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Development of a high-efficiency gene knockout system for *Pochonia chlamydosporia*

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ABSTRACT

The nematophagous fungus *Pochonia chlamydosporia*, which belongs to the family Clavicipitaceae (Ascomycota: Pezizomycotina: Sordariomycetes: Hypocreales), is a promising biological control agent for root-knot and cyst nematodes. Its biocontrol effect has been confirmed by pot and field trials. The genome sequence of the fungus was completed recently; therefore, genome-wide functional analyses will identify its infection-associated genes. Gene knockout techniques are useful molecular tools to study gene functions. However, cultures of *P. chlamydosporia* are resistant to high levels of a range of fungal inhibitors, which makes the gene knockout technique difficult in this fungus. Fortunately, we found that the wild *P. chlamydosporia* strain PC-170 could not grow on medium containing 150 μg ml^{-1} G418 sulfate, representing a new selectable marker for *P. chlamydosporia*. The neomycin-resistance gene (*neo*), which was amplified from the plasmid pKOV21, conferred G418-resistance on the fungus; therefore, it was chosen as the marker gene. We subsequently developed a gene knockout system for *P. chlamydosporia* using split-marker homologous recombination cassettes with resistance selection and protoplast transformation. The split-marker cassettes were developed using fusion PCR, and involved only two rounds of PCR. The final products comprised two linear constructs. Each construct contained a flanking region of the target gene and two thirds of the neo gene. Alkaline serine protease and chitinase were confirmed to be produced by *P. chlamydosporia* during infection of nematode eggs and could participate in lysis of the eggshell of nematode eggs. Here, we knocked out one chitinase gene, *VFPPC_01099*, and two protease genes (*VFPPC_10088*, *VFPPC_06535*). We obtained approximately 100 suspected mutants after each transformation. After screening by PCR, the average rate of gene knockout was 13%: 11% (*VFPPC_01099*), 13% (*VFPPC_10088*) and 15% (*VFPPC_06535*). This efficient and convenient technique will accelerate functional genomic studies in *P. chlamydosporia*.

Introduction

The nematophagous fungus *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) is one of the most studied egg-parasitic fungi (Zare and Gams, 2001; Siddiqui et al. 2009; Manzanilla-López et al. 2013). Willcox first reported it in 1974 as a fungal egg-parasite of plant-parasitic nematodes. Kerry found that the fungus made a contribution to the natural recession of *Heterodera* spp. in 1975 (Kerry, 1975). Since then, the fungus has been proved a biological control agent for *Meloidogyne incognita*, *Meloidogyne hapla*, *Meloidogyne javanica*, *Meloidogyne arenaria*, *Heterodera schachtii*, *Heterodera avenae* and *Heterodera glycines* (De Leij et al., 1993; De LeiJ and Kerry 1991; Ayatollahy et al. 2008; Zhang et al. 2009; Zhao, 2002; Chen and Dickson, 1996).

The main steps of *P. chlamydosporia* infection of nematode eggs were first observed on *M. arenaria* using a Scanning Electron Microscope. When it parasitizes *M. arenaria*, the fungus generates penetrative hyphae that hatch and colonize the eggs. Both the egg shell and larval cuticle are disrupted. The hyphae then readily proliferate within the eggs and larvae. Finally, infected larvae became totally necrotic and disintegrate internally (Morgan-Jones et al. 1983). Subsequently, the main steps of the mode of parasitism of *H.*

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glycines and *M. incognita* were observed (Chen and Dickson, 1996; Tahseen et al. 2005). Recently, Escudero used a GFP transformant Pc123gfp to analyze the *M. javanica* egg-infection process under the confocal laser microscopy. Using the GFP marker gene, they were able to observe the development of the entire infection process (Escudero and Lopez-Llorca, 2012).

Alkaline serine protease and chitinase are produced by *P. chlamydosporia* during infection of nematode eggs (Segers et al. 1994; Dackman et al. 1989). The functions of these enzymes were tested *in vitro*: they participate in lysis of the eggshell of M. incognita. The gene sequences of these enzymes were acquired by an anchored PCR technique and a genome walking technique, respectively (Zhang et al. 2009; Segers et al. 1996; Morton et al. 2003; Mi et al. 2010). However, there is a lack of direct evidence for the functions of these enzymes in the fungus, and this evidence could be acquired by homologous gene knockout technology.

Rothamsted Research was the first institute to develop a transformation system for *P. chlamydosporia*. Plasmids carrying the green fluorescent protein marker gene *gfp* and the hygromycin resistance gene *hph* were transformed into *P. chlamydosporia* isolate 10 using PEG-mediated protoplast transformation, and the transformants were screened on potato dextrose agar (PDA) containing 1 mg ml^{-1} hygromycin (Atkins et al. 2004). This system had the potential to investigate the expression of transgenes in *P. chlamydosporia* protoplasts; however, the genes were not maintained stably after regeneration. Subsequently, Maciá-Vicente et al. transformed a plasmid carrying *gfp* and the benomyl resistance gene *benA3* into *P. chlamydosporia* strain Pc123 using an *Agrobacterium tumefaciens*-mediated transformation protocol, and the transformants were screened on PDA containing 100 μg ml^{-1} benomyl (Maciá-Vicente et al. 2009). Although this method produced stable GFP transformants, constructing the plasmid is time consuming and includes many restriction enzyme digestions. Additionally, hygromycin and benomyl are not suitable screening agents for all *P. chlamydosporia* strains.

