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

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Abstract

The carotene producer *Mucor circinelloides* is the fungus within the Mucoromycota phylum with the widest repertoire of molecular tools to manipulate its genome. The initial development of an effective procedure for genetic transformation and later improvements have resulted in an expansion of available tools, which include gene replacement, inactivation of gene expression by RNA silencing, gene overexpression and functional genomics. Moreover, sequencing of its genome has given a definitive boost to these techniques making attainable the study of genes involved in many physiological or developmental processes, including carotenoid biosynthesis. Here, we describe in detail the latest molecular techniques currently used in *M. circinelloides* that have made it a valuable model for studying gene function within its phylum.

Key words: Transformation, Gene replacement, Gene overexpression, Gene silencing, RNAi, Functional genomics

1. Introduction

Early divergent lineages of fungi, also termed basal fungi, are receiving a growing attention since they can contribute to understand fungal evolution (1). Moreover, these fungal groups are evolutionary distant to the so-called higher fungi, Ascomycetes and Basidiomycetes, being their physiology, development, metabolism and ecology less characterized (2). *M. circinelloides,* informally referred to herein as *Mucor*, belongs to Mucorales, the largest order of the early divergent phylum Mucoromycota (1). *Mucor* is an emblematic species that has contributed importantly to a better knowledge of the biology of this phylum because of the development of several molecular tools to manipulate its genome, including genetic transformation (3, 4), gene replacement (5), RNA-mediated gene silencing (5) and overexpression of genes (6). The potential of these procedures has been amplified by the availability of *Mucor* genome sequence (7). Main aspects of *Mucor* biology that are currently receiving special attention and have been beneficiated by the existing molecular tools include responses to light (8, 9), sex determination and its evolution (10, 11), gene silencing or RNA interference (RNAi) (12), regulation of gene expression by endogenous small RNAs (esRNAs) (13), pathogenesis (14), lipid accumulation (15), and biosynthesis of carotenoids (9, 16, 17).

Mucorales are characterized for a fast growth and short life span which facilitates successful exploitation for the production of lipids and carotenoids. In fact, *Mucor* was the first microorganism used industrially for the production of an oil (18) while other Mucoral, *Blakeslea trispora*, is the main microorganism used for the production of carotenes on the industrial scale (19). Most of the information about the biosynthesis of carotenoids in Mucorales derived from *B. trispora* but also from the model *Phycomyces blakesleeanus* (20). However, both fungi exhibit the same the problem, the lack of molecular genetics tools to manipulate their genomes. This is in part compensated by the

availability of such tools in Mucor, which also have contributed to understand the regulation of carotenoid biosynthesis in Mucorales (8, 9, 17). Moreover, manipulation of genes involved in regulation of carotenoid biosynthesis and/or structural genes have produced strains that overaccumulate lycopene or β-carotene (16, 17, 21).

Molecular techniques that are of routine use in *Mucor* were gathered in a chapter of a previous book of this series (5), which should be consulted by researchers working with *Mucor*. Here, we complement it by updating some procedures and introducing novel techniques, such as gene overexpression and functional genomic screening.

2. **Materials**

1. *M. circinelloides* CBS277.49 (CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands).
2. *M. circinelloides* R7B (22) (see Note 1).
3. *M. circinelloides* MU402 (23) (see Note 2).
4. 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).
5. PS buffer: Mix 18.22 g of sorbitol and 20 mL of SPB. Adjust to 200 mL with double-distilled water (final sorbitol concentration: 0.5 M).
6. Lysing enzymes (L-1412, Sigma-Aldrich, St. Louis, MO, USA).
7. Chitosanase (C-0794, Sigma-Aldrich, St. Louis, MO, USA).

8. 0.5 M Sorbitol: Dissolve 22.77 g of sorbitol in 200 mL double-distilled water. Adjust to 250 mL with double-distilled water.

9. Gene Pulser Cuvette (0.2-cm electrode gap) (Bio-Rad, Hercules, CA, USA).

10. Bio-Rad Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA).

11. YPG: 3 g/L of yeast extract, 10 g/L of peptone, and 20 g/L of glucose (see Note 3).

12. YPGS: Add 91.1 g of sorbitol to the YPG medium before adjusting the volume to 1,000 mL with distilled water (final sorbitol concentration: 0.5 M) (see Note 3).

13. YPG-agar and YPGS-agar: YPG or YPGS with 15 g/L agar (see Note 3).

14. 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 niacin at a final concentration of 1 μg/mL (see Note 3).

15. YNBS: Add 91.1 g/L of sorbitol to the YNB medium before adjusting the volume to 1,000 mL with distilled water (final sorbitol concentration: 0.5 M) (see Note 3).

16. YNB-agar and YNBS-agar: YNB or YNBS with 20 g/L agar (see Note 3).

17. 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 niacin at a final concentration of 1 μg/mL (see Note 3).

