CHAPTER 29

Gene Transfer by Electroporation of Filamentous Fungi

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1. Introduction

The fungi encompass an enormous array of species, ranging from microscopic uninucleate, unicellular forms to multinucleate, coenocytic, highly differentiated macroscopic morphological forms. The wealth of diversity provided by a wide variety of life cycles, the availability of asexual and sexual modes of reproduction, and the presence of both haploid and diploid phases of the life cycle add further dimensions to this fascinating group of eukaryotic microbes. The tremendous potential of various taxa of filamentous fungi for biotechnological applications has yet to be realized. The filamentous fungi are emerging as a source of suitable hosts for expression of mammalian and other eukaryotic genes to yield products of commercial interest. They are ideally suited to this end by virtue of their normal mode of nutrition, based on the capacity for secretion of degradative enzymes, instrumental in conversion of complex growth substrates into simpler, readily utilizable derivatives that are absorbed by the growing fungal hyphae. Several species of filamentous fungi are known to produce pharmaceuticals, antibiotics, metabolites, phytohormones, and other industrially important products. There is a great deal of interest in the potential for the use of filamentous fungi as biocontrol agents—as antagonists of other fungal phytopathogens, as bioherbicides and bioinsecticides.

Species of Ascomycetes, such as *Neurospora crassa* and *Aspergillus nidulans,* are also of interest as useful model systems for investiga-

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tions of the molecular mechanisms of differentiation and regulation of eukaryotic gene expression. For virtually half a century of genetic research, *N. crassa*—along with the model prokaryote *Escherichia coli* and the eukaryote *Drosophila melanogaster*—has been the organism of choice for investigations of the fundamental principles of genetic recombination, eukaryotic genome organization and biochemical genetics, and regulation of metabolic pathways. The availability of a vast collection of auxotrophic and other mutants, together with the timely development of suitable experimental tools for molecular biological investigations, has rendered these model organisms invaluable for biological research.

Although some members of the Ascomycetes and Basidiomycetes are genetically well characterized, a vast majority of the fungal species, particularly the so-called imperfect fungi, lacking a sexual cycle, are not amenable to direct genetic analysis. The ability to carry out genetic manipulations with the objective of improving the yield of commercially important products of fungal species and to exploit filamentous fungi in biotechnology as host organisms for expression of eukaryotic genes is dependent on success in transferring cloned genes into fungal cells. Therefore, it is essential to have access to a variety of methods for transformation since individual species may require distinctive treatments, commensurate with the chemical composition of the cellular envelope. The use of spheroplasts in transformation experiments with filamentous fungi has been a common practice, ever since a suitable procedure was developed for *N. crassa* spheroplast formation by Case et al. *(1)*. Protocols based on slight modifications of the original method have been adapted successfully for use with several fungal species *(2)*. Although transformation of protoplasts results in high yields of transformants for some fungal species, notably *N. crassa,* the experimental procedure for preparation of protoplasts requires careful standardization of the individual steps. Often problems are encountered owing to the lack of reproducibility when different batches of commercial cell-wall-degrading enzyme preparations, such as Novozyme, are employed. Following transformation, regeneration of the cell wall is essential since protoplasts are extremely fragile. The success of the regeneration step, although vital in this procedure, can be variable. Furthermore, many of the fungal species do not yield protoplast preparations that are suitable for transformation experiments.

Consequently, attempts have been made to develop transformation protocols that do not require protoplasts. One of these procedures involves permeabilization of germinating *N. crassa* conidia by treatment with lithium acetate *(3)* followed by polyethylene glycol, adapted from a method originally developed for *S. cerevisiae* by Ito et al. *(4)*. However, this procedure has not been widely used, because lithium salts are found to be toxic for some fungal species and the overall transformation efficiencies have been reported to be generally low. A transformation system for filamentous fungi, independent of protoplast formation and potentially toxic alkali cations, is furnished by electroporation *(5)*.

2. Materials

2.1. Selectable Markers

The choice of dominant selectable markers depends on the availability of cloned marker genes that can be expressed in the recipient cells, yielding easily scorable phenotypic characteristics. Theoretically any fungal gene with a promoter region that is recognizable by the recipient's transcriptional apparatus can be employed. In this respect, the *N. crassa* gene encoding the benomyl-resistant mutant of β-tubulin *(6)* has proven to be a versatile marker for fungal species that are susceptible to benomyl, and it has been employed in the construction of cosmid vectors for genomic libraries.

