Improvement on genetic transformation in the nematode-trapping fungus *Arthrobotrys oligospora* and its quantification on dung samples

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Abstract

An improved DNA-mediated transformation system for nematode-trapping fungus *Arthrobotrys oligospora* based on hygromycin B resistance was developed. The transformation frequency varied between 34 and 175 transformants per μg linearized DNA and 93% of the transformants were stable for drug resistance when tested 100 randomly selected transformants. More than 2000 transformants were obtained by transformation of the fungus with pBChygro in the presence of *Hin*dIII and among them, one, YMF1.00110, which lost its ability of forming predacious structure, was isolated. Southern analysis showed that the plasmid DNA had integrated into the genome of all tested transformants (including YMF 1.00110) except one. The transformant tagged with *hph* gene could be re-isolated and quantified from dung samples based on the resistance of hygromycin B. All the results suggested that the method of restriction enzyme mediated integration (REMI) should facilitate not only the insertional mutagenesis for tagging and analysis genes of interest but also the ecological investigation of tagged fungi in a given environment.

Key words: *Arthrobotrys oligospora*, hygromycin B REMI

Introduction

The parasitic diseases of production animals are widely distributed in the world. Their economic significance has been demonstrated [1] and the losses caused by gastrointestinal nematodes can be considerable [2]. Due to increasing resistance of these nematodes to available anthelmintics, non-anthelmintic control alternatives are considerable. Nematode-trapping fungi have shown their potential as a biological control agent against these animal parasites. There have been some recent exciting advances in the control of nematode parasites of livestock, by exploiting the nematophagous properties of nematode-trapping fungi. Among these predators, *Arthrobotrys flagrans* (Dudd.) Sidorova, Gorlenko & Nalepina (= *Duddingtonia flagrans* (Dudd.) R. C. Cooke [3]) has been selected for commercial development, as this fungus easily produces large numbers of chlamydospores from culture and these resistance spores are capable of surviving passage through the gastrointestinal. Trials using *A. flagrans* to reduce parasitic nematodes of livestock are successful in Denmark [4], Sweden [5], United States [6, 7], Malaysia [8, 9] and Australia [10]. The biocontrol potential of nematode-trapping fungi of the genus *Arthrobotrys* has also been considered [11, 12]. To examine the biocontrol efficiencies or translation of agents in a given environment, a useful method for recovery and quantification of biocontrol strains is needed. Usually, the presence of distributed nematode-trapping fungi were semi-quantitatively assessed by the Petri dishes method [10, 13] or quantitatively determined by a most probable number technique [14]. However, in these methods we are not sure whether the recovery fungus derived from the agent or the original

habitat, and undoubtedly the issue will affect the analysis results. Furthermore, we lack an effective approach to understand the molecular basis of infection factors of nematode-trapping fungi.

Fortunately, transformed fungi containing reporter genes provide a new tool for monitoring the fate of biocontrol fungi. Some marker systems have been used in ecological studies and infection process of filamentous fungi using β-glucuronidase (GUS) reporter gene from *Escherichia coli* [15, 16], and the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* [17]. Hygromycin is an aminoglycosidic antibiotic that inhibits protein synthesis in prokaryotes and eukaryotes by interfering with translocation and causing misreading [18]. Hygromycin-resistance gene *(hph)* had been isolated and characterized from *E. coli* [19] and the vectors based on the *E. coli hph* gene were constructed for filamentous fungi [20]. Restriction enzyme mediated integration (REMI), a useful transformation method, was initially established in *Saccharomyces cerevisiae* [21] and then widely used in fungal tag and isolation of pathogenicity genes in fungi attacking plants [22]. Concerning the predacious fungi, Tunlid et al. [23] first developed the REMI system by successfully transforming *A. oligospora* with *hph* gene.

This study was carried out to develop a higher efficient DNA-mediated transformation system for nematode-trapping fungi based on the resistance to the hygromycin B and second, to determine whether this method could generate the transformants for ecological investigation and molecular analysis of morphogenesis.

