Reports

Isolation of fungal homokaryotic lines from heterokaryotic transformants by sonic disruption of mycelia

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Fungal hyphae—and in some cases, spores—are multi-nucleate. During genetic transformation of these spores or mycelia, only one nucleus generally receives the transferred T-DNA generating heterokaryotic colonies. Characterization of genetic changes, such as the effects of gene disruption in the transformants, requires purified homokaryotic lines. Hyphal tip transfer has conventionally been used to isolate homokaryons. We developed an alternative method for purification of fungal homokaryons from transformed heterokaryotic lines of Sclerotinia sclerotiorum. Ultrasound pulses were used to generate bi-septate, unicellular hyphal fragments that regenerate under selection to produce homokaryotic lines that can be easily identified using colony PCR. This technique facilitates the purification of transformed lines, which allows for routine genome manipulation, and should be adaptable for other filamentous fungi.

Introduction

Functional characterization of genes requires the development of mutant lines that disrupt or precisely alter the function of the resultant protein. In this context, simple and reliable genetic transformation systems are required. Traditionally, fungal spheroplasts or protoplasts were transformed by electroporation (1,2) or other means such as polyethylene glycol (3). However, protoplasts can be difficult to produce in some species and the activity of cell wall–degrading enzyme varies between lots and thereby requires ongoing optimization (4,5). As such, methods for the direct transformation of intact fungal mycelia or spores, such as biolistic transformation (6,7), are more desirable. de Groot and colleagues (8) reported the first use of Agrobacterium tumefaciens, historically used for plant transformation, as a vehicle for the introduction of DNA into fungi. This method is 100–1000 times more efficient than conventional transformation approaches and has been used with many filamentous fungal species (9,10).

Transformation of filamentous fungi often results in the uptake of the introduced DNA by only one of the nuclei, which leads to the formation of heterokaryons—cells in which two or more types of nuclei coexist. In addition, the plasmid DNA can integrate into the chromosome by either homologous or ectopic recombination (11). To perform reliable genetic analyses, homokaryotic clones must be isolated. In Neurospora crassa, this was achieved through multiple rounds of single spore isolation (12). The hypha of ascomycetes usually contains a single nucleus near the tip (13,14) and therefore hyphal tip transfer can be used for purification of genetic clones (15). Three to five rounds of hyphal tip transfer are usually required to isolate pure homokaryons and only trained individuals with excellent motor skills can perform this technique.

Sclerotinia sclerotiorum is an important plant pathogen that can infect over 400 different hosts, including important crops (16). Protocols for transformation of ascospores and mycelia using A. tumefaciens have been established (17,18); however, these tissues are multi-nucleate and methods to isolate homokaryons are lacking. The carpogenic production of homokaryotic ascospores requires 4–40 weeks based on the isolate (19). Here we provide an alternative technique for homokaryon isolation that uses ultrasound pulses to generate bi-septate, unicellular structures. Clones derived from mycelial fragments having a single nucleus are then identified using colony polymerase chain reaction (PCR).

Materials and methods

Fungal transformation and detection of homo- and heterokaryons
Sclerotinia sclerotiorum (Lib.) de Bary strain 1980 (No. 18683, ATCC, Manassas, VA, USA) was propagated in minimal salt–glucose (1% w/v) (MS-glu) broth as per Li et al. (20). Mycelia were transformed using A. tumefaciens strain AGL1 harboring the pCB301 binary vector (21) containing a MAP kinase gene (Smk3, SS1G_05445.1; www.BioTechniques.com) and hygromycin resistance cassette (hph). The transformation was carried out as per Liberti et al. (18). Briefly, mycelia grown in MS-glu media for 4 days in the dark were blended in a Waring blender (Model no. 33BL73; Torrington, CT, USA) for 10 s, pelleted by centrifugation and resuspended in 2 mL induction medium (IM) per 0.5 g mycelial wet weight. A. tumefaciens AGL-1 containing the transformation construct pCB301-Smk3 was grown in minimal media for 1 day at 27°C with aeration. This culture was used

