Chapter 5

Molecular Tools for Carotenogenesis Analysis in the Zygomycete *Mucor circinelloides*

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Abstract

The carotene producer fungus *Mucor circinelloides* is the zygomycete more amenable to genetic manipulations by using molecular tools. Since the initial development of an effective procedure of genetic transformation, more than two decades ago, the availability of new molecular approaches such as gene replacement techniques and gene expression inactivation by RNA silencing, in addition to the sequencing of its genome, has made *Mucor* a valuable organism for the study of a number of processes. Here we describe in detail the main techniques and methods currently used to manipulate *M. circinelloides*, including transformation, gene replacement, gene silencing, RNAi, and immunoprecipitation.

Key words: Mucor, Transformation, Gene replacement, Gene silencing, RNAi, Immunoprecipitation, Ubiquitylation

1. Introduction

Zygomycetes are a basal class of filamentous fungi that are receiving a growing attention due to their evolutionary distance to other fungi. In recent years, several molecular processes have been dissected, such as mechanisms involved in regulation of light responses (1, 2), determination and evolutionary implications of genes involved in sex determination (3, 4), methylation of their genomes (5), the mechanism of gene silencing (6–9), and the implication of endogenous small RNAs in gene regulation (10). They are also the main source of fungal carotenoids in industry, and could be considered as the most worthwhile alternative to applying molecular approaches to obtain carotene-overproducing strains of industrial interest.

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The main advantages of using filamentous fungi over yeast or bacteria are the high levels of produced metabolite and final biomass. Structural genes involved in the β-carotene biosynthesis in the zygomycetes *Blakeslea trispora* (11), *Phycomyces blakesleeanus* (12, 13), and *Mucor circinelloides* (14, 15) have been cloned and characterized. Among these, *M. circinelloides* has proved to be a good organism for a molecular approach to the study of carotenogenesis regulation. Several molecular tools, including genetic transformation (16, 17), gene replacement (18), and RNA-mediated gene silencing (19), are available for this fungus, allowing the manipulation of its genome. Furthermore, its genome sequence is already available (http://genome.jgi-psf.org/Mucci2/Mucci2.home.htmL). The availability of these molecular tools has opened new ways to study carotene biosynthesis regulation in *M. circinelloides* and is also contributing to the design of strategies for the study of many different processes by a growing number of research groups. Here we describe the procedures to obtain protoplasts and to transform *Mucor*, including a gene replacement method and the analysis of transformants, the construction of a specific vector to silence genes, procedures to isolate low molecular weight RNAs and, finally, the methods currently used to prepare cell lysate and to analyze specific proteins by immunoprecipitation.

2. Materials

2.1. Transformation, Gene Replacement, and Transformant Analysis

1. *M. circinelloides* CBS277.49 (CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands).
2. *M. circinelloides* R7B (20) (see Note 1).
3. SPB (0.1 M sodium phosphate buffer pH 6.5): 1.42 g of Na_2HPO_4 in a final volume of 100 mL double-distilled water (solution 1); 1.38 g of NaH_2PO_4 monohydrate in a final volume of 100 mL double-distilled water (solution 2). Take 100 mL of solution 2 and add solution 1 slowly until pH 6.5 is reached (about 53 mL).
4. PS buffer: Mix 18.22 g of sorbitol and 20 mL of SPB. Make up to 200 mL with double-distilled water (final sorbitol concentration: 0.5 M).
5. Lysing enzymes (Sigma-Aldrich, St. Louis, MO, USA).
6. Chitosanase RD (US Biological, Swampscott, MA, USA).
7. 0.5 M Sorbitol: Dissolve 22.77 g of sorbitol in 200 mL double-distilled water. Make up to 250 mL with double-distilled water.
8. Gene Pulser Cuvette (0.2-cm electrode gap) (Bio-Rad, Hercules, CA, USA).
9. Bio-Rad Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA).

10. YPG: 3 g/L of yeast extract, 10 g/L of peptone, and 20 g/L of glucose (see Note 2).
11. YPG-agar: YPG and 20 g/L agar (see Note 2).
12. YPGS: Add 91.1 g of sorbitol to the YPG medium before to adjust the volume to 1,000 mL with distilled water (final sorbitol concentration: 0.5 M).
13. YNB: 1.5 g/L of ammonium sulfate, 1.5 g/L of glutamic acid, 0.5 g/L of yeast nitrogen base (w/o ammonium sulfate and amino acids), and 10 g/L of glucose. After autoclaving add thiamine and niacine at a final concentration of 1 μg/mL (see Note 2).
14. YNB-agar: YNB and 20 g/L agar (see Note 2).
15. YNBS: Add 91.1 g/L of sorbitol to the YNB medium before to adjust the volume to 1,000 mL with distilled water (final sorbitol concentration: 0.5 M).
16. MMC: 10 g/L of casaminoacids, 0.5 g/L of yeast nitrogen base (w/o ammonium sulfate and amino acids), and 20 g/L of glucose. After autoclaving add thiamine and niacine at a final concentration of 1 μg/mL (see Note 2).
17. MMC-agar: MMC and 15 g/L agar (see Note 2).
18. MMCS: Add 91.1 g/L of sorbitol to the MMC medium before to adjust the volume to 1,000 mL with distilled water (final sorbitol concentration: 0.5 M).
19. DMSO (dimethyl sulfoxide).
20. Biotaq DNA Polymerase (Bioline Ltd, London, UK).
21. Mixture for colony PCR: 0.5 μL of Biotaq enzyme (5 U/μL), 2.5 μL of 10× Biotaq specific PCR buffer, 2.5 μL of 2 mM dNTP's, 1 μL of 50 mM $MgCl_2$, 1 μL of a 10 pmol/μL solution of each primer, and 15.25 μL of double-distilled water (see Note 3).

2.2. Gene Function Analysis by RNA Silencing

1. pr5: 5′-CCGCGGTCGACGGATCCTACGATGCGCCTG CTG-3′. Restriction site for *Bam*HI is underlined.
2. pr3a: 5′-CGGGGGTCGACTGAACGCGGAATACTTCAGG-3′. Restriction site for *Sal*I is underlined.
3. pr3b: 5′-GCGGGCTCGAGTTGCACCCACAAAGAATAG-3′. Restriction site for *Xho*I is underlined.
4. pBlueScript SK^+ (Stratagene, La Jolla, CA, USA).
5. pEUKA4 (21).
6. Trizol (Invitrogen, Carlsbad, CA, USA).
7. Diethyl pyrocarbonate (DEPC)-treated water: Add 1 mL of DEPC to 1,000 mL of double-distilled water (0.1% DEPC v/v) and stir at room temperature for at least 3 h to bring the DEPC into the solution. Let the solution incubate for 12 h at 37°C and autoclave for 15 min to remove any trace of DEPC (see Note 4).