Fusion PCR-based deletion methods using a split marker strategy is a more efficient method for homologous gene knockout. It only needs two rounds of PCR. The flanking region sequences of the targeted gene and the selectable marker are amplified in the first round, and the upstream region of the targeted gene is fused with the first two thirds of the selectable marker. In the same way, the downstream region of the targeted gene is fused with the last two thirds of the selectable marker. The two fragments containing flanking region sequences of the targeted gene and partial selectable marker can be used for direct transformation or to construct plasmids (Catlett et al. 2003). This method has been used previously for *Cochliobolus heterostrophus*, *Gibberella zeae* and *Magnaporthe oryzae*, but not for *P. chlamydosporia* (Catlett et al. 2003; Goswami et al. 2006; Kershaw and Talbot, 2009).

Here, we developed a high-efficiency gene knockout system for *P. chlamydosporia*, using fusion PCR-based deletion methods with split-marker strategy and PEG-mediated protoplast transformation. Using this method, we deleted three genes successfully: one chitinase gene *VFPPC_01099*, and two protease genes, *VFPPC_10088*, *VFPPC_06535*. The mutants were confirmed by PCR diagnosis and Southern hybridization analysis. The results suggested that this strategy is an efficient homologous gene knockout method for *P. chlamydosporia*, and should pave the way for high-throughput genetic analysis of this biological control agent.

Materials and methods

Strains and culture conditions

The *P. chlamydosporia* strain PC-170 used in the present study was originally isolated from *M. incognita* eggs and stored as a conidial culture at −80 °C in our laboratory. The strain was also registered in the Chinese General Microbiological Culture Collection Center (CGMCC 8860). The wild PC-170 strain was grown on PDA: (200 g potato, 20 g glucose, 15 g agar) l^{-1} for routine culturing. Modified minimal medium (MM: 1 g KH_2PO_4, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 2 g $NaNO_3$, 20 g Glucose, 200 g Sucrose, 1.5% agar) l^{-1} without antibiotic and T-top medium (T-top: 1 g KH_2PO_4, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 2 g $NaNO_3$, 20 g Glucose, 200 g Sucrose, 1% agar) l^{-1} supplemented with 150 μg ml^{-1} G418 sulfate (Amresco, OH, USA) were used to screen G418-resistant transformants (Pietro and Roncero, 1998). PDA medium supplemented with 150 μg ml^{-1} G418 sulfate were used to subculture the deletion mutants and investigate the mutants' stability. All cultures were grown at 28 °C in an incubator.

Construction of gene-disruption cassettes

The methodology of our fusion PCR and gene knockout technique is summarized in Fig. 2. All the primers used in the study are shown in Fig. 1. The plasmid pKOV21 was used as the template for amplifying the G418-resistance gene *neo*, which includes the cds, *Aspergillus nidulans trp*C promoter and *Aspergillus nidulans trp*C terminator sequences. Our laboratory in the Beijing Genomics Institute (BGI) has sequenced the whole-genome of *P. chlamydosporia* strain PC-170. The gene data is being processed, and will be released soon. Genomic DNA was extracted as previously described (Dean et al. 2005).

First round PCR

In PCR round 1, the upstream homologous arm (product (1)) and downstream homologous arm (product (4)) of the target gene were amplified separately using genomic DNA as the template. Meanwhile, the first two-thirds (*ne*, product (2)) and the last two thirds (*eo*, product (3)) of the G418-resistance gene *neo* were amplified separately using pKOV21 as the template. PCR reactions comprised 25 μl, containing 0.2 μM each primer, 2.5 μl ×10 PCR reaction buffer, 0.1 mM dNTPs, 2.5U EasyTaq DNA polymerase (TransGen Biotech, Beijing, China), template DNA as required, and sterile ddH_2O up to 25 μl. PCR conditions were as follows: 94 °C for 4 min; followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1.5 min; with a final incubation at 72 °C for 10 min. The PCR products were checked by 1% agarose gel-electrophoresis and purified using an EasyPure PCR Purification Kit (TransGen Biotech, Beijing, China) for subsequent experiments.

Second round PCR

In PCR round 2, the upstream homologous arm was fused to the partial split-marker *ne* by overlap extension; the templates were the upstream homologous arm and the *ne* fragment. The correctly targeted integration was region (1)+(2). The downstream homologous arm was fused to the partial split-marker *eo* by overlap extension; the templates were the downstream homologous arm and *eo* fragment. The correctly targeted integration was region (3)+(4). The PCR reactions(50 μl) comprised 1 μM each primer, 5 μl ×10 LA Taq Buffer II (Mg^{2+} Plus) (Takara Biotechnology, Dalian, China), 0.4 mM dNTPs, 2.5U Takara La Taq (Takara Biotechnology, Dalian, China), the ratio of the two fragments as templates was 1:1 and they were added as required, then add sterile ddH_2O up to 50 μl. PCR conditions were: 94 °C for 4 min; followed by 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 2.5 min; with a final incubation at 72 °C for 10 min. The PCR products were checked by 1% agarose gel electrophoresis and purified using an EasyPure Quick