to inoculate IM containing kanamycin (50 μM) and acetylosyringone (200 μM) and grown to an OD₆₀₀ of 0.3. Blended mycelia was diluted 1/100 in IM and mixed with an equal volume of A. tumefaciens, spread onto a cellophane membrane overlaid onto co-cultivation medium and incubated in the dark at 20°C for 3 days. Filters were transferred to MS-glu agar medium containing hygromycin (100 μg/mL), cefotaxime (200 μM), and moxalactam (100 μM) and incubated at 20°C for 3 days. Fungal colonies that were resistant to hygromycin after three rounds of subculturing were tested by PCR. Plugs containing colonies were transferred to minimal salt–glucose–hygromycin (MS-glu-hyg; 100 μg/mL) liquid medium and incubated as above for 3 days. Nucleic acids were extracted by transferring a 500 μL wet volume of mycelia to a 1.5 mL microcentrifuge tube and centrifuging at 13000 rpm for 2 min. The supernatant was removed and the tissue ground for 20 s using a pestle. Subsequently, 400 μL extraction buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the solution mixed with a vortex for 5 s and then centrifuged at 13000 rpm for 1 min. Three hundred microliters of the supernatant were transferred to a tube containing 300 μL isopropanol. The DNA precipitated at room temperature for 2 min followed by centrifugation for 5 min and 13000 rpm. The pellet was washed once with 75% ethanol, air-dried for 5 min, and dissolved in 50 μL ddH₂O. PCR was used to test for (i) homologous integration of the Smk3 gene into the Smk3 gene, and (ii) whether the resultant colony contained homo- or heterokaryotic mycelia. Homologous integration was examined using two sets of primers complementary to the hpb cassette and either the 5′ or 3′ untranslated region (UTR) of the Smk3 gene, both of which were outside of the region used to construct pCB301-Smk3. The first combination consisted of Pmnd Seq R (5′-GAGCCAAATATCGGAAACCCCGAGAA-3′) and Smk3 5′ (5′-CCCTCCAACCTCAACACCCTCAACACCACTCAAC-3′), while the second consisted of Pmnd Seq F (5′-GCAACGGACGTTGCTGAGGACGACGAGTCAGTAGGGTGACGTTA-3′). The presence of non-recombinant nuclei containing the wild-type Smk3 gene was evaluated using primers complementary to the 5′ UTR and 3′ UTR, again outside of the region used to construct pCB301-Smk3. The product amplified from the 5′ flanking region was sequenced to confirm that the construct had integrated properly. Amplification of a region of the Smk3 gene (encoding the ncs and ethylene-inducing peptide 1) (SSIG_03080.1; www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/) using the Smk3 F (5′-GAGCCAATATGCGACTGGATGTATGGTCAGTAGGGTGACGTTA-3′) and Smk3 3′ (5′-TCGGGAATCTTGTATCATACGCTGAGGACGACGAGTCAGTAGGGTGACGTTA-3′) primers served as control for the PCR. The PCR reaction mixture contained 2.5 μL 10× buffer, 2 μL dNTPs (5 mM each), 2 μL forward primer (2.5 μM), 2 μL reverse primer (2.5 μM), 0.5 μL Taq polymerase I, 2 μL containing 500 ng genomic DNA, and 14 μL ddH₂O. S. sclerotiorum wild-type DNA was used as a PCR control. The PCR conditions used were as follows: initial denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 7 min.

Isolation and analysis of homokaryotic lines
Five 3-mm plugs from the growing margin of colonies resistant to hygromycin after three rounds of subculturing were transferred to 25 mL of MS-glu-hyg medium in a 125 mL Erlenmeyer flask and incubated at 20°C for 24 h with shaking at 80 rpm. One hundred milligrams of mycelia (wet weight) were transferred to 900 μL MS-glu medium in a 1.5 mL microcentrifuge tube and subjected to a range of sonication periods to determine the optimal time for fragmentation. Ultrasound pulses did not exceed 10 s, and samples that were sonicated for longer than 10 s in total were allowed to cool for 10 s between pulses. A Misonix S-4000 sonicator (Qsonica, Newtown, CT, USA) with a frequency of 20 kHz set at 25 W was used. The degree of mycelial fragmentation was assessed by light microscopy. The concentration of intact bi-septate fragments (those bounded by single septae at their termini) was determined using a hemocytometer. Samples with the highest number of bi-septate fragments and the lowest number of multicellular hyphae were plated onto MS-glu-hyg selection medium and incubated at 20°C for 5 days. Mycelia and mycelial fragments were also stained with Calcofluor White (Invitrogen, Carlsbad, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions and viewed using a fluorescence microscope (Zeiss Imager Model Z1; Toronto, ON, Canada) to visualize septae and nuclei, respectively.