8. 4 M NaCl: 234 g of NaCl and double-distilled water to 1,000 mL.
9. PEG 8000: Poly(ethylene glycol) 8000 (Sigma-Aldrich, St Louis, MO, USA).
10. 50% PEG 8000 (w/v): 50 g of PEG 8000; bring the final volume to 100 mL with double-distilled water and autoclave.
11. 0.5 M EDTA pH 8.0: Add 186.1 g of disodium EDTA·2H_2O to 800 mL of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.
12. 1× TAE: Make a concentrated (50×) stock solution of TAE by weighing out 242 g of Trizma base and dissolving in approximately 750 mL distilled water. Carefully add 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0), and adjust the solution to a final volume of 1 L. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5. The working solution of 1× TAE buffer is made by diluting the stock solution by 50× in double-distilled water (final concentrations: 40 mM Tris acetate and 1 mM EDTA).
13. 1.5% Agarose gel: Add 1.5 g of RNase-free agarose to 100 mL 1× TAE. Heat the solution to boiling in the microwave to dissolve the agarose.
14. 3 M Sodium acetate pH 5: 24.61 g of sodium acetate (anhydrous) and 80 mL double-distilled water. Adjust to pH 5.0 with glacial acetic acid. Adjust volume to 100 mL with double-distilled water and autoclave.
15. 2× Formamide loading buffer: To prepare 20 mL, mix 19 mL of formamide (final concentration: 95% v/v), 720 μL of 0.5 M EDTA pH 8.0 (final concentration: 18 mM), 50 μL of 10% SDS (final concentration: 0.025% w/v), 5 mg of xylene cyanol (0.025% w/v), 5 mg of bromophenol blue (0.025% w/v), and 230 μL double-distilled water. Store at –20°C.
16. 10× TBE: 108 g of Trizma base, 55 g of boric acid, 9.5 g of EDTA, and double-distilled water to 1,000 mL. Adjust pH to 8.35.
17. 0.5× TBE: To prepare 1,000 mL, add 950 mL double-distilled water to 50 mL of 10× TBE.
18. AccuGel™ 19:1–40%: A 19:1 acrylamide to bisacrylamide stabilized solution (National Diagnostics, Atlanta, GA, USA).
19. 0.5× TBE/7 M urea/15% acrylamide gel: Mix in a 50-mL Falcon tube 16.8 g of Urea, 10 mL DEPC-treated water, 2 mL of 10× TBE, and 15.53 mL of AccuGel™ 19:1–40%. Stir at

room temperature to dissolve urea and add DEPC-treated double-distilled water to 40 mL. Add (in this order) 320 μL of 10% (w/v) ammonium persulphate (APS) and 42.6 μL TEMED. Mix well and pour immediately, using a pipette, into the assembled glass plate sandwich, following indications of the Protean II xi Cell Instruction Manual. Keep at 4°C. Polymerization time is about 30 min. After that, remove the comb and fill the wells with 0.5× TBE. With a syringe, remove the bubbles inside the wells. Then, the gel is ready to be assembled in the electrophoresis chamber.

20. Protean® II xi Gel System (Bio-Rad, Hercules, CA, USA).
21. 3 MM Whatman paper (Whatman Ltd, Maidstone, Kent, UK).
22. Hybond™-N+ (GE Healthcare, Waukesha, WI, USA).
23. Semidry electroblotter unit (Sigma-Aldrich, St Louis, MO, USA).
24. UV-Crosslinker (Hoefer, Inc., Holliston, MA, USA).
25. MAXIscript® T7 (Applied Biosystems/Ambion, Austin, TX, USA).
26. TE: 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA.
27. Sephadex G-50 (GE Healthcare, Waukesha, WI, USA).
28. Alkaline buffer: 0.317 g of Na_2CO_3 and 0.168 g of $NaHCO_3$, and double-distilled water to 25 mL (final concentrations: 120 mM Na_2CO_3 and 80 mM $NaHCO_3$). Autoclave, make aliquots, and store at –20°C.
29. Prehybridization/hybridization buffer: To prepare 20 mL of buffer, dissolve 1.4 g of SDS in 9.1 mL double-distilled water, by incubating at 65°C. Once dissolved, add 1.5 mL of 4 M NaCl, 1 mL of 1 M Na_2HPO_4–NaH_2PO_4, pH 7.1, 400 μL of 50× Denhardt's solution, and 8 mL of formamide (final concentrations: 7% SDS, 0.3 M NaCl, 0.05 M Na_2HPO_4–NaH_2PO_4, pH 7, 1× Denhardt's solution).
30. 50× Denhardt's solution: 1% (w/v) bovine serum albumin (BSA), 1% (w/v) of Ficoll 400, 1% (w/v) of polyvinylpyrrollidone (PVP) in double-distilled water.
31. 20× SSC solution: 175.32 g of NaCl and 88.23 g of trisodium citrate; adjust to pH 7 and bring to a final volume of 1,000 mL with double-distilled water.
32. 10% SDS solution: 20 g of SDS in a final volume of 200 mL double-distilled water. Resuspend at 50°C (do not autoclave).
33. SDS/SSC solution: Mix 100 mL of 20× SSC, 20 mL of 10% SDS, and 880 mL of double-distilled water (see Note 5).
34. Salmon sperm DNA (deoxyribonucleic acid sodium salt from salmon testes) (Sigma-Aldrich, St Louis, MO, USA).
35. Salmon sperm DNA 5 mg/mL: Weigh 100 mg of salmon sperm DNA and add DEPC-treated double-distilled water to

20 mL (final concentration: 5 mg/mL). If necessary, stir the solution on a magnetic stirrer for 2–4 h at room temperature to help the DNA to dissolve. Autoclave and dispense into aliquots in microfuge tubes. Store at −20°C.

36. Kodak BioMax™ MS film (Sigma-Aldrich, St Louis, MO, USA).
37. 1 M Tris–HCl pH 7.5 (100 mL): Mix 12.1 g of Trizma base with 80 mL ddH_2O. Adjust pH with HCl (pH is temperature dependent; make sure it is at room temperature before making final pH adjustments). Add dH_2O to 100 mL and autoclave.
38. RNase buffer: 400 μL 1 M Tris–HCl pH 7.5, 200 μL 0.5 M EDTA, 300 μL 4 M NaCl, and double-distilled water to 20 mL.
39. RNase A (Sigma-Aldrich, St Louis, MO, USA).