18. MMCS: Add 91.1 g/L of sorbitol to the MMC medium before adjusting the volume to 1,000 mL with distilled water (final sorbitol concentration: 0.5 M) (see Note 3).

19. MMC-agar and MMCS-agar: MMC or MMCS with 15 g/L agar (see Note 3).

20. GeneJET Gel Extraction Kit (ThermoFisher Scientific).

21. DMSO (dimethyl sulfoxide).

22. Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA).

23. *carRP*-F1: 5'-CCGGGCGCATTGTAGATAAACT-3'.

24. *carRP*-R1: 5'-GGGCATGTGTAACAGTGCATTGG-3'.

25. *pyrG*fwd: 5'-TGCCTCAGCATTGGTACTTG-3'.

26. *pyrG*rev: 5'-GTACACTGGCCATGCTATCG-3'.

27. *pyrG*ext: 5'-ATCCCACCAGAAGGAGTACATGG-3'.

28. *peuka-1*: 5'-CATGAAGTGTGAGACATTGCG-3'.

29. *pzrt1*: 5'-GGAGATGTGCCTTGATGATATGCTCT-3'.

30. pMAT1476 (see Note 4).

31. pMAT1477 (see Note 5).

32. pMAT1552 (see Note 6).

33. pMAT1702 (see Note 7).

34. Mixture for overlapping PCR: 1 μL of Herculase II Fusion DNA polymerase, 10 μL of 5X Herculase II reaction buffer, 0.5 μL of a 100mM solution of dNTPs (25 mM each), 2 μL of a 10 μM solution of each primer, 100 ng of each upstream and downstream fragments, 200 ng of *pyrG* marker fragment and adjust to 50 μL of double-distilled water.

35. Lysis Buffer: to prepare 50 mL of buffer dissolve 23.6 g of Guanidine thiocyanate (118.16 g/L) in 25 mL of double-distilled water. Once dissolved add: 2.5 mL of 1 M Tris-HCl pH 7.0 (121.14 g/L), 2 mL of 0.5 M EDTA pH 8.0 (186.12 g/L) and 0.05 mL of Triton X-100. Add double-distilled water to 50mL (final concentrations: 4 M guanidine thiocyanate, 50 mM Tris-Hcl pH 7.0, 20 mM EDTA and 0.1 % Triton X-100).

36. DNA Binding Solution: First, wash diatomaceous earth (silica particles) by resuspending 5 g in 50 mL of water and allowing them to settle. Decant the supernatant after 30 s and repeat this step twice keeping the supernatant. Centrifuge at 1,400 x g for 1 min, discard the supernatant and resuspend in 25 mL of acetone. Centrifuge at 1,400 x g for 1 min, discard the supernatant and dry the silica particles at 37ºC for 24 hours. To prepare 50 mL of buffer dissolve 23.6 g of Guanidine thiocyanate in 25 mL of double-distilled water. Once dissolved add 2.5 mL of 1 M Tris-HCl pH 7.0, 2 mL of 0.5 M EDTA pH 8.0 and 1 g of diatomaceous earth previously filtrated. Add double-distilled water to 50 mL (final concentrations: 4 M guanidine thiocyanate, 50 mM Tris-HCl pH 7.0, 20 mM EDTA and 20 g/L diatomaceous earth).

37. Wash Buffer: to prepare 50 mL of buffer mix 2.5 mL of 4 M NaCl, 1 mL of 0.5 M EDTA pH 8.0, 2.5 mL of 1 M Tris-HCl pH 7.4 and 25 mL of absolute ethanol. Add double-distilled water to 50 mL (final concentrations: 200 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4 and 50% ethanol).

38. Elution Buffer: Add 0.5 mL of Tris-HCl pH 8.5 to 45 mL of double-distilled water (final concentration 10 mM Tris-HCl pH 8.5).

3. Methods

3.1. Transformation

Genetic transformation of *Mucor* is based on the use of auxotrophies since this fungus is naturally resistant to antibiotics and antifungals commonly used in Ascomycetes and Basidiomycetes. Three different auxotrophies are used for genetic transformation of *Mucor*, methionine (24), leucine (3) and uridine (25). However, the most widely used strains are auxotrophs for leucine and uridine that are complemented with *leuA1* and *pyrG* genes respectively**.** All plasmids that complement auxotrophies in *Mucor* are self-replicative, making necessary the use of linear fragments in transformation to favor chromosomal integration. The procedure described below works for both self-replicative plasmids and linear integrative DNA fragments.

1. Collect fresh spores from mycelium grown in YPGS pH 4.5 for 4-6 days at 26 ºC in the light. Release spores from the sporangia by pouring 10 mL/plate of sterile distillated water on the mycelium and spreading it with a Drigalski spatula. Take the water with the spores by using a pipette and put in a conical tube.

2. Wash the spore solution by centrifugation for 5 min at 440 × g. Resuspend the spores in 10 mL of sterile distillated water and repeat the centrifugation. Resuspend the pellet in 5 mL of sterile distillated water and store the spore solution at 4 ºC. Count the spores using a hemocytometer and light microscope.

