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Transformation of Filamentous Fungi by Microprojectile Bombardment

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Introduction

The vast majority of transformation protocols for filamentous fungi are based on permeabilizing cell membranes with polyethylene glycol (PEG) or electroporation. Both methods typically require preparing protoplasts or osmotically sensitive cells prior to transformation (Herzog *et al.*, 1996; Goldman *et al.*, 1990; a detailed review of fungal transformation systems is given by Lemke and Peng, 1995). Over the last few years, microprojectile bombardment has become a powerful tool for transformation of intact cells, particularly for the transformation of fungal strains that do not yield sufficient numbers of regenerating protoplasts or species that cannot be grown in culture, such as obligate plant pathogens. Biolistic® transformation of *Saccharomyces cerevisiae* cells was optimized using the PDS-1000/He system (Heiser, 1992). In addition, several filamentous fungi have been transformed by means of microprojectile bombardment (Table 1). In this study, the PDS-1000/He system was used for stable nuclear transformation of an *Aspergillus nidulans* strain deficient in pyrimidine synthesis by selection for prototrophic transformants. The effect of conidial density, helium pressure, and size of microprojectiles on transformation frequency were investigated. Transformation frequency, mitotic stability of transformants, and Southern blot hybridization patterns were compared between conidia transformed by particle bombardment and protoplasts transformed by chemical (PEG-mediated) transformation. In addition, optimized conditions for microprojectile bombardment were compared with literature data on Biolistic transformation of other fungal species.

Materials and Methods

Strain and plasmid

Aspergillus nidulans strain A773 (*pyrG89*, *wA3*, *pyroA4*) was obtained from the Fungal Genetics Stock Center, Dept. of Microbiology, University of Kansas Medical Center, Kansas City, Kansas, and maintained on solid YG medium [0.5% yeast extract, 2% glucose, 2% agar, pH 6.3] supplemented with 0.12% uracil and 0.12% uridine. Plates were incubated for 6–8 days at 37 °C at which time conidia were harvested in 0.9% sterile saline. The resuspended conidia were sonicated in an ultra sound sonicator for 1 min prior to being used for protoplast formation or for microprojectile bombardment.

Plasmid pRG-1, a 5 kb plasmid containing the *Neurospora crassa pyr4* gene cloned into pUC9 was used for transformation experiments. This plasmid has been shown to complement the *pyrG89* mutation in *A. nidulans* (Ballance *et al.*, 1983; Waring *et al.*, 1989).

Biolistic transformation

Intact conidia were spread onto 10 ml of solid YG medium (in a 100 x 15 mm petri dish), briefly air-dried under sterile conditions, and used for microprojectile bombardment within 1–3 hours. Each plate was bombarded twice. Tungsten particles (M5, M10, or M17 particles, Bio-Rad Laboratories, Hercules, California) were prepared and coated with plasmid DNA as described by Daniell (1993). The following were added in order to a 1.5 ml microcentrifuge tube: 25 µl tungsten particle suspension (1.5 mg in 50% glycerol), 5 µl plasmid DNA (0.5 µg/µl), 25 µl 2.5 M $CaCl_2$, and 5 µl 1 M spermidine free base. After each addition the suspension was vigorously vortexed and incubated on ice for 10 min. The DNA-coated tungsten particles were then

Table 1. Nuclear Transformation of Filamentous Fungi by Microprojectile Bombardment

Species	Target	Selection	References
Aspergillus nidulans	conidia	Prototrophy	Herzog *et al.* 1996, Fungaro *et al.* 1995
Trichoderma harzianum	conidia	Hygromycin resistance	Lorito *et al.* 1993
Gliocladium virens	conidia	Hygromycin resistance	Lorito *et al.* 1993
Phytophthora ssp.	mycelia	Hygromycin resistance and GUS activity[3]	Bailey *et al.* 1993
Uromyces appendiculatus[1]	uredospores	GUS[3]	Li *et al.* 1993, Bhiari and Staples 1992
Uncinula necator[2]	hyphae	Benomyl resistance	Smith *et al.* 1992
Neurospora crassa	conidia	Prototrophy	Armaleo *et al.* 1990
Botryotinia fuckeliana	conidia	Hygromycin resistance	Hilber *et al.* 1994
Paxillus involutus	mycelia	Hygromycin resistance and GUS activity[3]	Bills *et al.* 1995