2.3. Cell Lysate Preparation, Immunoprecipitation, and Ubiquitilation Analysis

1. Semidry electroblotter unit (Sigma-Aldrich, St Louis, MO, USA).
2. Mini-Protean 3 Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
3. Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO, USA).
4. Benzamidine (Sigma-Aldrich, St Louis, MO, USA).
5. Sodium deoxycholate (Sigma-Aldrich, St Louis, MO, USA).
6. Igepal CA-630 (Sigma-Aldrich, St Louis, MO, USA).
7. Lysis buffer (washing buffer 1): 2.5 mL of 1 M Tris–HCl pH 7.5 (121.14 g/L), 1.875 mL of 4 M NaCl (233 g/L), 0.5 mL of Igepal CA-630, and 0.25 g of sodium deoxycholate. Add double-distilled water to 50 mL (final concentrations: 50 mM Tris–HCl, pH 7.5; 150 mM sodium chloride; 1% Igepal CA-630; 0.5% sodium deoxycholate).
8. Protein A-Agarose Fast Flow (Sigma-Aldrich, St Louis, MO, USA).
9. Washing buffer 2: 2.5 mL of 1 M Tris–HCl pH 7.5 (121.14 g/L), 6.25 mL of 4 M NaCl (233 g/L), 0.05 mL of Igepal CA-630, and 0.025 g of sodium deoxycholate. Add double-distilled water to 50 mL (final concentrations: 50 mM Tris–HCl, pH 7.5; 500 mM sodium chloride; 0.1% Igepal CA-630; 0.05% sodium deoxycholate).
10. Washing buffer 3: 2.5 mL of 1 M Tris–HCl pH 7.5 (121.14 g/L), 0.05 mL of Igepal CA-630, and 0.025 g of sodium deoxycholate. Add double-distilled water to 50 mL (final concentrations: 50 mM Tris–HCl, pH 7.5; 0.1% Igepal CA-630; 0.05% sodium deoxycholate).
11. 5× Protein loading buffer: 375 microL 3M Tris–HCl pH 6.8, 2.5 mL of glycerol, 0.25 g of SDS, 0.0025 g of bromophenol blue, and 0.0125 g of dithiothreitol (DTT), add double distilled water to 5 mL (final concentrations: 0.225 M Tris–HCl

pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25% DTT).

12. ProtoGel™ 30%: A 37.5:1 acrylamide to bisacrylamide stabilized solution (National Diagnostics, Atlanta, GA, USA).
13. 7% Resolving gel: 1 mL of 3 M Tris–HCl pH 8.85, 1.04 mL of ProtoGel™ 30%, 1.96 mL of double-distilled water, and 40 μL of 10% SDS. Add (in this order) 32 μL of 25% (w/v) APS and 4 μL of TEMED. Mix well and pour immediately following indications of Subheading 3.
14. Stacking gel: 0.5 mL of 0.5 M Tris–HCl pH 6.8, 0.4 mL of ProtoGel™ 30%, 1.1 mL of double-distilled water, and 20 μL of 10% SDS. Add (in this order) 16 μL of 25% (w/v) APS and 4 μL of TEMED. Mix well and pour immediately following indications of Subheading 3.
15. 10× Running buffer: 60.56 g of Tris, 144.14 g of glycine, and 10 g of SDS. Add distilled water to 1,000 mL and adjust to pH 8.3 (final concentrations: 0.5 M Tris–HCl, 1.92 M glycine, 1% SDS).
16. BenchMark prestained protein ladder (Invitrogen, San Diego, CA, USA).
17. Amersham ECL Western Blotting System (GE Healthcare, Waukesha, WI, USA).
18. Protran nitrocellulose membrane (Whatman Ltd, Maidstone, Kent, UK).
19. Plastic wrap.
20. Western blotting buffer: 5.8 g of Trizma base, 2.9 g of glycine, 200 mL methanol, 0.37 g of SDS, and distilled water to 1,000 mL. Adjust to pH 8.8 (final concentrations: 48 mM Trizma base, 29 mM glycine, 20% methanol, 0.037% SDS).
21. 10× PBS buffer: 81.82 g of NaCl, 2.46 g of KCl, 14.2 g of Na_2HPO_4, and 2.45 g of KH_2PO_4. Add double-distilled water to 1,000 mL (final concentrations: 1.4 M NaCl, 33 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4).
22. PBST buffer: 100 mL of 10× PBS, 900 mL double-distilled water, and 1 mL of Tween 20.
23. Mouse anti-ubiquitin monoclonal IgG_1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).
24. Blocking buffer: Dissolve 0.5 g of nonfat dry milk in 10 mL of PBST buffer (final concentration: 5%).
25. ECL™ Western Blotting System (GE Healthcare, Waukesha, WI, USA).
26. Hyperfilm ECL (GE Healthcare, Waukesha, WI, USA).
27. Multigrade paper developer.
28. Rapid fixer.

3. Methods

3.1. Protoplasts Preparation and Transformation

1. Collect fresh spores (no more than 1 week old) and resuspend them in YPG medium pH 4.5. Adjust the final spore concentration to 10^7 spores/mL.
2. Incubate overnight at 4°C, without shaking.
3. Incubate the spores at 26°C with shaking (300 rpm), until germ tube length becomes about four times the swollen spore diameter. This usually takes 3–4 h.
4. Wash the cells twice by centrifugation in PS buffer pH 6.5 at 340 × *g* for 5 min.
5. Resuspend the pellet in 4 mL of PS buffer. Transfer the germinated spore solution to a 50-mL Erlenmeyer flask.
6. Add 5 mg of lysing enzymes dissolved in 1 mL PS buffer, and 100 μL (0.15 U) of Chitosanase RD (dissolved in PS buffer). Incubate at 30°C with gentle shaking (60 rpm) for about 90 min (see Note 6).
7. Transfer the 5 mL solution to a screw cap centrifuge tube and fill the tube with cold 0.5 M sorbitol. Wash twice by centrifugation in cold 0.5 M sorbitol at 91 × *g* for 5 min.
8. Resuspend the pellet gently in 800 μL of cold 0.5 M sorbitol. This 800 μL solution allows for eight different transformation experiments.
9. Each tube of transformation mixture must contain 100 μL protoplast solution and 10 μL DNA sample (1 μg total DNA for circular plasmid or 3 μg total DNA for lineal fragments; DNA must be dissolved in double-distilled water). Use as a control 10 μL of double-distilled water instead of DNA.
10. Mix and transfer to the electroporation cuvette.
11. Apply an electrical pulse using the following conditions: field strength of 0.8 kV, capacitance of 25 μF, and constant resistance of 400 Ω.
12. Immediately after the pulse, remove the cuvette and add 1 mL cold YPGS 4.5. Keep on ice until all cuvettes have been pulsed.
13. Transfer the liquid of each cuvette to 1.5-mL microcentrifuge tubes.
14. Incubate for 1 h at 26°C and 100 rpm.
15. Centrifuge at 91 × *g* for 5′ and gently resuspend the pellet in a final volume of 400–600 μL YNBS 4.5.
16. Inoculate plates of the adequate medium containing 0.5 M sorbitol with 200 μL solution (see Notes 7 and 8).
17. Incubate in the dark at 26°C for 3–4 days.

3.2. Gene Replacement

To obtain null mutants by gene replacement, it is necessary to design replacement fragments (RF) to disrupt the target gene by homologous recombination. These RF must contain a selectable marker gene flanked by DNA sequences (about 1 kb each) upstream and downstream of the target gene (Fig. 1a). In *M. circinelloides* two main marker genes, *leuA1* and *pyrG*, are normally used. The 5′ and 3′ flanking regions and the marker gene with its promoter sequence can be obtained by PCR amplification, using the appropriated primers, and then connected by an overlapping PCR (see Note 9). The RF thus obtained can be cloned in a vector to obtain enough DNA for transformation, although the recombinant plasmid must be digested to release a linear RF to be used for transformation. It is recommended to apply the following rapid PCR amplification procedure (colony PCR) to detect if gene replacement has been successful (Fig. 1b).