3. Take an aliquot of spores containing 2.5×10^8 (no more than 1 week old) and centrifuge for 5 min at 440 × g. Resuspend the pellet in 25 mL of YPG medium pH 4.5 (final spore concentration to 10^7 spores/mL), supplemented with 200 µg/ml of uridine when a uridine auxotroph is going to be transformed.

4. Incubate overnight at 4°C, without shaking.

5. Incubate the spores at 26°C with shaking (300 rpm) until most of them are germinated having a germ tube length shorter than the swollen spore diameter (Fig. 1). This usually takes 3-4 h.

6. Wash the cells twice by centrifugation in 5 mL of PS buffer pH 6.5 at 91 × g for 5 min.

7. Resuspend the pellet in 4 mL of PS buffer. Transfer the germinated spore solution to a 50-mL Erlenmeyer flask.

8. Add 5 mg of lysing enzymes dissolved in 1 mL PS buffer, and 0.3 µL (15 U/mg) of Chitosanase previously diluted in 50 µL PS buffer. Incubate at 30°C with gentle shaking (60 rpm) for about 90 min (see Note 8).

9. 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.

10. Resuspend the pellet gently in 800 µL of cold 0.5 M sorbitol. This 800 µL solution allows four or eight different transformation experiments depending on the type of DNA used (see next step).

11. Each tube of transformation mixture must contain 100 µL protoplast solution and 10 µL DNA sample (1-3 µg total DNA in double-distilled water) for transformation experiments with circular plasmid or 200 µL protoplast solution and 20 µL DNA sample (3-6 µg total DNA) for linear fragments. Include a negative control with either 10 µL or 20 µL of double-distilled water instead of DNA, respectively.

12. Mix and transfer to the electroporation cuvette.

13. Apply an electrical pulse using the following conditions: field strength of 4 kV/cm, capacitance of 25 μF, and constant resistance of 400 Ω.

14. Immediately after the pulse, remove the cuvette and add 1 mL cold YPGS 4.5. Keep on ice until all cuvettes have been pulsed.

15. Transfer the liquid of each cuvette to 2.0-mL microcentrifuge tubes.

16. Incubate for 1 h at 26°C and 100 rpm.

17. Centrifuge at 91 × g for 3 min and gently resuspend the pellet in a final volume of 400-600 μL YNBS 4.5.

18. Inoculate plates of the adequate medium containing 0.5 M sorbitol with 200 μL protoplast solution (see Note 9).

19. Incubate in the dark at 26°C for 3-4 days to obtain the transformants (see Note 10)

3.2. Gene Replacement

Generation of null mutants by gene replacement requires the designing of replacement fragment (RF) that disrupts the target gene after homologous recombination (HR). This RF must contain a selectable marker gene flanked by upstream and downstream DNA sequences (approximately 1 kb long each) of the target gene (Fig. 2). The construction of the RF based on *pyrG* marker, which is the preferred marker for disruption, and selection of transformants are described below. The same procedure can be applied for RFs based on *leuA1* gene (see Note 11).

1. Set up independent PCR reactions to amplify 1-kb sequence of both upstream and downstream flanking regions of the target gene using genomic DNA (gDNA) as template. Upstream sequence is amplified using primers Ufwd and Urev*pyrG*, whereas downstream sequence is amplified using primers Dfwd*pyrG* and Drev (see Note 12).
2. Amplify the marker gene and its promoter sequence by PCR from either gDNA or any appropriate vector containing the *pyrG* gene, such as pMAT1702 (14), using primers *pyrG*fwd and *pyrG*rev.
3. Check correct amplification of all fragments by agarose gel electrophoresis. Purify the PCR fragments from agarose slabs using a commercial kit, such as GeneJET Gel Extraction Kit.
4. PCR fragments from the upstream region, selectable marker and downstream region are mixed in 1:1:1 molar proportion and join together by overlapping PCR using primers Ufwd and Drev (Fig. 2) (see Note 13).
5. Check correct amplification of the RF by agarose gel electrophoresis and purify it as described in step 4.
6. The RF thus obtained is used to transform *Mucor* using the protocol described in Subheading 3.1 (see Note 14).
7. Grow initial transformants, which are usually heterokaryons, for several vegetative cycles in selective media to select for homokaryons, which are expected to harbor the mutation in all their nuclei (see Note 10).
8. Correct disruption of the gene has to be confirmed by PCR or/and Southern hybridization (see Subheading 3.4).