Materials and methods

Fungal culture and media

The model species in various studies of nematode-trapping fungi, *A. oligospora* YMF1.00056, was used. This fungus was isolated originally from soil and maintained on corn meal agar (CMA) medium at 4 °C.

Transformation vector

Plasmid pBChygro was provided by the Fungal Genetics Stock Centre of USA, contains the *hygB* gene from *E. coli, cpc-1* promoter from *Neurospora crassa* and *trpC* terminator from *Aspergillus nidulans*.

Transformation protocol

Transformation was performed based on the methods of Tunlid et al. [23] and Lu et al. [24] with modifications. Briefly, ca 10^8 conidia of *A. oligospora* from 14 days old cultures on TGA medium (l^{-1}: 10 g tryptone, 10 g glucose, 18 g agar) were inoculated in 50 ml of TG medium (TGA medium without agar) on a rotary shaker (165 rpm), After incubation at 28 °C for 36 h, the mycelium was harvested and washed with 50 ml of MN solution (0.3 mol/l MgSO$_4$, 0.3 mol/l NaCl). About 0.5 g of wet mycelium was suspended in 5 ml MN buffer containing 5 mg/ml snailase (Beijing Jingke Company, China) and 5 mg/ml cellulase (Yakult Honsha Co. Ltd., Japan). After incubation on a shaker (180 rpm) at 28 °C for 5 h, the protoplasts were passed through 4-layer of tissue to remove the undigested mycelium and precipitated by centrifugation (8000 rpm) for 10 min at room temperature. After washing with 5 ml KTC buffer (1.2 mol/l KCl, 10 mmol/l Tris–HCl, pH 7.5, 50 mmol/l CaCl$_2$), the pelleted protoplasts were resuspended in 500 μl KTC and used for transformation immediately.

For transformation, 100 μl protoplasts (ca 8.0×10^7) was mixed with 10 μg of linear vector DNA (linearized by *Hin*dIII), and incubated for 30 min on ice. Then 250 μl of PTC (10 mmol/l Tris–HCl, pH 7.5, 50 mmol/l CaC1$_2$, 10–50% W/V PEG3350) were added and mixed gently. After incubation at 28 °C for 20 min, 100 μ1 of protoplast mixture was added to 10 ml of PDSSA medium (potato dextrose agar, PDA, supplying with 10 g/l molasses and 0.4 mol/l saccharose) containing 200 μg/ml of hygromycin B (Yunke Bioproduct Company), previously kept at 48 °C, then poured into Petri dishes. The plates were incubated at 28 °C for transformants growth.

For REMI, the restriction enzyme *Hin*dIII was added to the transformation mixture just prior to the addition of vector DNA.

Southern analysis of transformants

Genomic DNA was extracted from liquid grown mycelium by the method of Persson et al. [25]. Probe label and hybridization were conducted using Digoxigenin–dUTP Kits (Boehringer

Mannheim) and Nylon membrane (positively charged, Cat. No. 1209299, Roche) according to the instructions.

Mitotic stability analysis

Transformants were isolated and incubated on CMA plates containing 200 μg/ml of hygromycin B. The stability of hygromycin B resistance of transformants was tested according to the method of Tunlid et al. [23].

Selection of morphological mutagenesis transformants

All the obtained transformants were incubated on water agar (WA) medium for the examination of morphological modification and the ability of forming predacious structure. Briefly, a 2 cm^2 piece of agar in the center of the plate was removed from a 7-day-old culture to create an open space. About 200 nematodes (*Panagrellus redivivus*) were added to the free space after the mycelia emerged from the cut margin. The potential of trap formation was determined after incubation for further 3 days at 28 °C. Those transformants which showed morphological deficiency were selected.