Transformants were also examined to determine whether they were able to produce transcript from the disrupted Smk3 gene (Smk3). For reverse transcription PCR (RT-PCR) analysis, mRNA was converted to cDNA using the Superscript First Strand Synthesis System (Invitrogen) followed by PCR using the Smk3 F (5′-TTATGATATGGTGATAGGGTG-3′) and Smk3 υ (5′-GCCTCTGTGGTGCTGAGG-3′) primers and Smk3 υ (5′-GGCTCGAGTTAGATTTGTGCCTCTG-3′) primers served as control for the PCR. The PCR reaction mixture contained 2.5 μL 10× buffer, 2 μL dNTPs (5 mM each), 2 μL forward primer (2.5 μM), 2 μL reverse primer (2.5 μM), 0.5 μL Taq polymerase I, 2 μL containing 50 ng genomic DNA, and 14 μL ddH₂O. S. sclerotiorum wild-type DNA was used as a PCR control. The PCR conditions used were as follows: initial denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 7 min.

Results and discussion
In order to provide a proof-of-principle that homokaryotic strains can be generated using this technique, the Smk3 gene was chosen for the generation of a gene replacement strain. Smk3 is homologous to the Botrytis cinereavbp5 gene, which encodes a MAP

![Figure 1. Transformation of S. sclerotiorum using A. tumefaciens.](image-url)
kinase involved in cell wall formation and response to contact with solid surfaces (22) and is therefore important for pathogenicity. *S. sclerotiorum* mycelial fragments were transformed using the *A. tumefaciens* delivery method with a construct designed to disrupt the Smk3 open reading frame. Five transformed colonies were tested, all of which were found to possess a mixture of nuclei containing the Smk3v cassette and the wild-type Smk3 allele (Figure 1). In all of the transformants, the Smk3v cassette had integrated through homologous recombination. Amplification of the single-copy gene SsNep1 was used as an internal control for gene copy number. A similar amount of SsNep1 product was amplified from each of the samples tested. This demonstrated that the ratio of Smk3v to wild-type Smk3 product could be used to estimate the relative proportion of each type of nucleus in the line tested. Those lines with the least amount of wild-type nuclei (i.e., the highest ratio of Smk3v to Smk3 product) were selected for development of homokaryotic lines.

Previous reports described that sonication depending on the dose applied could be used to separate compartmentalized units of bacterial cells (23) or break up mycelial aggregates to allow extraction of cell components (24). In this study, mycelia from individual colonies were fragmented by sonication to generate bi-septate fragments. A range of sonication times were applied to select the optimal sonication conditions (Figure 2). For *S. sclerotiorum*, two 10-s sonication pulses were sufficient to produce the maximal number of intact bi-septate fragments. Post-sonication quality assurance using the microscope was necessary since inadequate sonication periods are counterproductive (they can lead to multicellular structures that would develop heterokaryotic colonies). Sonication for longer periods led to complete destruction of the mycelia (Figure 2) and colonies could not be regenerated. While ultrasound intensities twice that used here led to chromosome non-disjunction in diploid *Aspergillus nidulans* nuclei, it was not mutagenic (25), and therefore this procedure is unlikely to introduce background point mutations.

Hyphal tip isolation has been traditionally used for the purification of homokaryons. Staining with Calcofluor White and DAPI revealed that the bi-septate fragments and hyphal tips were multi-nucleate. In the synchronous model for mitosis, a wave of mitosis progresses from the nucleus closest to the tip so that sister nuclei located nearest the tip are of the same genotype (26). Branch formation and mitosis are also closely coordinated, and new branches acquire one or a few seed nuclei that subsequently undergo mitosis. In either case, this increases the likelihood that new hyphal tips or branches will possess nuclei of the same genotype. The karyotype of colonies regenerated from the mycelial fragments on selective medium was established using PCR to amplify the two Smk3 alleles. Of the colonies examined, all contained mycelia having nuclei with only the Smk3v allele and were presumably homokaryotic (Figure 3). This indicates that the multiple nuclei observed in the bi-septate fragments and hyphal tips were derived from the same progenitor nucleus and that the original heterokaryotic colonies contained a mixture of homokaryotic mycelia containing nuclei with either the wild-type or Smk3v allele. Sequencing the region flanking the disruption cassette confirmed that it had faithfully integrated into the Smk3 gene via homologous recombination. The absence of Smk3 gene expression as demonstrated using RT-PCR provided confirmation of the homokaryotic state. In addition, the Smk3v lines exhibited growth phenotypes expected to be associated with disruption of this gene including lack of sclerotia formation and reduced virulence which was not the case for the heterokaryotic lines (data not shown).

In summary, sonic disruption of mycelia for homokaryon isolation is a versatile method that requires only limited skill and can generate unicellular structures far quicker than traditional hyphal tip transfer. Typically, we found that homokaryotic clones could be isolated from heterokaryotic transformants within 4 days. While this method has only been tested with *S. sclerotiorum*, it is reasonable to assume that it could be easily adapted to other filamentous fungi.

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Competing interests
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