1. After one or two rounds of spore recycling of transformants, pick up a small amount (about 1 cm^2) of transformant mycelium (without agar) with a tweezer, freeze on liquid nitrogen and resuspend in 50 μL DMSO (see Note 10).
2. Set up amplification reactions with 1.25 μL of the DMSO solution and PCR mixture for a final volume of 25 μL. Depending on primer length and sequence, the annealing temperature could vary between 53 and 55°C. A standard program is 30 cycles, each one consisting of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min/kb. Finally, analyze the PCR results by gel electrophoresis.
3. Continue the spore recycling only with those transformants showing a successful gene replacement.

3.3. Silencing Vector Construction

RNA silencing, or RNA interference (RNAi), is being increasingly considered as a worthwhile alternative for manipulating fungal gene expression, since it does not imply sequence DNA modification and, probably most important, it allows to study genes whose lack of expression could be lethal for the organism. To silence a gene, it is necessary designing the adequate "silencing vector", and also demonstrate that silencing is being produced. Below are explained the methods used in *M. circinelloides* to construct a "silencing vector" and to detect the small RNA molecules (siRNAs) that are the hallmarks of RNA silencing, which in the case of *M. circinelloides* are 21- and 25-nt long (Fig. 2).

The "silencing vector" contains two main features. One of them is a selectable marker gene in *Mucor*, for instance the gene *leuA1*, which complements the *leu*$^-$ auxotrophy of R7B strain and its derivatives (see Note 8). The other one is a construction (transgene) containing inverted repeated sequences of the gene to be silenced cloned under a strong promoter, as the *gpd1* promoter. The plasmid pEUKA4 (21) contains the *leuA1* gene and the *gpd1*

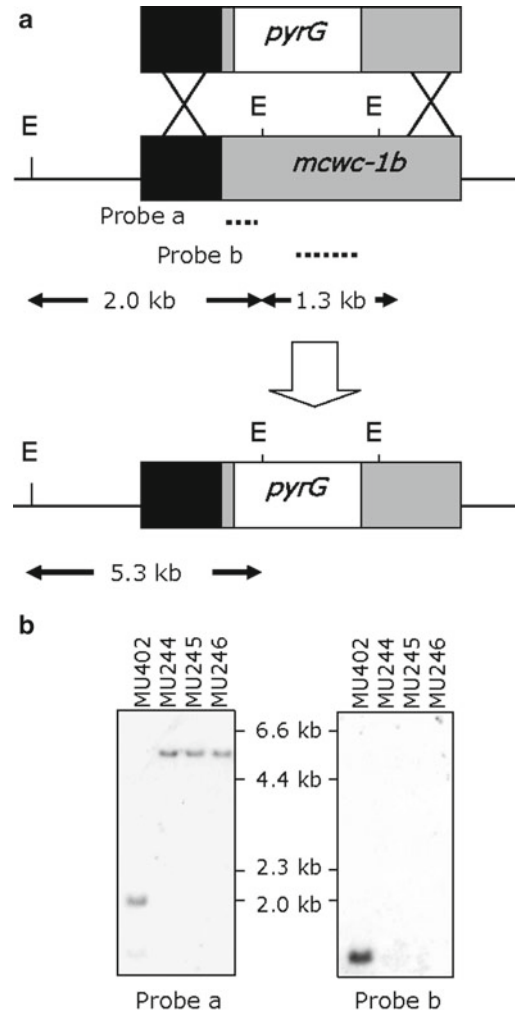

Fig. 1. (**a**) Genomic structure of *mcwc-1b* wild-type locus and after homologous recombination of the replacement fragment. Positions of the probes used and expected sizes of *Eco*RI fragments detected by the probes are indicated. E, *Eco*RI. (**b**) Genomic DNA (0.5 μg) from recipient strain in transformation (MU402) and *mcwc-1b* knockout mutants (MU244, MU245, and MU246) was digested with *Eco*RI and hybridized with probe *a* (0.7 kb *Bam*HI fragment of the *mcwc-1b* gene), and subsequently with probe *b* (1 kb *Eco*RV–*Apa*I fragment of the *mcwc-1b* gene (2)). The positions and sizes of the fragments of the DNA molecular weight marker (λ *Hind*III) are indicated in the middle of both Southern blots.

promoter. Transgene sequences must be displayed as an inverted repeat (see Note 11). Figure 2 outlines an example of "silencing vector" construction. In this case, the transgene contains sequences of the *M. circinelloides carB* gene (accession number AJ238028), a gene involved in the biosynthesis of β-carotene. Thus, silencing of this gene produces albino transformants, due to the lack of *carB* (phytoene dehydrogenase) gene activity.

The construction of the silencing vector is as follows:

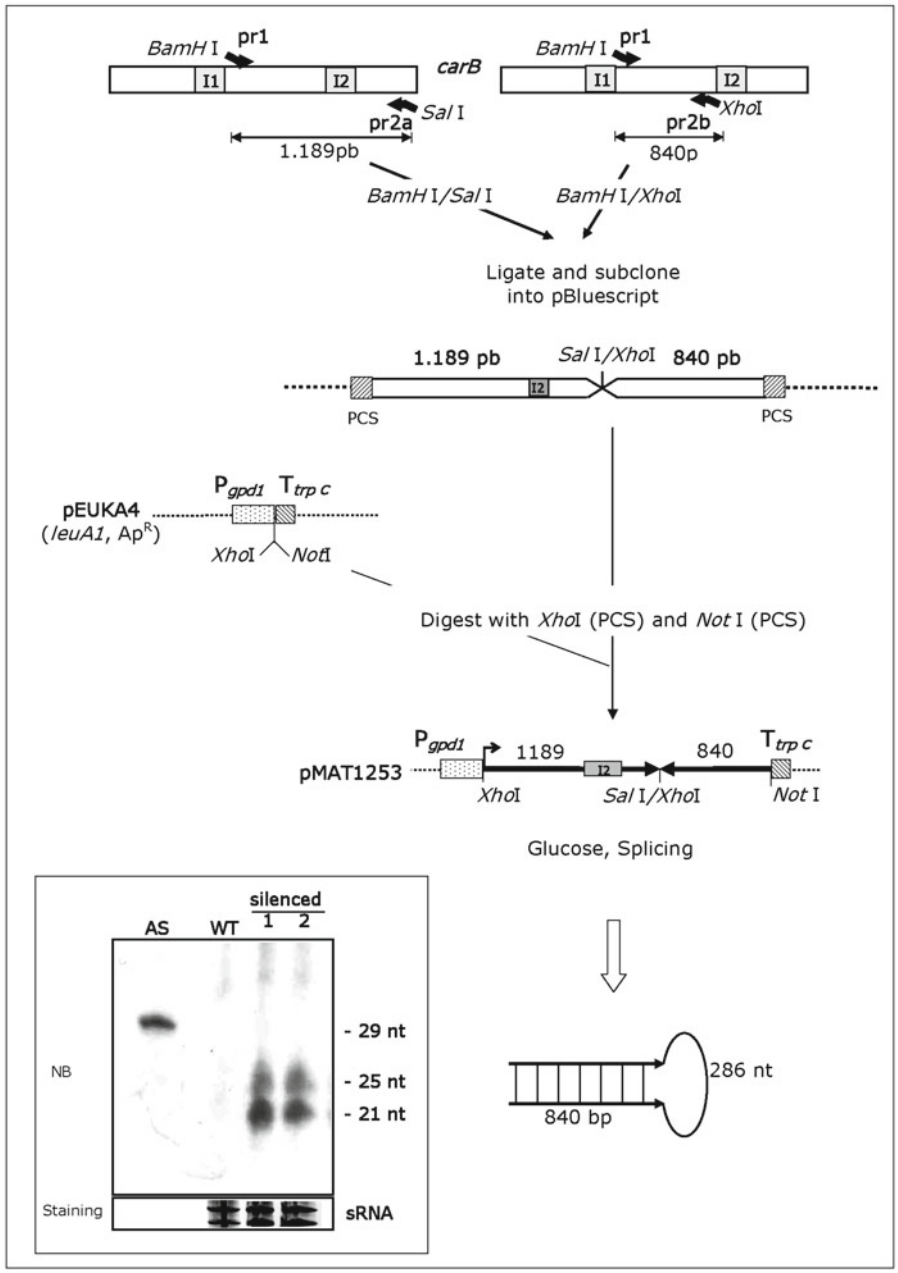

Fig. 2. Schematic representation of the silencing plasmid pMAT1253. This plasmid contains the inverted-repeat transgene for silencing the *carB* gene. *pr1*, *pr2a,* and *pr2b* are the primer used for PCR amplification of *carB* fragments included in the inverted repeat. Promoter (P), terminator (T), and introns (I1 and I2) are represented as *boxes*. The stem-and-loop region size of the hpRNA obtained upon transcription is shown. Only relevant restriction sites are indicated. Inset: Northern blot analysis of low molecular weight RNAs (50 μg) isolated from cultures of the wild-type strain (WT) and from two silenced transformants (1 and 2) carrying the hpRNA-expressing plasmid pMAT1253, grown for 24 h in liquid medium. Ten picomoles of 29-mer DNA oligonucleotide in antisense orientation (AS) was used as a size marker and to control the hybridization specificity. The RNA blot (NB) was hybridized with a hydrolyzed *carB* antisense-specific riboprobe, which corresponded to a 1,662-bp DNA fragment of the *carB* sequence that extends from position +863 to the end of the *carB* gene (6). The predominant RNA species in the small RNA samples (sRNA) were stained with ethidium bromide after size separation by agarose gel electrophoresis.

1. Use the PCR primers pr1 (forward) and pr2a (reverse) to amplify a 1,189-bp fragment, which contain *carB* sequences including intron 2.
2. Use the PCR primers pr1 (forward) and pr2b (reverse) to generate an 840-bp fragment, which contains *carB* sequences overlapping with those present in the 1,189-bp fragment.
3. Digest the 1,189-bp fragment with *Sal*I and *Bam*HI.
4. Digest the 840-bp fragment with *Xho*I and *Bam*HI.
5. Ligate both fragments with the pBlueScript vector restricted with *Bam*HI (*Sal*I and *Xho*I are compatible; this ligation produces, among others, a fragment that contains *carB* sequences with opposite orientations).
6. Select the correct construction by restriction analysis.
7. Digest this construction with *Xho*I and *Not*I (restriction sites present into the PCS sequence) to excise the 2-kb inverted repeat fragment.
8. Digest the pEUKA4 plasmid with *Xho*I and *Not*I.
9. Ligate the resulting fragment with the 2-kb fragment. The new plasmid contains the 2-kb inverted repeat under the *gpd1* promoter.
10. Use the silencing construction to transform the adequate recipient strain, following the protocols described in Subheading 3.1.

3.4. Low Molecular Weight RNAs (sRNA) Isolation

1. Incubate a 500-mL Erlenmeyer flask with 50 mL YNB medium pH 4.5 inoculated with 5×10^4 spores/mL, at 26°C for 48 h, shaking at 150 rpm.
2. Isolate the mycelium by filtration and dry well with filter paper.
3. Weigh about 100 mg of mycelium; add liquid nitrogen and grind it on a mortar and pestle to a fine powder.
4. Transfer immediately the powder to an ice cold 50-mL Falcon tube and add 1.5 mL Trizol. Vortex vigorously to resuspend the powder completely. Transfer the solution to a 2-mL centrifuge tube and centrifuge at 10,000 × *g* for 10 min at 4°C.
5. Keep the supernatant at room temperature for 5 min.
6. Add 300 μL of chloroform.
7. Vortex vigorously for 15 s.
8. Keep at room temperature for 3 min.
9. Centrifuge at 10,000 × *g* for 15 min at 4°C.
10. Take the aqueous phase and add 750 μL of isopropanol. Incubate for 2 h on ice.
11. Centrifuge at 10,000 × *g* for 10 min at 4°C.

12. Wash the pellet with 1.5 mL of 70% ethanol, centrifuge at 10,000 × *g* for 5 m at 4°C, and dry it for 15 min at 37°C.
13. Resuspend the dried pellet in 300 μL of DEPC-treated double distilled water and maintain it at 65°C for 10 min.
14. Add 50 μL of 4 M NaCl and 40 μL 50% PEG 8000. Mix well and incubate on ice for 30 min.
15. Centrifuge at 10,000 × *g* for 10 min at 4°C. The supernatant contains the low molecular weight RNA (small RNAs). The pellet, which contains the rRNA and mRNA, can be resuspended in 50 μL of 50% formamide and electrophoresed on 1.5% agarose gel (at 150 V for 30 min), as a control of the RNA integrity.
16. To purify the low molecular weight RNA, add to the supernatant 3 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate pH 5, keep at –20°C overnight. After that, centrifuge at 10,000 × *g* for 30 min at 4°C.
17. Wash the pellet with 2 volumes of 80% ethanol, centrifuge at 10,000 × *g* for 10 min at 4°C, and dry it at 37°C for 15 min.
18. Resuspend the dried pellet in 20 μL of DEPC-treated water.
19. The concentration of the RNA preparation is quantified by spectrophotometric analysis.
20. Samples can be frozen for long-term storage at –80°C.

3.5. Electrophoresis of Small RNAs and Blotting

1. Mix the RNA sample (50–60 μg) with an equal volume of 2× formamide loading buffer, with a maximum final volume of 40 μL.
2. Incubate the samples at 65°C for 5 min (to open RNA secondary structures) and then place them on ice for 5 min.
3. Load the samples in a 40 mL 0.5× TBE/7 M urea/15% acrylamide gel.
4. Electrophorese at 300 V for 3 h in a Protean II Cell System, until the bromophenol blue (the faster-migrating dye) is about 1 cm of the end. If possible, use the central lanes, to avoid the "smiling" effect. The low molecular weight RNA localizes between the two dye bands.
5. After electrophoresis is complete, remove the upper glass. Mark positions of the markers.
6. Divide horizontally the gel in two parts, the cutting line being the upper dye band (xylene cyanol). The upper part of the gel contains the fraction of small RNAs (sRNA), as tRNA, 5S rRNAs, etc., which will be used as a loading control.
7. Incubate the upper part of the gel with 1× TBE and 5 μL of ethidium bromide, shaking at 60 rpm for 20 min. Wash with 1× TBE and incubate again at 60 rpm for 20 min. The gel is now ready to take a picture using UV light.