A vector constructed with the hygromycin phosphotransferase gene of *E. coli,* pCSN44 *(7),* has proven to be suitable in many instances, the prime requisite being the susceptibility of the target fungus to hygromycin B. Alternatively, appropriate selectable, homologous marker genes can be used in conjunction with auxotrophic mutants as recipients for transforming DNA. A good example of this approach uses the qa-2 gene of *N. crassa,* encoding the catabolic dehydroquinase gene and the recipient strain R-206A, which is a double mutant, deficient in the catabolic as well as the biosynthetic dehydroquinase (*see* Notes 1–3 and Table 2). Table 1 lists the selectable markers and the plasmids employed by us using the protocols described in this chapter.

2.2. Recipient Cell Type and Pretreatment

The electroporation-based transformation procedure, originally developed by us for use with *N. crassa (5,8),* has been found to be readily applicable to other species of filamentous fungi, such as *Aspergillus*

Table 1
Species of Filamentous Fungi, Plasmids
for Electroporation and Selectable Markers Employed

Recipient species/strains	Selectable markers	Plasmids	Reference
Neurospora crassa			
Wild type (74-OR23-IVA)	*E. coli* hygB^r	pCSN44	*8*
	N. crassa ben^r	pBEN	
Auxotrophic mutant R-206A	*N. crassa* qa-$^{2+}$	Bsqa	*5,8*
Pencillium urticae (NRRL 2159A)	*E. coli* hygB^r	pCSN44	*8*
Aspergillus oryzae (ATCC 14895)	*N crassa* ben^r	pBEN	*8*
Beauveria bassiana (ATCC 7159)	*N. crassa* ben^r	pBEN	Unpublished

Table 2
Genes and Plasmids Used for Transformation of Germinated Conidia of *N. crassa*

Gene	Cotransformed Selectable marker	Phenotype conferred
N. crassa gdh-1 (NAD$^+$-glutamate dehydrogenase	*N. crassa* ben^r	Benomyl resistance
	E. coli hygB^r	Hygromycin B resistance
N. crassa hspe-1	*N. crassa* ben^r	Benomyl resistance
	E. coli hygB^r	Hygromycin B resistance
N. crassa hsps-1	*N. crassa* qa-$^{2+}$	Dehydroquinase production
Human mtIIA (metallothionein gene family)	None	Cadmium chloride resistance

oryzae, Leptosphaeria maculans, Penicillium urticae, and *Beauveria bassiana (B. sulfurescens).* With sporulating species, it is feasible to use sexual or asexual spores—macro- or microconidia, ascospores, and pycnidiospores. Although intact spores, with walls of varying thickness and chemical composition, are not suitable unless spheroplasts are formed first, spores during early stages of germination are excellent recipients for exogenous DNA, following brief pretreatment with a suitable enzyme (*see* Notes 4 and 5).

2.3. Solutions and Reagents for Growth of Fungi and Electroporation

1. Electroporation buffer: 1 m*M* HEPES buffer, pH 7.5, 50 m*M* mannitol.
2. β-glucuronidase (Sigma [St. Louis, MO] type H-1) is supplied in powder form. It is a partially purified preparation from *Helix pomatia* containing 300–400 U/mg β-glucuronidase activity and 15–40 U/mg of sulfatase activity (*see* Note 4).
3. Trace element solution: Dissolve successively in 95 mL water: 5.0 g citric acid · $1H_2O$, 5 g $ZnSO_4$ · $7H_2O$, 1.0 g $Fe(NH_4)_2(SO_4)_2$ · $6H_2O$, 0.25 g $CuSO_4$ · $5H_2O$, 0.05 g $MnSO_4$ · $1H_2O$, 0.05 g H_3BO_3 (anhydrous), and 0.05 g Na_2MoO_4 · $2H_2O$. Adjust total volume to 100 mL. Store at room temperature with 1 mL chloroform.
4. Biotin stock solution: Dissolve 5.0 mg in 100 mL of 50% ethanol. Store at –20°C in 2.5-mL aliquots.
5. Fries' medium: In a total volume of 500 mL, dissolve 2.5 g $(NH_4)_2$ tartrate; 0.5 g NH_4NO_3, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$ · $7H_2O$, 0.05 g $CaCl_2$, 0.05 g NaCl, 0.05 mL biotin solution, 0.05 mL trace element solution. Sucrose (1.5%) may be used as the carbon source.
6. Vogel's minimal medium (for 50X stock): Dissolve successively in 750 mL distilled water: 124 g sodium citrate · $2H_2O$, 250 g KH_2PO_4 (anhydrous), 100 g NH_4NO_3 (anhydrous), 5 g $CaCl_2$ · $2H_2O$ (dissolve separately in 10 mL water and add slowly), 2.5 mL biotin stock solution, 5.0 mL trace element solution. Adjust final volume to 1 L. Store at room temperature with 5 mL chloroform as a preservative. The pH of the 1X strength medium will be close to 5.8; adjustment is not necessary.
7. Czapek-Dox medium is available from Difco, Detroit, MI.
8. Mineral medium: For 1 L of medium, mix 2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.3 g $ZnSO_4$, 0.01 g $FeSO_4$ · $7H_2O$ dH_2O to 900 mL. Autoclave, and then add 100 mL of 30% (w/v) glucose presterilized by filtration.
9. Plasmid DNA: CsCl-purified or relatively crude, "miniprep" quality DNA is satisfactory.