3.3. Stable gene overexpression

Mucor strains that overexpress a particular gene can be generated by genetic transformation using a self-replicative plasmid containing the coding region of the gene under either its native or a heterologous strong promoter (26). However, these strains must be maintained under selective pressure to reduce plasmid loss. Thus, integration of a copy of the gene in *M. circinelloides* genome is recommended to provide a stable overexpression. This can be achieved by generating a RF containing both a selectable marker and a copy of the gene under the control of an appropriate high-expressing promoter, flanked by upstream and downstream sequences designed to target the whole construction to a particular genomic locus. Current *Mucor* vectors (see Notes 4, 5 and 6) for overexpressing genes are designed to disrupt *carRP* locus because its disruption blocks colored-carotenoid production, rendering albino colonies that allow a fast screening for transformants with the construction in the *carRP* locus. In case of studies concerning carotenoid biosynthesis, new vectors that target a locus unrelated to carotenogenesis should be developed. The construction of an overexpression construction based in pMAT1477 (Fig. 3) and selection of transformants are as follows:

1. Set up a PCR reaction to amplified the coding region of the gene to be overexpressed using appropriate primers with added restriction sites for *Xho*I and/or *Sac*II at their 5' ends to facilitate cloning.
2. The PCR product is digested with *Xho*I and/or *Sac*II and cloned in pMAT1477 previously cut with the same enzymes.
3. Transform *Escherichia coli* and select clones with recombinant plasmids.
4. Recombinant plasmids are cut with the restriction enzyme *Cfr*9I to release the whole construction, which is used to transform *Mucor* protoplasts following the procedure described in Subheading 3.1 (see Note 15).

5. Pick initial transformants, which are usually heterokaryons, in selective media pH 4.5 to favor the generation of albino patches, which reveal integration in *carRP* locus.
6. Harvest spores from the albino patches by scratching them using a micropipette tip or small laboratory spatula.
7. Dip the tip or spatula in 0.5 ml of sterile distillated water and shake it to release the spores.
8. Plate the spores in selective media pH 3.2 and incubate for 2-3 days at 26 ºC. Collect spores from white colonies for subsequent plating. After repeating this process from 1 to 3 times, colonies that produce only albino descendants are obtained, indicating that they are homokaryons for the integration of the overexpressing construction.
9. Confirm the correct integration of the whole construction in the *carRP* locus by PCR or/and Southern hybridization (see Subheading 3.4).

3.4. Rapid genomic DNA purification and analysis of transformant DNA

Identification of knockout mutants with successful gene replacement or strains with an integrated overexpressing construction require confirmation by PCR or/and Southern hybridization. These analyses need a straightforward and quick procedure to purify genomic DNA from selected transformants. The method described below, which is based on the silica binding method developed by Carter and Milton (27), works with high reliability in *Mucor*.

1. Weigh about 100 mg of mycelium and freeze it in liquid nitrogen.

2. Grind the frozen mycelium with a mortar and pestle until a fine powder is obtained.

3. Transfer the powder to a 2-mL microcentrifuge tube, avoiding tissue thawing.

4. Immediately add 500 µL of Lysis Buffer. Vortex until the mycelium is completely resuspended and centrifuge at 13,000 x g for 5 min.

5. Transfer the supernatant to a new 2-mL tube without disturbing the cell-debris pellet.

6. Add 1 mL of DNA Binding Solution. Ensure that the binding buffer is mixed completely before proceeding because silica particles precipitate quickly.

7. Mix the tube content by inversion during 5 min at room temperature to allow DNA binding to silica particles. Centrifuge for 1 min at 13,000 x g. Discard the supernatant with a micropipette.

8. Resuspend the pellet in 700 µL of Washing Buffer. Centrifuge for 1 min at 13,000 x g. Discard the supernatant with a micropipette. Repeat this step.

9. Add 700 µL of acetone. Centrifuge for 1 min at 13,000 x g. Discard the supernatant with a micropipette.

10. Repeat the centrifugation to remove any residual acetone from the pellet.

11. Dry the pellet by putting the tube open in an oven for 5 min at 37 ºC

12. Add 50 µL of Elution Buffer and incubate at 55 ºC for 5 min. Centrifuge 1 min at 13,000 x g.

13. Collect the supernatant containing the genomic DNA. Transfer it to a new 2 mL tube and store the purified DNA at -20ºC. DNA concentration should be between 20 and 50 ng/µL (see Note 16).

Once genomic DNA is isolated from transformants and wild-type strains, gene replacement can be detected by PCR using discriminatory primers. For instance, a PCR reaction can be set up using a reverse primer of the selectable marker (*pyrG*ext) and forward primer (Uext) that anneals upstream to the flanking region used to generate the replacement fragment (Fig. 2); thus assuring that PCR amplification can only occur if gene replacement has taken place by HR. It is recommended to apply the following rapid PCR amplification procedure:

1. Set up PCR amplification reactions with 8% of DMSO in a final volume of 25 µL using 1 µL of gDNA and primers pyrGext and Uext. Use Herculase II Fusion DNA polymerase and follow supplier's recommendations.

2. Run a PCR program with an initial denaturation step at 95 ºC for 5 min followed by 30 cycles, each one consisting of 95 °C for 30 s, 55-60 °C (depending on primer length and sequence) for 30 s, and 72 °C for 30 s/kb. A final step of 5 min a 72 ºC is included to finish all DNA chains.