8. Cut six pieces of 3 MM Whatman paper, about 1 cm larger than the lower part of the gel.
9. Cut one piece of Hybond™-N+ nylon transfer membrane slightly bigger than the lower part of the gel.
10. Place the membrane on top of the gel, removing the bubbles by rolling with a sterile pipette.
11. Pre-wet three pieces of Whatman paper with 1× TBE and place on top of the membrane, removing again the bubbles.
12. Invert the plate carefully to remove the gel off the glass.
13. Place it on the bottom (anode) electrode plate of the semidry electroblotting unit. The order must be: bottom plate, paper, membrane, and gel. Now place the last three pieces of 1× TBE pre-wet filter paper on top of the gel and then, the upper (cathode) electrode plate on top of the stack.
14. Run at 3 mA/cm^2 gel for 1 h.
15. After transfer, fix the RNA by ultraviolet cross-linking (0.120 J/cm^2, 2 min) in a UV-Crosslinker (see Note 12).

3.6. Riboprobe Preparation

1. The riboprobe is synthesized using the in vitro transcription kit MAXIscript. The source of DNA template for the riboprobe synthesis can be either a T7 promoter-containing vector or a PCR product. In the first case, the DNA sequence to be transcribed must be cloned in the polylinker region, downstream the T7 polymerase promoter. The recombinant plasmid must be linearized by cutting on the polylinker region with the appropriate enzyme, downstream the sequence to be transcribed. Thus, the polymerase produces a transcription unit (riboprobe) that extends from the T7 promoter to the cleavage site. If the DNA template is a PCR product, the primers designed to amplify the DNA must include a 5′ T7 promoter sequence. Use 1 μg of DNA template for the transcription reaction. All the components needed for the transcription reaction and instructions are supplied in the kit.
2. Place the T7 RNA polymerase on ice (it is stored in glycerol and will not be frozen at –20°C), and vortex the 10× transcription buffer and ribonucleotide solutions until they are completely in solution. Once they are thawed, store the ribonucleotides on ice, but keep the 10× transcription buffer at room temperature (the spermidine in the 10× transcription buffer can coprecipitate the DNA template if the reaction is assembled on ice).
3. Assemble the transcription reaction at room temperature, in a RNase-free Eppendorf-like tube, by adding the DNA template (1 μg), nuclease-free water to a 20 μL final volume, 1 μL of 10 mM ATP, 1 μL of 10 mM CTP, 1 μL of 10 mM GTP, and 2 μL of 10× transcription buffer.

4. Then add 2 μL of the T7 polymerase, but do not mix it with the other components by keeping the liquid in the tube wall. Then add finally 5 μL of α^{-32}P UTP (800 Ci/mmol).
5. Gently flick the tube and then microfuge briefly to collect the reaction mixture at the bottom of the tube.
6. Incubate at 37°C for 10 min.
7. To remove the DNA template, add 1 μL of RNase-free DNaseI (included in the kit) and incubate at 37°C for 15 min.
8. Add 1 μL of 0.5 M EDTA to stop the reaction.
9. Add 300 μL of 1× alkaline buffer and incubate at 60°C for 3 h (see Note 13).
10. Add 20 μL of 3 M sodium acetate pH 5.0 to stop the hydrolysis reaction.
11. Prepare Sephadex G-50 spin columns: Resuspend and equilibrate Sephadex G-50 with 2 volumes of TE, then wash with several volumes of TE. Place the resuspended and washed resin in 1.5 volumes of TE in a glass bottle and autoclave. Store at 4°C until use. Rinse a 1–3 mL spin column thoroughly with DEPC-treated double-distilled water; frits may be pre-installed, or made by plugging the bottom of a 1-mL syringe with a support such as siliconized glass beads. Pipet 1–3 mL of the prepared, well mixed resin into the washed spin column. Place the column in a 15-mL plastic centrifuge tube and spin at 360 × *g* for 10 min in a centrifuge with a swinging bucket rotor. Place the end of the spin column containing the spun resin into an appropriate microfuge tube (typically 0.5 mL) and insert the assembly into a new 15-mL centrifuge tube. Load 20–100 μL of the sample onto the center of the resin bed (dilute sample with nuclease-free water or TE if necessary), and spin at 360 × *g* for 10 min. The eluate collected in the microfuge tube should be approximately the same volume as the sample loaded onto the column, and it will contain about 75% of the nucleic acid applied to the column.
12. The riboprobe solution can now be used for hybridization or stored at 4°C.

3.7. Riboprobe Hybridization

1. Membranes must be prehybridized, in a hybridization oven, with 20 mL of prehybridization/hybridization buffer containing 400 μL of denatured salmon sperm DNA (stock 5 mg/mL: denature by boiling at 100°C for 10 min, and then cooled on ice; final concentration: 100 μg/mL). Incubate at 30°C for 2–3 h.
2. Heat all the riboprobe solution at 95°C for 10 min and chill on ice. Then, add the riboprobe to the hybridization solution and incubate overnight at 30°C in the hybridization oven.

3. After hybridization, wash the membranes with the SDS/SSC solution at 50°C for 20 min. Repeat two more times.
4. Expose the membranes to the Kodak BioMax™ MS film (high sensitive) at −70°C.
5. If necessary, remove nonspecifically bound riboprobe by washing the membranes with 20 mL of RNase buffer containing 10 μg/mL of RNase A, at 37°C for 1 h. Wash vigorously the membranes using SDS/SSC solution at room temperature for 3 min to remove RNase.

3.8. Cell Lysate Preparation

Blue light regulates carotene biosynthesis in the fungus *M. circinelloides*, and this response has been demonstrated to be controlled by *crgA* and *mcwc-1c* gene. CrgA shows characteristics of ubiquitin ligases and represses carotenogenesis in the dark, whereas *mcwc-1c*, one of three *white collar 1*-like genes present in *M. circinelloides*, is required for its light induction. The effect of *crgA* on carotenogenesis is mediated by *mcwc-1b*, another *white collar 1*-like gene, which acts as a carotenogenesis activator. CrgA is involved in proteolysis independent mono- and di-ubiquitylation of MCWC-1b, which results in its inactivation. Proteolysis independent ubiquitylation is a novel and interesting mechanism of regulation of carotenogenesis. In this section, we describe the procedures to prepare cell lysates of *M. circinelloides*, to inmunoprecipitate proteins (MCWC-1b) and to the specific detection of ubiquitylated proteins (Fig. 3).

1. Inoculate 2.5×10^5 spores over cellophane paper in a 9-cm diameter plate with the proper agar medium at pH 4.5. Grow for about 18 h (inoculation of a high amount of spores per plate allows production of enough young mycelium in a short time).
2. Separate the mycelium from the cellophane paper and dry well with filter paper.
3. Weigh about 100–150 mg of mycelium and grind it with liquid nitrogen on a mortar and pestle to a fine powder.
4. Place the powder to a microcentrifuge tube and add 1 mL of ice cold lysis buffer (washing buffer 1) containing a 1:50 dilution (20 μL) of the Protease Inhibitor Cocktail, and 10 μL of 100 mM benzamidine. Keep 30 min on ice.
5. Centrifuge at $13{,}000 \times g$ for 30 min at 4°C.
6. Recover the supernatant using a pipette.
7. The supernatant contains the fraction of total proteins. At this point can be used for western blotting, or frozen for long-term storage at −80°C.