1. Obligate plant pathogen, transient gene expression 2. First obligately plant-pathogenic fungus transformed 3. Gus = β-glucuronidase

pelleted in a microfuge for 10 seconds. After removing the supernatant, the tungsten particles were washed with ethanol, resuspended in 30 µl ethanol, and 8 µl loaded onto each of three macrocarrier discs for bombardment in the PDS-1000/He instrument. Target distance (6 cm), vacuum (28 in Hg) and other parameters were identical to conditions optimized for nuclear transformation of *S. cerevisiae* cells (Heiser, 1992). The bombardment pressure was varied from 400 to 1,600 psi. Bombarded plates were incubated at 37 °C for 48 hours. Control plates were bombarded with tungsten particles which were prepared as described above but not coated with plasmid.

Formation and transformation of protoplasts

PEG-mediated transformation of protoplasts was performed as described by Herzog *et al.*, 1996.

DNA isolations and Southern blot analysis

DNA was isolated from transformed and non-transformed mycelia by the procedure of Raeder and Broda (1985). Samples of DNA were digested with *Pst*I which cuts pRG-1 at a single site (Waring *et al.*, 1989). Southern blot hybridizations were carried out as outlined by Sambrook *et al.* (1989). Plasmid DNA probes were radioactively labeled using the Random Primed DNA Labeling Kit from United States Biochemical (Cleveland, Ohio).

Results and Discussion

In initial experiments using M10 tungsten particles, a helium pressure of 1,200 psi, and other bombardment parameters were used as optimized for nuclear transformation of *S. cerevisiae* (Heiser, 1992). The spore density was varied from 10^6–10^8 conidia per plate. When at least 10^7 conidia were plated, colonies with a prototrophic phenotype were found, indicating that the *pyrG89* mutation of the pyrimidine auxotrophic *A. nidulans* strain was successfully complemented by transformation with pRG-1 plasmid DNA (Figure 1). The first transformants were visible 24–36 hours after bombardment. After 48 hours, transformants were counted and transferred onto fresh selective plates for further study.

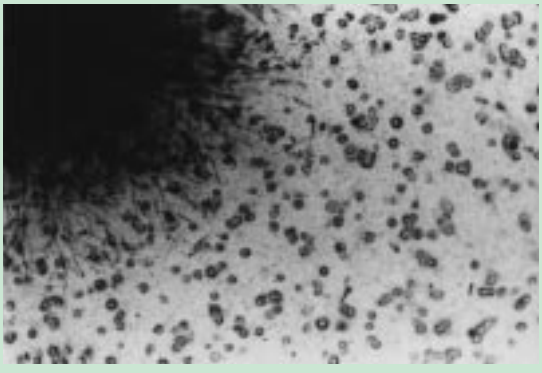

Fig. 1. A section of a plate 48 hrs after bombardment of 10^7 *A. nidulans* conidia bombarded with tungsten particles coated with pRG-1. Mycelium of a putative transformant is visible in the upper left corner on a background of non-transformed conidia. Tungsten particles are also visible near the edges of the growing colony. Magnification: approximately 150x.