3. Analyze the PCR results by agarose gel electrophoresis**.** Transformants showing positive amplification should be analyzed by Southern hybridization to confirm proper integration.

3.3. Silencing vectors and functional genomic screenings

RNAi is an alternative procedure to knocking out genes in order to study gene function, particularly useful in the case of essential genes. The silencing vectors used in *Mucor* so far are self-replicative and harbor two main elements: a selectable marker (*leuA1* or

pyrG) and a gene construction that triggers silencing. The triggering construction (transgene) can contain either inverted repeated sequences of the gene to be silenced cloned under a strong promoter, or a gene fragment under the control of inverted strong promoters. The first type of plasmids is ideal to silence a specific gene because it transcribes hairpin RNA structures that produce the highest silencing frequencies (nearly 95% of the colonies) (28). The second type is particularly useful when a large number of genes has to be analyzed, as in functional genomic screenings, because construction of a hairpin producing vector for each gene is not feasible. Detailed procedures to construct the first type of silencing vectors and analyze small RNAs were previously reported (5). This section describes the procedure to generate a genomic library using the second type of silencing vectors, in particular vector pMAT1702 (see Note 7), and its use to perform functional genomic screenings in *Mucor* to look for genes involved in a particular process (Fig. 4). This strategy has been used to identify virulence determinants in mucormycosis (14), an opportunistic fungal infection of humans caused by Mucorales.

1. Digest vector pMAT1702 with *Xho*I and fill in with dCTP-dTTP to avoid self-ligation (29).
2. Partially digest *Mucor* gDNA with *Sau*3A and electrophorese it in an agarose gel.
3. Purify fragments of 0.5-4 kb from the agarose gel using a commercial kit such as GeneJET Gel Extraction Kit.
4. Fill in the purified fragments with dGTP-dATP to avoid self-ligation (29).
5. After ligation of *Mucor* DNA fragments and vector, transform *E. coli* to obtain a number of transformants higher than 73,680, which ensures that the probability that any particular fragment is found in the library is higher than 99 % (see Note 17).

6. Collect all *E. coli* transformants using Luria-Bertani (LB) medium to create pools with around 10,000 colonies.
7. Purify plasmid DNA from each pool (see Note 18).
8. Plasmid DNA from each pool is used to transform *Mucor* (see Subheading 3.1) to obtain a number of transformants above of the confidence level of 99 %. Phenotypes can be screened at this point or spores from each pool can be harvested to perform later screenings.

3.4. Identification of the genes responsible for phenotypes selected in functional genomic screenings

The self-replicative nature of pMAT1702 facilitates the identification of the sequences responsible for the phenotypes selected in screenings of silenced transformants. The procedure is as follows:

1. Purify gDNA from the selected transformant following the procedure described in Subheading 3.4.
2. Set PCR reactions using Herculase II Fusion DNA Polymerase amplification and primers *peuka-1* and *pzrt1* to amplify the DNA fragments cloned in pMAT1702 (Fig 4A) (see Note 19).
3. Sequence the PCR fragments using primers *peuka-1* and *pzrt1* and confront their sequences with the genome sequence of *Mucor* (http://genome.jgi.doe.gov/Mucci2/Mucci2.home.html) to identify the gene silenced in each transformant (see Note 20).

NOTES

1. *M. circinelloides* R7B, the standard strain used as wild type, is a leucine auxotroph derived from *M. circinelloides* CBS277.49 (22).

2. *M. circinelloides* MU402, the standard strain used for transformation, is a leucine and uridine auxotroph derived from R7B strain (23).

3. For regular growth, adjust pH to 4.5 all described media with 1 M HCl or 1N NaOH before autoclaving. When controlled colony growth is required (for viability counts, in transformation plates, 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 *M. circinelloides*. MMC is a rich medium that allows the growth of leucine auxotrophs, but not uridine auxotroph, being frequently used in transformations selecting *pyrG* gene. To avoid hydrolysis of agar, double-strength solutions of agar and the other media components are autoclaved separately, and mixed after cooling at 50°C. When it is required, uridine or leucine is supplemented to the corresponding media at a final concentration of 200 μg/mL and 20 μg/mL, respectively.

4. pMAT1477 (Fig. 3) contains *leuA1* as a selectable marker, followed by promoter of *Mucor zrt1* gene without a translation start codon and a cloning site for *Xho*I and *Sac*II restriction enzymes. This whole construction is flanked by upstream and downstream sequences of the *carRP* locus, to favor HR after transformation (6).

5. pMAT1476 comprises all pMAT1477 elements except for *zrt1* promoter and is designed to clone the target gene with its native promoter in *Nhe*I, *Xho*I or *Sac*II sites (6).

6. pMAT1552 derives from pMAT1477 by replacing the *leuA* marker by *pyrG* gene. As a consequence of this replacement, this plasmid lacks *Sac*II as cloning site.

7. pMAT1702 is a high-throughput silencing vector that harbors a multiple cloning site (MCS) flanked by two convergent strong promoters (P*gpd1* and P*zrt1*) of *Mucor* (Fig. 4A).