3.9. Immunoprecipitation

1. Transfer the supernatant to a microcentrifuge tube.
2. Add 1–5 μg of purified antibody against MCWC-1b (primary antibody) (see Notes 14 and 15).

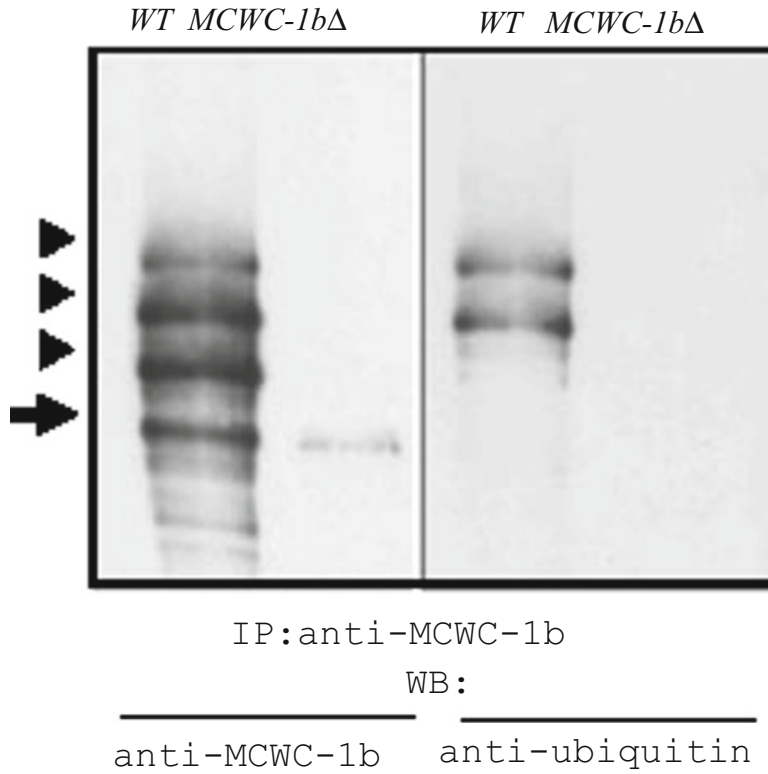

Fig. 3. Identical aliquots of crude lysates from mycelia grown in dark (18 h on YNB pH 4.5) of the wild-type strain of *M. circinelloides* (WT), and the null *mcwc-1b* mutant transformed with pLEU4 to complement its leucine auxotrophy (22), were immunoprecipitated using anti-MCWC-1b monospecific antibodies. Western blots assays were subsequently performed using the same antibody (*left*) or polyclonal antibody against human ubiquitin (*right*). The anti-MCWC-1b antibody detected an unspecific band as it also appeared in the *mcwc-1bΔ* mutant (*arrow*), and specific proteins with apparent molecular weights of 106, 115, and 125 kDa, respectively (*arrowheads*). The anti-ubiquitin antibody detected the ubiquitylated forms of the MCWC-1b protein.

3. Incubate overnight at 4°C on a rotating wheel.
4. Add 30 μL of 50% aqueous suspension Protein A-Agarose Fast Flow and keep at 4°C for 4 h. The Protein A-Agarose tube must be vortex vigorously, before taking the aliquot with a cutoff pipette tip.
5. Centrifuge at 13,000 × *g* at 4°C for 60 s.
6. Discard the supernatant and add 1 mL of cold washing buffer 1 to the pellet. Incubate at 4°C for 20 min on a rotating wheel. To discard supernatant in steps 6, 9, 12, and 14, use a 1-mL pipette, and avoid pipetting pellet protein A-agarose beads.
7. Centrifuge at 13,000 × *g* at 4°C for 60 s.
8. Repeat steps 6 and 7.

9. Discard the supernatant and add 1 mL of cold washing buffer 2 to the pellet. Incubate at 4°C for 20 min on a rotating wheel.
10. Centrifuge at 13,000 × *g* at 4°C for 60 s.
11. Repeat steps 9 and 10.
12. Discard the supernatant and add 1 mL of cold washing buffer 3 to the pellet. Incubate at 4°C for 20 min on a rotating wheel.
13. Centrifuge at 13,000 × *g* at 4°C for 60 s.
14. Discard the supernatant and add to the pellet 60 μL of cold washing buffer 3 and 15 μL of protein loading buffer. Shake well and incubate at 100°C for 5 min. After that, keep on ice.
15. Centrifuge at 13,000 × *g* at 4°C for 60 s.
16. Use a 100-μL pipette to remove the supernatant and transfer it to a clean centrifuge tube, avoid pipetting protein A-Agarose bead pellet.
17. The supernatant should contain the fraction of proteins reacting against the antibody. At this point can be used for SDS-PAGE and western blotting, or frozen for long-term storage at −80°C.

3.10. Western Blotting: Gel Assembly

1. Assemble the glass cassette and casting stand of the Mini-Protean 3 Cell, or any equivalent system.
2. The gel is divided into an upper "stacking" gel of low acrylamide percentage and low pH (6.8) and a resolving gel with a pH of 8.8.
3. Prepare the 7% resolving gel (see Note 16).
4. Take up the gel with a Pasteur pipette, put the pipette on the edge of glass, hold it at a 90° angle and begin filling to 2–2.5 cm below the top of the smallest glass plate.
5. Carefully, add 800 μL of double-distilled water on top of the acrylamide solution by using a 1,000-μL pipette.
6. Let sit for 30–45 min until the gel is polymerized.
7. Invert the apparatus and remove the water. Blot with Whatman paper strips to remove any water.
8. Prepare the stacking gel.
9. Pour in stacking gel as the resolving gel.
10. Insert comb into gel. Let sit for 10 min, checking polymerization.
11. Remove comb when stacking gel is polymerized.
12. Remove the gel cassette sandwich from the casting frame and place it into the electrode assembly with the short plate facing inward.

13. Slide the gel cassette sandwich and electrode assembly into the clamping frame.
14. Press down the electrode assembly while closing the two cam levers of the clamping frame.
15. Lower it into the mini tank and fill it with 1× running buffer.

3.11. Western Blotting: Samples Preparation and Running Gel

1. Total extract: add to a microcentrifuge tube 20 μL of total protein extract and 5 μL of 5× protein loading buffer.
2. Inmunoprecipitated proteins: add to a microcentrifuge tube 30 μL of total immunoprecipitation extract.
3. Marker: add to a microcentrifuge tube 7–10 μL of BenchMark prestained protein ladder.
4. Boil samples at 100°C during 5–10 min.
5. Quick spin (30 s) at 4°C.
6. Load them on gel.
7. Run gel at 200 V for 45–60, until the sample dye reaches the bottom of the gel.