Re-isolation of hph gene tagging transformant from dung

A stable transformant, YMF1.00153, which showed similar morphology and pathogenicity as the wild type, was chosen for this experiment. The conidia were harvested from 2 weeks old colonies by gently washing the plates with sterile water. The concentration of conidia was estimated as 1×10^5 using a haemocytometer. Dung sample was collected from compost of a farm close to Songming county of Yunnan, P.R. China. The conidia were thoroughly mixed with dung at the required numbers (0, 50, 500 conidia/g dung). Triplicate samples of each treatment were applied. After deposited at 25 °C for 30 days, the released fungus was re-isolated by sprinkling 2 g of subsample on a petri dish containing 20 ml of selective medium (CMA containing 200 mg hygromycin B, 50 mg streptomycin sulphate and 50 mg ampicillin). After incubation for 7–14 days, the entire surface of the dish was observed under dissecting microscope to count the number of conidiophores bearing conidia. For quantification, one conidiophore was artificially taken as a colony, and the colony-forming unit (CFU) per gram dung was used for fungal quantification.

Results

Transformation

The protoplast yield of *A. oligospora* was 0.3–0.8×10^7/ml and the protoplast regeneration frequency was 32–56% on PDSSA medium. The regeneration frequency reduced to 5–10% when mixed with 10–50% PEG 3350. On CMA and PDSSA media, 150 μg/ml of hygromycin B can completely inhibit the regeneration of protoplasts and the growth of the wild type.

Transformants became visible after incubation for 4–7 days at 28 °C. The transformation frequency mainly was affected by the concentration of restriction enzyme and PEG. When varying the concentration of *Hin*dIII at 0, 10, 20, 30, 40, 50, 60 units per transformation reaction, the frequencies were 7, 25, 62, 175, 22, 18 and 19 transformants/μg of DNA (maintaining the concentration of PEG3350 as 20%). The addition of *Hin*dIII increased the frequency by 25-folds over the control without enzyme (repeated thrice with similar results). The frequencies using PEG 3350 at a concentration of 10%, 20%, 30%, 40% and 50% (the concentration of *Hin*dIII was 30 U) were 42, 175, 23, 6 and 2 transformants/μg DNA (repeated twice). There was no obvious difference on transformation frequency when using PEG3350, PEG4000 or PEG6000. Furthermore, the using of linearized pBChygro DNA increased the transformation frequency by more than 10-folds over treatment with circular DNA (repeated thrice). With the optimum protocol the frequencies varied between 34 and 175 transformants/μg of DNA. Totally, more than 2000 transformants were obtained by transformation of the fungus with pBChygro in the presence of *Hin*dIII.

Mitotic stability analysis and the selection of morphological mutagenesis transformants

A high percentage (93%) of transformants was mitotically stable when examined 100 randomly

selected transformants. They showed a similar growth rate and morphology with their wild type on non-selective medium and retained their stable resistance level to hygromycin B.

One mutant, YMF1.00110, which did not form predacious structure even if induced by nematodes, was isolated (repeated thrice with similar results). This isolate grew slowly compared to its wild type. On S medium (tryptone 10 g, glucose 10 g, agar 18 g, water 1000 ml) the mutant attains a colony of 0.7 cm diameter after incubation for 7 days at 28 °C, and that of the wild type was 9 cm.

Southern analysis

Twelve randomly selected transformants (lanes 1–12 in Figure 1a) and the transformant YMF1.00153 (lane 13 in Figure 1a) which was used in quantification test were analyzed by Southern blots to confirm integration of the vector DNA into the genome. Undigested genomic DNA (10–15 μg was loaded in each lane) of 12 transformants hybridized to the probe labelled by Digoxigenin–dUTP and provided a signal in a high molecular mass zone, which indicated that the plasmid had integrated into the fungal genome (Figure. 1a). One transformant not showed the hybridization band, which suggested it not be labelled (lane 4 in Figure. 1a) or the plasmid DNA not integrated into its genome.

The undigested genomic DNA of YMF1.00110 (which lost its ability to form predacious structure) showed one band of 14.3 kb (3–4 lanes in Figure. 1b) hybridised to the probe. Two bands of 10, and 6.8 kb (1–2 lanes in Figure. 1b) are generated when the genomic DNA digested with HindIII (the enzyme used in REMI), which suggests an integration of at least two copies pBChygro and the integration pattern should be a little complex.