3. Methods

3.1. Electroporation Protocol for Neurospora crassa

1. Germinate conidia from 7-d-old 50-mL cultures of the wild-type strain (74A) or 15-d-old 50-mL cultures of strain R-206A (auxotrophic for aromatic amino acids) in 0.5X Fries' medium *(9)*, containing the appropriate growth supplements, for 2 h at 30°C, while shaking at 150 rpm in a rotary shaker. Culture R-206A in a medium containing a carbon source supple-

mented with 80 µg/mL each of L-phenylalanine, L-tyrosine, and L-tryptophan, 2 µg/mL of *p*-aminobenzoic acid, and 0.2 µg/mL inositol (*see* Note 6).
2. Immediately after the emergence of the germ tubes, add 1 mg/mL solid β-glucuronidase (Sigma: type H1), and continue the treatment for another 2 h under the conditions specified in step 1 (*see* Notes 4 and 7).
3. Wash the germinated conidia free of the growth medium by centrifugation at 3000*g* for 5 min, and transfer to the electroporation buffer. Repeat the washing step three times by centrifugation, and resuspension of the conidial pellet.
4. Suspend the pellet in 0.5–1.0 mL of the electroporation buffer, and to 100-µL conidial suspension, add 1–2 µg of transforming DNA (in 1 or 2 µL). Chill the mixture on ice for 15 min, and subject to electroporation at room temperature. Controls, without added DNA, are treated in exactly the same manner.
5. Pulse once with a field strength of 12.5 kV/cm; capacitance, 25 µF; resistance, 400 Ω. This will yield a time constant of about 5 ms.
6. Immediately following electroporation, add 1 mL of minimal medium (with supplements, if the recipient strain is auxotrophic) to the electroporation cuvet, suspend the contents rapidly, transfer the mixture to a sterile test tube, and incubate for 2–3 h at 30°C, while shaking. This step is important for optimal recovery of transformants. Optimal recovery time should be determined empirically for each species/strain.
7. Finally, select the transformants by plating appropriate dilutions of the spore suspension on a suitable selection medium. The choice of the selection medium will depend on the plasmid/gene employed for transformation (*see* Note 8 *[10]*).
8. Fungal colonies appear within 48 h on benomyl plates and within 72–96 h on hygromycin B plates. Transfer individual colonies to agar slants containing the selection medium (*see* Notes 9 and 10 *[11–13]*).

3.2. *Electroporation Protocol for* Aspergillus oryzae

1. Prepare a dense spore suspension using a 10-d-old culture in 25 mL of Czapek-Dox liquid medium. Grow the culture at 30°C for 6 h with shaking or until germ tube emergence is evident by microscopic examination (*see* Note 6).
2. Add β-glucuronidase (1 mg/mL), and continue incubation while shaking under the same growth conditions for an additional 2 h.
3. Centrifuge the germinating spore suspension at 5900*g* in sterile glass centrifuge tubes for 10 min.
4. Gently remove the supernatant containing the enzyme using a sterile Pasteur pipet, resuspend the germinated spores in the electroporation buffer, and centrifuge at 5900*g* for 10 min. Resuspend the spores in the

Table 3
Experimental Conditions and Results of Electrotransformation of Filamentous Fungi with a Dominant Selective Marker

	Fungal species		
Conditions/results	*N. crassa*	*P. urticae*	*A oryzae*
Number of conidia ($\times 10^6$)	3.0–6.0	8.0	2 5
Percent viability	54–57	~68	52–55
Pretreatment			
β-glucuronidase	+	+	+
Chitinase	–	–	–
Selectable marker	Hygromycin B resistance	Hygromycin B resistance	Benomyl resistance
Transformation efficiency (stable transformants/ μg DNA)	$\sim 1.8 \times 10^3$	$\sim 2.6 \times 10^3$	$1–6 \times 10^2$

same buffer, and repeat the washing step two more times to ensure complete removal of β-glucuronidase. After the final washing step, suspend the pellet in 500 μL of the electroporation buffer.