8. Cell wall digestion and appearance of protoplasts are monitored with a light microscope (protoplasts are identified because cell wall refringence is lost). This usually takes about 90 min.

9. Usually, each transformation mixture is spread on three plates.

10. The self-replicative nature of the plasmids used for *Mucor* transformation causes that only a proportion of the spores produced by initial transformants contain plasmid. Moreover, these transformants have to be grown in selective medium to avoid plasmid loss. In the case of integrative transformation with linear DNA fragments, the presence of several nuclei in the protoplast results in heterokaryotic transformants, which have to be grown for several vegetative cycles in selective media to increase the proportion of transformed nuclei. Since the two main selectable markers used in *Mucor* are genes *leuA1* and *pyrG*, the proportion of the Leu+ or PyrG+ spores is used as an indicator of DNA integration. When PyrG+ transformants must be selected, mainly when the recipient strain is a double mutant *pyrG*$^{-}$ *leu*$^{-}$ (MU402), the selective medium has to be MMC, because otherwise the mycelial growth is very poor and spore production scarce. When Leu+ transformants are selected, the selective medium has to be YNB. Once transformants have grown, spores from each transformant are plated on minimal medium YNB pH 3.2 with and without leucine or MMC with and without uridine 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 is denoted by the gradual increase in the proportion of Leu+ or PyrG+ spores throughout the cell cycles. Homokaryotic transformants showing 100% stable Leu^+ or Ura^+ spore proportion are usually obtained after 2-4 vegetative cycles.

11. RFs based on *leuA1* marker are constructed using the 4.4-kb fragment derived from plasmid pLEU4 (30), which contains the *leuA1* wild-type allele with its own promoter.

12. Gene specific primers (Ufwd, Drev, Uext, Urev*pyrG* and Dfwd*pyrG*) should have 18-25 nucleotides, 40-60 % GC content, melting temperature of approximately 65 ºC and 1-3 G/C bases at the 3' end. Primers Urev*pyrG* (5'-CAAGTACCAATGCTGAGGCA[N]-3') and Dfwd*pyrG* (5'-CGATAGCATGGCCAGTGTAC[N]-3') include *pyrG* sequences and 18-25 nucleotides of the target sequence in the 3' end (Fig. 2).

13. The program for overlapping PCR (Table 1) has the feature of including intervals above and below annealing temperature that have to course at a slow pace of approximately 0.3 ºC/s. After the 20^{th} cycle, elongation time can be increased by 10 s per cycle to counteract possible loss of polymerase activity.
14. RF can be further reamplified by PCR with primers Drev and Ufwd (Fig. 2) to obtain enough linear DNA for genetic transformation. Alternatively, the RF can be cloned in a standard cloning vector, such as pUC series vectors, and released in linear form by specific enzymatic restriction.

15. Alternatively, the whole construction can be amplified by PCR using primers *carRP*-F1 and *carRP*-R1, though it is not recommended due to the high probability that the polymerase introduces mutations in the gene.

16. Before using DNA, centrifuge briefly the DNA to avoid that remaining silica particles interfere with further reactions.

17. Confidence level is calculated according to the formula: N = ln(1-P)/ln(1-f); where "N" is the necessary number of recombinants, "P" is the desired probability that any fragment of the genome is represented in the library at least one time, and "f" is the fractional proportion of the genome in a single recombinant. "f" can be further shown to be f = i/g, where "i" is the insert size and "g" is the genome size (31). Thus, in the case of *Mucor*, which has a genome size of 3.6×10^7 bp, for fragments of 2.25 kb average, the number of transformants has to be higher than 73,680 for a P = 0.99.

18. Plasmid DNA aliquots of each pool can be store at -20 ºC.

19. An alternative procedure to identify the DNA cloned in pMAT1702 consists in transforming *E. coli* with gDNA purified from the selected *Mucor* transformants. Then, the plasmid is isolated from *E. coli* transformants and sequenced with primers *peuka-1* and *pzrt1*.

20. It is possible that more than one *Mucor* gene is found in a recombinant pMAT1702. In these cases, each gene must be amplified separately and cloned again in pMAT1702. The resulting plasmids are individually transformed into *M. circinelloides* and the phenotype of silenced transformants will be analyzed to identify the gene responsible for the phenotype observed in initial transformants (14).

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Figure Caption

Fig. 1. Spores preparation in the moment to be treated with lysing enzymes and Chitosanase to produce protoplasts.

Fig. 2. Schematic representation of a replacement fragment obtained by overlapping PCR. Arrows represent primers for PCR amplification (see Note 12), U and D are upstream and downstream flanking regions of the target gene, and *pyrG* is the selectable marker. HR is checked by PCR amplification with primer Uext and *pyrG*ext. HR sites are marked with X.