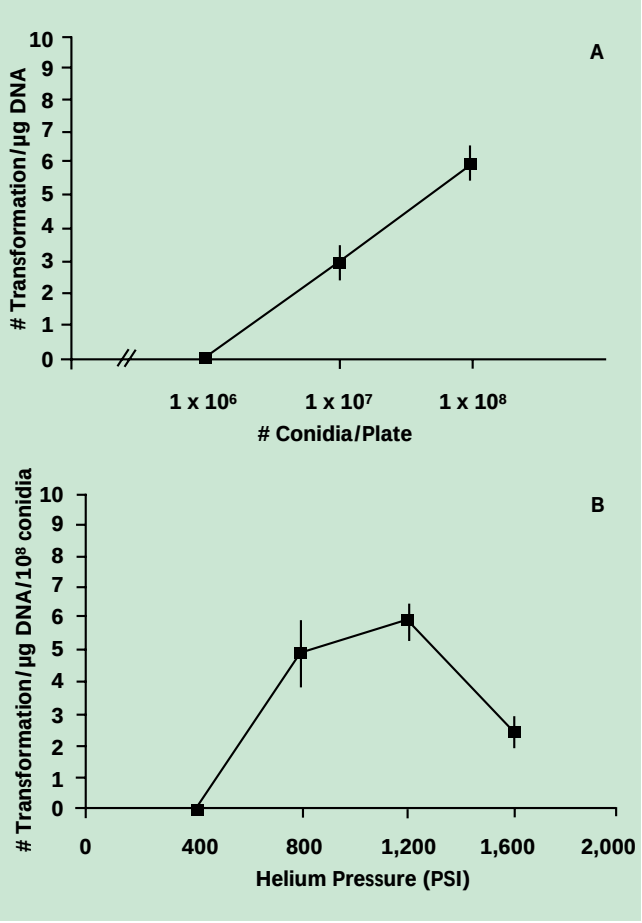

Fig. 2. *A. nidulans* transformants as a function of (A) the number of *A. nidulans* conidia per plate bombarded at a helium pressure of 1,200 psi, and (B) helium pressure, bombarding 10^8 conidia per plate. Mean values of at least five independent experiments with M10 tungsten particles (mean diameter of 0.7 µm) are shown. Vertical bars indicate standard deviations. Modified from Herzog *et al.*, 1996, with permission from Springer Verlag, Berlin.

Conidial density was found to be a crucial parameter in optimizating transformation frequency (Figure 2A). In subsequent experiments, 10^8 conidia were spread on each plate. Further increasing the spore density, overincubation of plates, or prolonged storage of conidial suspensions prior to the transformation experiment resulted in the occurrence of background colonies. Transformants were found under a wide range of helium pressures (800–1,600 psi, Figure 2B) with a reduced transformation frequency at 1,600 psi, possibly due to increased cell death. No transformants were produced by bombardment at 400 psi. No significant difference in transformation frequency was observed when M5 (mean diameter 0.4 µm), M10 (0.7µm) and M17 (1.1 µm) tungsten particles were compared (data not shown). Particles made of material other than tungsten were not tested. The addition of an osmotic stabilizer to the media (1 M sorbitol) did not increase the transformation efficiency. This result is consistent with the results of others who reported that fungal spores or mycelia were transformed in the absence of an osmoticum (see references in Table 1).

Table 2. Optimized conditions for Biolistic transformation of *Aspergillus nidulans* compared to data given by Lorito *et al.* for *T. harzianum* and *G. virens*

Variable	*A. nidulans*	*T. harzianum* and *G. virens*
Conidia/plate	10^8	10^7
Helium pressure	1,200 psi	1,200 psi
Chamber vacuum	28 inches Hg	29 inches Hg
Target distance	6 cm	6 cm
Mean diameter of microprojectiles	0.4–1.1 µm	1.1 µm
Microprojectiles material	tungsten	tungsten
Reference	Herzog *et al.* 1996	Lorito *et al.* 1993

Microprojectile bombardment resulted in an overall transformation frequency (6 ± 0.5 transformants/µg DNA) that was somewhat lower than the frequency observed by chemical transformation of protoplasts (20 ± 4 transformants/µg DNA, Herzog *et al.*, 1996). However, only about 30% of the transformants obtained from regenerated protoplasts were stable transformants (for further details see Herzog *et al.*, 1996). On the other hand, among Biolistic transformants, 60–70% were mitotically stable. Therefore, the frequency with which stable *A. nidulans* transformants were obtained was similar for both techniques (4 transformants/µg DNA for Biolistic transformation, 6 transformants/µg DNA for chemical transformation). The reason for this difference may be because fungal protoplasts are typically enucleate to multinucleate, while conidia of *A. nidulans* are uninucleate (Bennett & Klich, 1992). Consequently, in a protoplast, only one of several nuclei might be transformed, thus resulting in a lower proportion of stable transformants compared to Biolistic transformation of *Aspergillus* conidia. A more detailed analysis of this phenomenon is given by Lorito *et al.* (1993) who made similar observations during their study of *T. harzianum* and *G. virens*.