5. Transfer 100 μL of spore suspension to a sterile 1.5-mL tube, and add 1 μL of transforming DNA (1–5 μg) (*see* Note 1). Incubate the mixture on ice for 15 min. Prepare control samples without added transforming DNA.
6. Transfer the mixture of the spore suspension and the transforming DNA to an electroporation cuvet, and pulse once with 11–12.5 kV/cm, capacitance, 25 μF. A time constant of 4.6–4.8 ms will result.
7. Add 1 mL of an appropriate growth medium, and incubate the suspension while shaking for 30–60 min. This step is necessary to ensure proper recovery of transformants (*see* Note 11).
8. Finally, plate appropriate dilutions of the spore suspension on selective medium. For average transformation efficiencies *see* Table 3.

3.3. Protocol for Electroporation of Hyphal Fragments

In the case of filamentous fungal species where spore formation is either inefficient or the spores are not suitable for electroporation for other reasons, it is feasible to use hyphal fragments directly in electroporation experiments. An example is the following protocol that we have used with *Beauveria* species.

1. Prepare the initial inoculum by transferring approx 3-mm (approx 50 mg wet wt) of hyphal mass from a slant, using a sterile platinum loop, to 25 mL of mineral medium *(14)* in a 125-mL Erlenmeyer flask.

2. Allow the culture to grow at room temperature while shaking at 100 rpm on a rotary shaker for 48 h. During this time, the hyphal clusters increase in mass at a slow rate.
3. Transfer 100–200-µL vol of the medium with the fungal material to sterile 1.5-mL microcentrifuge tubes, and vortex at a moderate speed (e.g., setting of 4 of 5) for approx 30 s to fragment the hyphae. Pellet the mycelial fragments by centrifugation at 12,000*g* for 10 min at room temperature.
4. Remove the supernatant, and wash the pelleted fungal cells three times with sterile distilled water to remove the salts in the growth medium. Washing is carried out by resuspension of the cells followed by centrifugation. Following the final wash, suspend the cells in 500 µL of distilled water by vortexing (*see* Note 12).
5. To a sterile electroporation cuvet, add 5 µg plasmid DNA (1 µg/µL), 200 µL of the fungal cell suspension, and 200 µL sterile distilled water, and mix the contents gently. Pulse once with a field strength of 12.5 kV, capacitance, 25 µF, resistance, 400 Ω. This will produce a time constant of about 5 ms.
6. Dilute the electroporated mixture with 1 mL of mineral medium, transfer to a sterile 1.5-mL tube, and incubate at room temperature for 2 h. Plate appropriate volumes of the suspension (50–200 µL) on medium containing 1.5% agar.
7. After the surface has dried, overlay the plates with 5–6 mL of soft agar (0.7% agar + *B. bassiana* liquid medium containing 2 or 5 µg/mL benomyl), and incubate the plates at room temperature. Benomyl-resistant colonies will arise within 3–4 d. No colonies should be observed on controls electroporated in the absence of plasmid DNA and plated subsequently on benomyl-containing medium. Transformation efficiencies are estimated on the basis of the number of benomyl-resistant colonies recovered/µg of input plasmid DNA (*see* Note 13).

4. Notes

1. Cotransformation along with the dominant selectable gene has proven to be an effective mode of introducing target DNA into the recipient cells. The frequency of uptake of target DNA (nonselectable marker) along with the selectable marker is often sufficiently high for most purposes.
2. The overall efficiency of electrotransformation, assessed by determination of the average number of stable transformants recovered/µg of input DNA varies depending on the selectable marker and the host species/strain (Table 2). For instance, the auxotrophic strain R-206A of *N. crassa,* when used as the recipient with qa-2 DNA, resulted in a low yield of transformants (21 stable transformants/µg DNA), whereas the wild-type host strain was transformed with a markedly greater efficiency.