Fig. 3. Schematic representation of the overexpressing vector pMAT1477. *carRP* boxes represent flanking regions for integration in the *carRP* locus, *leuA1* is the selectable marker and P*zrt1* is the promoter of gene *zrt1*. Restriction sites for designated enzymes are shown.

Fig. 4. Schematic diagram of the RNA silencing vector pMAT1702 (A) and the platform for functional genomics in *Mucor* (B). P*zrt1*, *Mucor zrt1* promoter; P*gpd1*, *Mucor gpd1* promoter; *pyrG*, is the selectable marker; amp^R, ampicillin-resistant gene. Asterisks indicate unique restriction sites in the vector.

Table 1. Overlapping PCR program.

Step	Temperature	Duration
1. Denaturation	95 ºC	3 min
2. Denaturation	95 ºC	30 s
3. Drop	70 ºC	1 s
3-4 Interval	Temperature rate: 0.3 ºC/s	
4. Annealing	60 ºC	30 s
4-5 Interval	Temperature rate: 0.3 ºC/s	
5. Elongation	72 ºC	30 s/kb (add 10 s per cycle after 20_{th} cycle)
Repeat steps 2-5 for 30 cycles		
6. Elongation	72 ºC	5 min

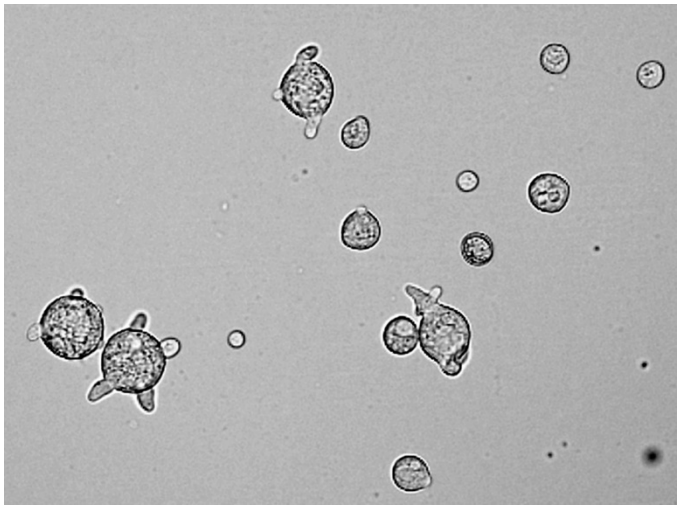


Figure 1.

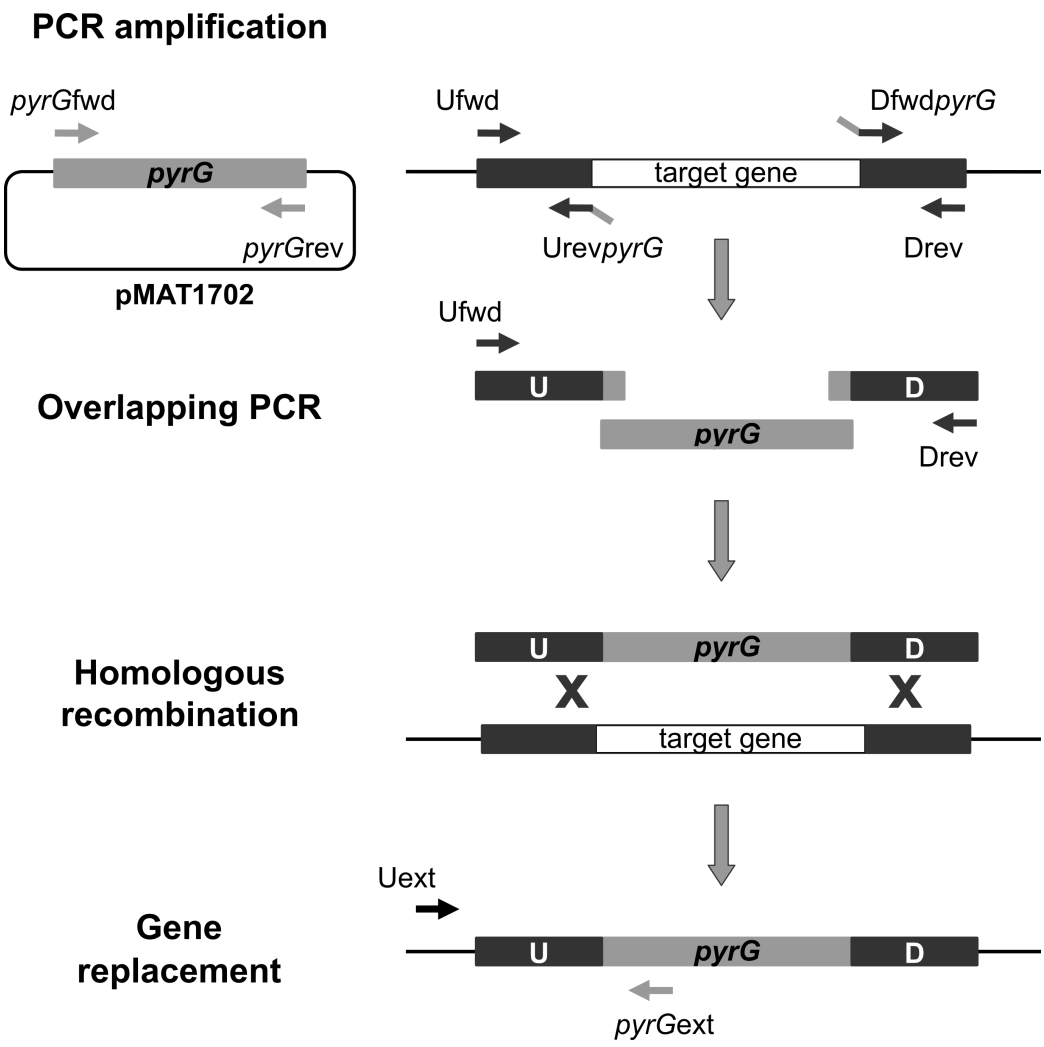
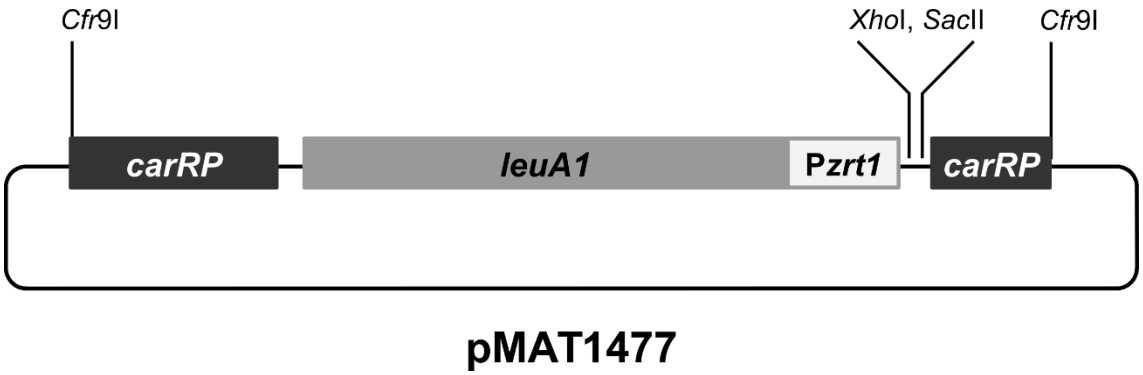