3.12. Western Blotting: Transfer, Antibody Reaction, and Detection

1. Cut six pieces of 6×9 cm 3 MM Whatman paper.
2. Cut one piece of 5×9 cm Protran nitrocellulose membrane.
3. Pre-wet three pieces of Whatman paper with transfer buffer and place on the bottom (anode) electrode plate of the semi-dry electroblotter unit, removing bubbles by rolling with a pipette.
4. Place the membrane on top of the filter paper pack, removing bubbles carefully.
5. Gently nudge gel off plate with spatula, and place on top of the membrane (see Note 17). Remove bubbles carefully.
6. Cutoff stacking gel/wells with razor or spacers.
7. Pre-wet three pieces of Whatman paper with western blotting buffer and place them, one by one, on top of the gel, removing again the bubbles.
8. Place the upper (cathode) electrode plate on top of the stack.
9. Run at 200 mA for 45 min.
10. Use the prestained marker as control of transfer.
11. Block membrane with 10 mL blocking buffer at room temperature for 1–2 h or overnight at 4°C.
12. Wash with blocking buffer for 10 min at room temperature.
13. Add a 1/2,500 dilution of primary antibody (see Note 15) (3 μL in 7.5 mL of blocking buffer; dilution could vary,

depending of the antibody) and 1/200 dilution of anti-ubiquitin antibody.

14. Gently sake at room temperature for 1–2 h or overnight at 4°C.
15. Discard the blocking buffer and wash three times with 10 mL PBST for 10 min each.
16. Add 10 mL of blocking buffer with a 1/2,500 dilution of secondary antibody (supplied in the ECL kit).
17. Gently shake at room temperature for 1–2 h.
18. Discard the blocking buffer and wash three times with 10 mL of PBST for 10 min each.
19. Place the membrane in double-distilled water.
20. Remove the membrane and place it on a piece of plastic wrap.
21. Distribute a mix of 0.750 mL ECL Reagent 1 and 0.750 mL of Reagent 2 over the membrane. Cover with the plastic wrap and expose the membrane to a high performance chemiluminescence film into a cassette for 5 min. If necessary, expose to another film for a longer time.
22. Develop the film with a 1:10 dilution of multigrade paper developer, and fix with rapid fixer.

4. Notes

1. *M. circinelloides* R7B, the standard strain used as wild type, is a leucine auxotroph derived from *M. circinelloides* CBS277.49 (21).
2. For a normal growth using YPG, YPG-agar, YNB, YNB-agar, MMC, and MMC-agar, adjust pH to 4.5 with 1 M HCl before autoclaving. When colony growth is required (for viability counts, etc.) the media are adjusted to pH 3.2 with 1 M HCl before autoclaving. YPG and YNB are, respectively, the complete and minimal media for *Mucor*. To avoid hydrolysis of agar, double-strength solutions of agar and the other media components were autoclaved separately, and mixed after cooling at 50°C.
3. The Biotaq DNA polymerase is supplied with the 10× PCR buffer and the 50 mM $MgCl_2$ solution.
4. DEPC is a strong RNase inhibitor, and causes irritation to eyes, skin, and mucous membranes. It is a suspected carcinogen. Use it in the fume hood, wear gloves, and avoid getting DEPC on your skin.

5. When adding SSC and SDS make sure to add water first to dilute one before adding the other or a precipitate will be formed.
6. The cell wall digestion, and the appearance of protoplasts, is monitored with a light microscope (protoplasts are identified because the cell wall refringence is lost). This usually takes about 90 min.
7. Usually, each transformation mixture is spread on three plates.
8. Plasmids used for *Mucor* transformation are all self-replicative. For this reason, and because the initial transformants are usually heterokaryons due to the presence of several nuclei in the protoplasts, these transformants must be grown in selective medium for several vegetative cycles to increase the proportion of transformed nuclei. Since the two main selectable marker genes used in *M. circinelloides* are genes *leuA1* and *pyrG*, the proportion of the Leu^+ or $PyrG^+$ spores is used as an indicator of transformation or DNA integration. When $PyrG^+$ transformants must be selected, mainly when the recipient strain is a double mutant *pyrG*$^-$ *leu*$^-$, the selective medium must be MMC, because other way the mycelial growth is very poor and spore production scarce. When Leu^+ transformants have to be selected, the selective medium must be YNB. Once transformants have grown, spores from each transformant are plated on minimal medium YNB pH 3.2 with and without leucine (20 μg/mL) or MMC with and without uridine (200 μg/mL) to determine the proportion of Leu^+ or $PyrG^+$ spores, respectively. For each cycle, spores from each transformant are harvested from colonies grown under selective pressure, that is, without leucine or uridine. Integration (or efficient transformation) is denoted by the successive increase in the proportion of Leu^+ or $PyrG^+$ spores throughout the cell cycles. Usually, in case of DNA integration, after 2–4 cycles, homokaryotic transformants showing 100% stable Leu^+ or $PyrG^+$ spores can finally be obtained.
9. In our hands, gene replacement based on the RF homologous integration works better when the ends of the linear RF correspond to sequences of the target gene and not to vector sequences used for cloning. This can be achieved either by digesting the recombinant plasmid with restriction enzymes that cut appropriate restriction sequences present into the 5′ and 3′ ends of the RF, or by including the adequate restriction sequences in the design of primers used for amplification of the flanking regions of the target gene.
10. It is recommended to use the rapid PCR amplification procedure after the percentage of transformed spores is higher than 50%.
11. The highest frequency of gene silencing is obtained when using an inverted repeat transgene as a silencing trigger. This type of

construction produces, upon transcription, a hairpin RNA molecule (hpRNA), which contains sequences corresponding to the target gene. Because both the frequency and stability of gene silencing in *Mucor* are highly dependent on the level of hpRNA transcription, the strong *gpd1* promoter, which is active during the fungal vegetative growth and is regulated by the carbon source, can be used to drive transgene transcription. A spliceable intron must be included in the loop region, since the presence of introns increase the silencing efficiency in *M. circinelloides*. The hairpin stem length should be longer than 500–600 bp, and the loop after intron elimination around 300 bp.

12. It is not convenient to allow the membrane to dry completely prior to UV cross-linking. To avoid that, introduce the membrane on the top of the three wet pieces of filter paper, with the side of the blot with the bound RNA exposed to the UV light source. A 15-cm Petri dish can be used to place the membrane into the unit.
13. The alkaline buffer is used to hydrolyze the riboprobe to small RNA fragments (average size of 50 nt) to obtain a greater resolution in the hybridization.
14. This amount of antibody refers to purified antibody, and it should be calibrated according to each antibody and experimental condition. If nonpurified antibody is used (i.e., serum preparation) this calibration is necessary.
15. Monospecific antibodies against MCWC-1b are generated by immunization of rabbits with a 119-amino acid MCWC-1b peptide (from I193 to C311), which is expressed fused to a six histidine tag and purified by Nickel affinity chromatography (2).
16. Good resolution of MCWC-1b protein requires a 7% acrylamide resolving gel. Proteins with different molecular weights may require other acrylamide percentages.
17. Wet your finger with transfer buffer to manipulate the gel.

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