3. The optimal electroporation parameters were found to be comparable for all the filamentous fungal species tested (Table 3). The empirically determined combination of high field strength (12.5 kV/cm; the maximum output of our instrument) and capacitance value of 25 μF resulted in <50% reduction in viability of the treated cells.
4. A gentle treatment of the germinating spores with a suitable hydrolytic enzyme will often suffice to attain the requisite weakening of the cell walls. For this purpose, an appropriate enzyme should be selected in view of the chemical composition of the cell wall of the particular species. Commercial preparations containing cellulase, β-glucanase, β-glucuronidase, and chitinase can be used alone or in combination, depending on the target species. In this procedure, enzymatic treatment is brief to permit a slight weakening of the cell wall without extensive degradation. Protoplast formation is not required for success of electroporation. In fact, protoplasts, being extremely fragile, are susceptible to damage by the electroporation treatment.
5. For asexual spores (conidia), treatment with the mycolytic enzyme β-glucuronidase, at a concentration of 1 mg/mL, was found to be adequate. It is advisable to monitor the effect of the enzyme on cell viability and the rate of germination by frequent microscopic examination. Under the specified conditions, β-glucuronidase treatment was not detrimental for hyphal growth or cell viability, and spheroplast formation was not observed. However, if it is found necessary to use a higher concentration of β-glucuronidase and/or prolonged exposure, a reduction in viability may result. Treatment of pycnidiospores of *Leptosphaeria maculans* with thick, pigmented, chitinous spore walls requires the concomitant use of chitinase and β-glucuronidase *(8)*.
6. The age of the starting material is a critical factor in the yield of transformants. Since wild-type and mutants strains often differ widely in their rate of growth, the most suitable age should be determined empirically for each species and strain.
7. For each strain, the progress of germination should be monitored microscopically. For faster-growing strains, incubation intervals should be adjusted accordingly. Enzymatic treatment should not be excessively long; normally 1–2 h will be sufficient.
8. For *N. crassa,* we use Vogel's minimal medium *(10)* with 1% sorbose, 0.1% glucose, 0.1% fructose, and 1.5% agar (supplemented with 2 μg/mL benomyl for selection of resistant transformants). If hygromycin B-resistance is used as a selectable marker, the *N. crassa* conidial suspension is plated on the sorbose-glucose-fructose-agar medium without hygromycin B. The plates are incubated for 24 h, after which a 0.7% soft-agar overlay containing 200 μL/mL hygromycin B is applied. This two-step procedure

has been found to improve the yield of transformants, since a combination of sorbose and hygromycin B may prove to be lethal for the early stages of hyphal growth.

Alternatively, for selection of hygromycin B-resistant transformants of wild-type *N. crassa,* electroporated cells can be plated on δ Vogel's minimal medium—0.05% yeast extract—0.05% casein hydrolysate—0.01% fructose—1.5% agar, supplemented with 200 μg/mL hygromycin B. Following incubation for 24 h at 30°C, the plates are layered with 5 mL agar containing the same growth medium without hygromycin B.

9. Average values for transformation efficiency are given in Table 3.
10. We have utilized the above procedure for transformation of *N. crassa* germinating conidia using plasmids harboring heterologous genes, such as the human mtIIA *(11),* as well as homologous *N. crassa* genes. Efficient cotransformation was obtained for genes encoding NAD-specific glutamate dehydrogenase, *gdh*-1 *(12),* heat-shock protein 80, *hspe*-1 *(13),* 70-kDa heat-shock-protein, *hsps*-1 *(15),* and qa-2 *(1),* with benomyl-resistant β-tubulin *(6)* and hygromycin phosphotransferase *(7)* genes as selectable markers (Table 2). Southern blot hybridization showed the integration of introduced DNA at ectopic sites in the host genome *(8).*
11. Longer recovery times may be required if the yield of transformants is low. It may be necessary to adjust the recovery conditions for other species/strains of *Aspergillus.*
12. In contrast with germinating spores, treatment with β-glucuronidase is not necessary for successful transformation of hyphal fragments. Following transformation, hyphal fragments can regenerate readily, and a considerable simplification of the procedure is achieved. If hyphal fragments are used, however, care should be exercised in determining the appropriate stage of hyphal growth that yields the best results. Younger hyphae are best suited for electroporation without β-glucuronidase pretreatment.
13. Estimates of hyphal transformation efficiencies are approximate, since accurate counts of hyphal fragments are difficult owing to the variation in fragment size and the possibility of aggregation.
14. These electroporation procedures are simple, convenient, and inexpensive methods for DNA-mediated transformation of filamentous fungi. Neither spheroplast formation nor the use of potentially toxic chemicals is required; germinated spores and hyphal fragments are used directly. Furthermore, relatively crude preparations of plasmid DNA, such as those generated by "minipreps," yield satisfactory results. The yield of transformants is sufficiently high for most genetic manipulations and biotechnological applications.
15. The above protocol can be adapted for use with virtually any filamentous fungal species, provided appropriate selectable markers are available.

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