediated transformation (ATMT). The *Aspergillus nidulans gpdA* promoter (*gpdAp*) and *trpC* terminator (*trpCt*) were amplified from plasmid pAN7 (Mao et al., 2015a,b) with primer pair 1 + 2 and 3 + 4, respectively, and assembled into *Kpn*I-digested pFGL815N by One Step Cloning Kit (Vazyme, China) to give rise to plasmid pFGL-ANgpdAp. G418-resistance gene *neoR* was amplified with primer 5 + 6 from plasmid pBI101 (Yang et al., 2008) and assembled into *Nco*I-digested pFGL-ANgpdAp for plasmid pFGL-neoR. Then *aurA* promoter (*aurAp*) (Fig. S6) and *aurC* terminator (*aurCt*) were amplified from *C. arbuscula* genomic DNA with primer pairs 7 + 8 and 9 + 10, respectively, and assembled into *Hin*dIII/*Sma*I-digested pFGL-neoR for plasmid pFLG-aurAp, together with one *Bam*HI site and FLAG tag introduced between the promoter and terminator. *C. arbuscula gpdA* promoter (*gpdAp*) (Fig. S7), *tef1* promoter (*tef1p*) (Fig. S8) and *tubC* promoter (*tubCp*) (Fig. S9) were amplified with primers 11 + 12, 13 + 14 and 15 + 16, and assembled into pFLG-aurAp, which has been digested with *Bam*HI/*Hin*dIII to remove *aurAp*, for plasmid pFGL-gpdAp, pFGL-tef1p and pFGL-tubCp, respectively. And *aurC* was amplified from fungal genome with primer 17 + 21, 18 + 21, 19 + 21 and 20 + 21, and assembled into *Bam*HI-digested pFGL-aurAp, pFGL-gpdAp, pFGL-tef1p and pFGL-tubCp, respectively, to create plasmid pFGL-aurAp-aurC, pFGL-gpdAp-aurC, pFGL-tef1p-aurC and pFGL-tubCp-aurC (Fig. S1).

For plasmid pFGL-tubCp-RFP construction, *tubCp* and mcherry DNA fragment (*rfp*) were obtained by PCR with primer pairs 15 + 22 and 23 + 24 from *C. arbuscula* genomic DNA and pFA6a-link-yoPA-mCherry-Kan (Lee et al., 2013), respectively, and co-assembled into *Bam*HI/*Hin*dIII-digested pFGL-neoR (Fig. S2).

For *aurA* disruption by ATMT, the upstream and downstream homologous regions of *aurA* were amplified with primer pairs 25 + 26 and 27 + 28, respectively, and assembled into pFGL-neoR, which was initially digested with *Eco*RI/*Kpn*I, followed by *Bam*HI digestion for sequential assembly of the two homologous fragments and *neoR* to create plasmid pFGL-neoR-aurA L + R (Fig. S3).

2.3. Agrobacterium tumefaciens-mediated transformation (ATMT) of C. arbuscula

ATMT of *C. arbuscula* was performed as described previously (Groot et al., 1998; Michielse et al., 2008) with some modifications. Spores of *C. arbuscula* were inoculated on PDA at 25 °C for 1 week and then conidiospores were collected, washed with sterile water plus 0.1% Tween-20, and diluted into 10^7 spores/ml. Plasmids were introduced to *A. tumefaciens* by electro-transformation (Gouka et al., 1999), and bacterial cells were cultured on LB plate (50 μg/ml streptomycin and 50 μg/ml kanamycin) at 28 °C for 2 days. The correct clone was confirmed by colony PCR, and inoculated in liquid LB medium (50 μg/ml streptomycin and 50 μg/ml kanamycin) at 28 °C with 200 rpm agitation. The overnight culture was centrifuged, washed with sterile water once and diluted in 5 ml liquid induction medium (IM) (Michielse et al., 2008) containing 400 μM acetosyringone (AS) to OD_{600} = 0.1–0.15 and continued for culture to OD_{600} = 0.8. About 100 μl of bacterial culture was mixed with 100 μl of *C. arbuscula* 10^7/ml spores, and 200 μl of *Agrobacterium*-conidiospore mixture was spread on the solid IM overlaid with sterile cellophane for further incubation at 25 °C for 48 h in darkness. The cellophane was then transferred to the selection PDA plate (300 μg/ml cefotaxime sodium, 100 μg/ml G418), which was incubated at 25 °C for 7 days for colony appearance. Three repeated experiments were performed and the data were collected from three plates with the same condition.

2.4. Reverse transcription-PCR (RT-PCR)

RT-PCR was performed with RNA prepared from *C. arbuscula* mycelia grown on PDA for 1 week or 4 weeks. The RNA extraction was performed as described previously (Mao et al., 2015a,b). PCR was performed with Phusion® High-Fidelity DNA Polymerase (New England Biolabs) in the presence of 25 ng of reverse transcribed RNA. Primers were listed in Table S3.

2.5. Microscopy

The *C. arbuscula* strain expressing red fluorescence protein (RFP, mcherry) under the control of *tubCp* was inoculated on PDA supplemented with 100 μg/ml G418, and grown at 25 °C for 5 days. The