Analysis of genomic DNA by Southern blot hybridization showed that Biolistic transformants were indistinguishable from transformants isolated previously following PEG-mediated transformation (Ballance *et al.*, 1983). Furthermore, microprojectile bombardment often resulted in the delivery of several copies of the pRG-1 plasmid per conidium. Tandem repeat integration of the whole plasmid with varying copy number (Figure 3, lanes 7–9) as well as integrations at multiple sites had taken place (Figure 3, lanes 5 and 6). Such integration is thought to result from a single cross-over event (type I or type II as illustrated by Lemke and Peng, 1995) between the plasmid and chromosomal DNA.

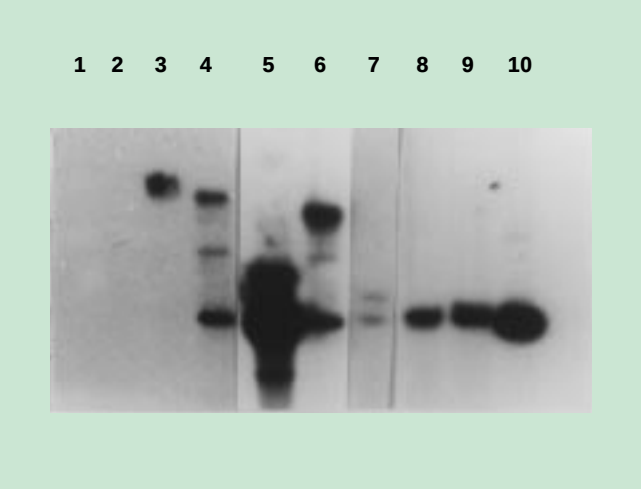

Fig. 3. A Southern blot of total DNA isolated from stable transformants produced by particle bombardment or PEG-mediated transformation. DNA was isolated, digested with restriction enzyme, separated on agarose gels, transferred to nylon membrane, and probed with ^{32}P-labelled pUC9 DNA as described in Materials and Methods. Lanes 1 and 2, DNA from a non-transformed *A. nidulans* strain A773 colony. Lanes 3 and 4, DNA isolated from a PEG-mediated transformant. Lanes 5–9, DNA isolated from transformants produced by particle bombardment. Lane 10, pRG-1 DNA. Lanes 1, 3, undigested DNA. Lanes 2 and 4–10, *Pst*I-digested DNA. 10 µg of fungal DNA were loaded per lane. Marginal markers designate 1-kb DNA ladder fragments (Gibco BRL). Modified from Herzog *et al.*, 1996, with permission from Springer Verlag, Berlin.

Conclusions

Aspergillus nidulans conidia are an ideal target for transformation by microprojectile bombardment due to their uninucleate character and the ease with which conidial density can be controlled and transformants selected and quantified. Since *A. nidulans* conidial suspensions are a heterogeneous mixture of spores (with a minimum diameter of 3 µm and various stages of maturity, cell wall thickness and composition), and tungsten particles are extremely heterogeneous in size, similar transformation frequencies over a relatively wide range of conditions might not be surprising. These findings are consistent with the study by Lorito *et al.* (1993) who demonstrated that the number of conidia per plate significantly affected transfor-

mation efficiency of *T. harzianum* and *G. virens* conidia, whereas changes in the helium pressure (800 vs. 1,200 psi) did not affect transformation efficiencies. Bombardment conditions optimized for *T. harzianum* and *G. virens* are compared in Table 2. Transformation frequencies for each of these species by particle bombardment or chemical transformation were comparable (Lorito *et al.*, 1993; Herzog *et al.*, 1996). Integration of several copies of the entire plasmid at multiple sites and/or as tandem repeats, as evident from the Southern blot, was found to be the predominant fate of introduced DNA in stable transformants for both conidia transformed by particle bombardment and protoplasts transformed by PEG-mediated transformation (Figure 3, lanes 3–9; Waring *et al.*, 1989).

These results show that microprojectile bombardment is a very efficient method for transformation of intact *Aspergillus* conidia. A major advantage is that pre-treatment of cells prior to transformation is unnecessary. The conditions in Table 2 can be used as a starting point for optimizing transformation of other filamentous fungi. Additionally, particle bombardment can be an especially effective approach for transformation of obligately parasitic fungi that require transformation of intact cells.

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