Re-isolation of hph gene tagging transformant from dung

On the selective medium, the *hph* gene tagging transformant, YMF1.00153, grew normally. All of the bacteria, actinomycetes and most of fungi could not grow on the medium except a few species of *Verticillium, Penicillium* and *Paecilomyces*. The released fungus began forming conidiophores and conidia after incubation for 7 days and the number of conidiophores did not increase after 14 days. The average CFUs of the tagging isolate at the dung samples (0, 50, 500 conidia/g dung) were 0, 17.67 and 21.33, respectively.

Discussion

Restriction enzymes have been used to facilitate integration of transforming DNA. The mechanism of REMI elevating transformation frequency has been suggested that the enzymes cut genomic DNA to provide sites for the integration of transforming DNA. In the transformation of fungi, it has been proved that using REMI can increase the transformation frequency notably [22]. Michael et al. [26] found that *Bam*HI and *Not*I increased the number of transformants by 2–5 and 29–46 folds over the control without enzyme. Tunlid et al. [23] first transformed the nematode-trapping fungus *A. oligospora* with 5 plasmids including the pBChygro which we used in this study and obtained a frequency of 1–6 transformants/μg of DNA. Based on this work, we increased frequency to 34–175 transformants/μg of DNA by optimizing the factors of protoplast/ DNA ratio, the concentration of PEG, the quantity of restriction enzyme added. Tunlid et al. [23] increased the frequency from 2 to 5 transformants/
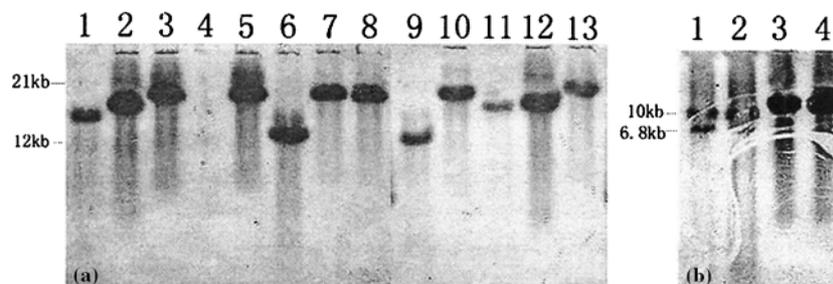

Figure 1. Southern blot analysis of transformants. (a) Tagged efficiency analysis of 13 transformants; (b) integration events of transformants YMF1.00110.

µg of DNA when transforming *A. oligospora* in the presence of the enzyme *Hin*dIII. In our study, the enzyme *Hin*dIII also obviously increased the number of transformants.

REMI provide a method to disrupt genes randomly by plasmid insertion and the subsequent cloning of these genes by plasmid rescue in *E. coli* [27]. The predacious structures (trap) of nematode-trapping fungi have two characteristics, which distinguish trap cells from normal vegetative hyphal cells: a fibrillar extracellular adhesive which is used to capture the nematodes, and dense bodies which are involved in the penetration and digestion of the nematode [28]. Though the morphological development of traps [29, 30] and the causes regulating the rapid-inflation process of rings [31, 32] have been demonstrated, up to now, a gene coding or regulating the trap structures has never been cloned. But then, these molecular studies would be carried out by rescuing genes from the trap-lacking mutant generated by REMI.

While studies on pathogenic nematode biocontrol have been ongoing for decades, there were few effective commercial formulations available to field use. The causes that lack of enough knowledge on the ecology of agent in field, including germination, proliferation, control efficiency and population variation, should be included [33]. Low temperature scanning electron microscopy technique [34] and fluorescence microscopy observation technique [35, 36] have been developed to visualize these fungi and demonstrate their activity in natural habitat. However, knowledge of the role of biocontrol fungi in nature is incomplete due to the lack of suitable techniques for their quantitative estimation though some indirect techniques have been developed [14, 37, 38]. This is the first work to quantify the tagging strain of nematode-trapping fungi from a substrate based on the drug resistance. Of course, in this quantification method, the rationality of taking a conidiophore as a colony needs to be re-considered.

Acknowledgements

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