Figure 2.



pMAT1477

Figure 3.

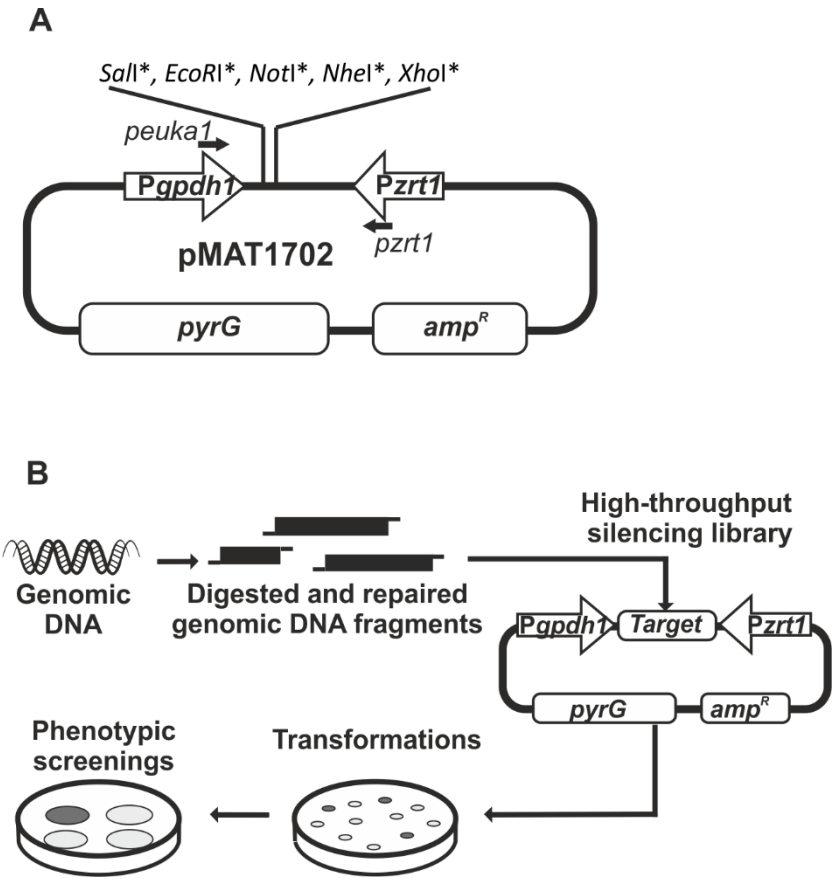


Figure 4.