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Abstract

DNA-mediated transformation in *Neurospora crassa* results in the formation of heterokaryons comprising a mixture of both transformed and untransformed nuclei. This makes it imperative to resolve the transformed nuclei from the untransformed ones. The present methods for purifying the transformed nuclei from primary transformants include repeated plating of macroconidial transformant to enrich the transformed nuclei or producing uninucleate microconidia. Both these methods are labour intensive and time consuming. We report here a simple and efficient method of purifying the transformed nuclei by the induction of microconidiation in an otherwise non-microconidiating strain using a *mcm* mutant strain.

Use of mutant *mcm* strain in purification of *Neurospora crassa* transformants resistant to hygromycin

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DNA-mediated transformation in *Neurospora crassa* results in the formation of heterokaryons comprising a mixture of both transformed and untransformed nuclei. This makes it imperative to resolve the transformed nuclei from the untransformed ones. The present methods for purifying the transformed nuclei from primary transformants include repeated plating of macroconidial transformant to enrich the transformed nuclei or producing uninucleate microconidia. Both these methods are labour intensive and time consuming. We report here a simple and efficient method of purifying the transformed nuclei by the induction of microconidiation in an otherwise non-microconidiating strain using a *mcm* mutant strain.

Ever since the first successful report of genetic transformation in *Neurospora crassa* (Mishra 1979 *J. Gen. Microbiol.* **133**: 255-259), the technique has continuously improved. Genetic transformation has been used as a tool to clone a number of genes by complementation (Vollmer and Yanofsky 1986 *Proc. Natl. Acad. Sci. U.S.A.* **83**: 4869-4873), as well as to reveal new phenomena such as RIP (Selker *et al.*1987 *Cell* **51**: 741-752), and quelling (Romano and Macino 1992 *Molec. Microbiol.* **6**: 3343-3353). Further studies have shown that competence for transformation is a nuclear rather than a cellular phenomenon (Grotelueschen and Metzenberg 1995 *Genetics* **139**: 1545-1551 and Pandit and Russo 1992, *Mol. Gen. Genet.* **234**:412-422) and ectopic integration of transforming DNA was rare among transformants selected for gene replacement (Miao *et al*. 1995 *Genetics* **139**: 1533-1534). Because of this property, the introduction of exogenous DNA results in the generation of a heterokaryon comprising a mixture of transformed and untransformed nuclei in the same cytoplasm. Such a heterogenous system is less amenable for further studies and thus it becomes a prerequisite to obtain a homokaryotic transformant from the heterokaryon to carry out genetic and biochemical characterisation of the transformants. Several methods have been developed to purify the transformant nuclei.

One of the earliest methods involved the purification of the transformed nuclei by passing through a sexual cross, but the method is generally unsuccessful because of the RIP phenomenon (Selker *et al.*1987 *Cell* **51**: 741-752), which inactivates both the ectopic and endogenous copies of the gene of interest. Alternatively, vegetative purification employs the use of multinucleate macroconidia as well as the uninucleate microconidia. In case of macroconidia, purification of the transformed nuclei is done by a laborious method involving repeated plating of macroconidia of transformants and selecting for the transforming DNA phenotype from single macroconidial isolates. Such isolates are then verified for the absence of the untransformed nuclei. An easier approach, which includes looking for occasional homokaryons among random macroconidial isolates, has also been used.

Uninucleate microconidia provide another alternative of purifying the transformants. Certain genotypes of *N. crassa*, *e.g.*, the *fluffy* strain of *N. crassa* (FGSC 45), selectively produce only microconidia. This strain was transformed with a plasmid carrying gene for the benomyl-resistance. The transformed strain was allowed to produce microconidia and individual (homokaryotic) transformed colonies were obtained. (Rossier *et al*. 1985 *Curr. Genet.* **10**: 313-320). Ebbole and Sachs (1990 *Fungal Genet. Newsl.* **27**:17-18), induced the primary transformant to produce microconidia which were then plated on selective medium to purify the transformed nuclei. The availability of the *mcm* mutant (Maheshwari 1991 *Exp. Mycol*. **15**: 346-350) and its ability to produce microconidia gave a major advantage over the previous methods. Since then, microconidia have been used in many different ways to purify the transformed nuclei. A *mcm* mutant strain (Maheshwari 1991 *Exp. Mycol*. **15**: 346-350) was used to generate transformable spheroplasts (Royer and Yamashiro 1992 *Fungal Genet. Newsl.* **39**: 76-77). The cellophane method (Pandit and Maheshwari, 1994 *Fungal Genet. Newsl.* **40**: 64-65) was used to purify transformed nuclei from the untransformed nuclei (Margolin *et al.* 1997 *Fungal Genet. Newsl.* **44**: 43-36). In other cases the *mcm* gene has been incorporated in the parent strain, so as to purify the transformed nuclei subsequently (Pitchaimani and Maheshwari 2000 *Fungal Genet. Newsl*. **47**: 89-91). All these methods have their own limitations; for example, it is a time consuming process to introduce the *fluffy* or *mcm* gene into the parent strain before transformation. The transformation of microconidial spheroplasts is limited by their low viability (12%). Here we report the use of a mutant *mcm* strain to purify wild type *N. crassa*, which has been transformed with the *hph* gene.

Grigg (*Neurospora Newsl*. **7**: 12-13) observed that a normal macroconidial strain could be induced to form microconidia if combined in a heterokaryon with a microconidial strain with the nuclear ratio (1:20 to 1: 40 respectively) in favour of the microconidial strain. We have used this observation to design a simple and efficient method to purify the transformed nuclei. We have generated a heterokaryon by mixing the impure transformant with an excess of a *het*-compatible *mcm* strain containing a selection marker and growing the heterokaryon to conidiation. Macroconidia from such a heterokaryon were shaken overnight to obtain microconidia and plated to isolate the transformed nucleus for hygromycin resistance in pure homokaryotic form. Thus, an otherwise non-microconidiating transformant is induced by the *mcm* counterpart to produce microconidia.

Generation of hygromycin resistance transformants: The wild type strain of *N. crassa* OR23-I V *A* (FGSC 2489) was transformed by electroporation (Gene Pulsar II, BIORAD) with the plasmid pCSN44 containing the *hph* gene which encodes for hygromycin resistance (Staben *et al*. 1989 *Fungal Genet. Newsl*. **36:** 79-81). Macroconidia from a week-old culture grown on Vogel's Medium N (Davis and de Serres 1970 *Methods Enzymol*. **A17**: 79-143) containing 1.5% (w/v) sucrose were harvested by using sterile distilled water, and washed twice with 1M sorbitol. Approximately 1×10^8 conidia were mixed with 1µg of plasmid DNA in 40 µl of 1M sorbitol and electroporation was performed according to the method of Margolin *et al*. (*Fungal Genet. Newsl*., **44:** 34-36). The electroporated macroconidia were plated on sorbose plating medium (Davis and de Serres 1970 *Methods Enzymol*. **A17**: 79-143) without any selection pressure. After 20 hrs of growth at 34°C, the plates were overlaid with soft agar (0.7% agar) supplemented with hygromycin B (Sigma Chemical Company, MO, USA) at a concentration of 150 µg/ml for selection of the hph^+ transformants and further incubated at 34°C. Transformant colonies started appearing after five days of incubation, and they grew out of the soft agar. These colonies were transferred to slants of Vogel's Medium N supplemented with 1.5 % (w/v) sucrose and 150 µg/ml of hygromycin B. Out of fifty colonies that were picked and subcultured, forty were found to be hygromycin resistant. The presence of the transforming *hph* gene was confirmed by Southern analysis using linearised pCSN44 as a probe (data not shown). The primary transformants confirmed by Southern analysis were used for the next step of purification.

Purification of the primary transformant: To purify the hygromycin resistant transformants, a strain of *N. crassa*, *al-1; mcm arg-5* was constructed using the following parent stocks: *al-1* (JH 216, FGSC 3714); *arg-5* (P9125), and *mcm* (RM124-2A, FGSC 7455). Macroconidia of the constructed strain and that of the primary transformant were mixed in a ratio of 2:1 respectively, and centrifuged to form a pellet. The pellet was transferred to agar slants of Vogel's Medium N with 1.5 % (w/v) sucrose and grown at 34°C to allow the formation of heterokaryon. After 7-10 days of growth, the macroconidia were harvested and inoculated in liquid Vogel's N Medium with 1.5 % (w/v) sucrose and grown at 22°C with shaking at 240 rpm to produce microconidia. Abundant microconidia were produced because of the expression from the *mcm* mutant counterpart of the heterokaryon (Maheshwari 1991 *Exp. Mycol*. **15**: 346-350). Since both the transformant nuclei and the *mcm* mutant nuclei share a common cytoplasm in the heterokaryon, the *mcm* nuclei induce the microconidiation of the transformant nuclei as well (presumably through a diffusable factor). As a result, the microconidia formed would contain a mixed population of transformed and untransformed nuclei.

The microconidia were separated from the germinated macroconidia by filtering the culture suspension through a synthetic fabric, ThermoLam Plus (Metzenberg 1989 *Fungal Genet. Newsl.* **36**: 83) and plated on sorbose plating medium containing 150 µg/ml hygromycin B. After 24 hrs of growth at 34°C, another layer of soft agar containing hygromycin B (150 µg/ml) was overlaid. This would select only the homokaryotic hygromycin -resistant transformants. Colonies appearing after seven days were transferred to slants of Vogel's Medium N with 1.5 % sucrose and 150 µg/ml of hygromycin B. The examination of these purified cultures showed that they grew more vigorously as compared to the primary transformants. Moreover, the purified transformants showed a higher tolerance to hygromycin (600 µg/ml) as compared to the primary transformants. The presence of the *hph* gene in these purified transformants was further confirmed by Southern analysis (not shown).

This simple and easy method can be used to purify primary transformant of any genotype without any further genetic modification.

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