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A novel improved method for *Aspergillus nidulans* transformation

Marina Koukaki [1], Eleni Giannoutsou [1], Amalia Karagouni, George Diallinas *

Department of Botany, Faculty of Biology, University of Athens, Panepistimioupolis, Athens 15781, Greece

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Abstract

We systematically investigated the efficiency of *Aspergillus nidulans* transformation using protoplasts prepared from different stages of conidiospore germination and young mycelium. Using standard integrative plasmids, increased transformation yields were obtained with protoplasts isolated from a specific stage coincident with germ tube emergence. This increase ranged, on the average, from two- to eightfold depending on different plasmids used. Transformation efficiencies with a replicative plasmid were similar to those obtained using previously described methods. Although this observation suggests that elevated transformation efficiencies might be due to increased efficiency of recombination between plasmid and genomic sequences, we cannot exclude other factors associated with the particular developmental stage used. In the course of this study, we also examined the effect of other parameters that might enhance transformation yields. The method described is also significantly easier and faster than other current methods.
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Keywords: *Aspergillus nidulans*; Transformation; Replicative and integrative plasmid; Conidiospore germination

1. Introduction

Application of virtually all molecular genetic approaches to filamentous fungi has been achieved since the development of transformation protocols for *Aspergillus nidulans* and *Neurospora crassa* in the early 1980s (Fincham, 1989; van den Hondel and Punt, 1991). It appears possible that all fungal species that can be grown in culture, including those of medical, agricultural or biotechnological importance, can be genetically transformed. However, unlike the case in unicellular yeasts, transformation of filamentous fungi is hampered by the relatively low frequencies obtained. The high transformation efficiency obtained in yeast is due to the development of a variety of autonomously replicating plasmids as vectors of gene transfer. For long, the lack of analogous vectors in filamentous fungi led to the development of transformation systems based on the stable homologous or heterologous chromosomal integration of plasmid sequences. The relative frequencies of different systems seem to vary significantly according to both the recipient strain, the selection marker used or whether the transforming DNA is linear or supercoiled (Fincham, 1989; van den Hondel and Punt, 1991; Bird and Bradshaw, 1997). A critical step towards the development of improved transformation systems in filamentous fungi has been the description of an autonomously replicating plasmid that transforms *A. nidulans* at 100-fold increased frequencies (Gems et al., 1991;

* Corresponding author. Tel.: +30-210-7244649; fax: +30-210-7274702.
E-mail address: diallina@biol.uoa.gr (G. Diallinas).
[1] Both authors contributed equally to this work.

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Aleksenko et al., 1996; Aleksenko and Clutterbuck, 1997). For several filamentous fungi, present-day frequencies are now sufficiently high to allow direct cloning of genes by complementation of the corresponding mutants. However, current protocols are still inefficient for more sophisticated manipulations such as gene replacement (Bird and Bradshaw, 1997; Chaveroche et al., 2000) or the use of filamentous fungi as model systems for cloning by genetic complementation genes from organisms with more complex genomes such as plants or animals. Electroporation and biolostic transformation have proved inefficient (Meyer et al., 2003). Efficient *Agrobacterium tumefaciens*-mediated transformation of several commercially important filamentous fungi has recently been described and promises to be an important tool for fungal reverse genetics (de Groot et al., 1998). A drawback of T-DNA-mediated transformation is that it always occurs via random heterologous integration and cannot be used for gene replacements.

A. nidulans transformation protocols have essentially remained unchanged since they first appeared in 1983 (Tilburn et al., 1983; Ballance et al., 1983). All protocols described are based on the biochemical removal of the cell wall for the preparation and isolation of protoplasts from young mycelium, introduction of exogenous DNA by protoplast fusion in the presence of polyethylene glycol and calcium ions, and regeneration of transformants in the appropriate selective media. Protoplast fusion allows the uptake and the subsequent integration of plasmid DNA into the genome. Fused protoplasts have multiple nuclei but usually a single nucleus is transformed. In *A. nidulans*, the heterokaryon is easily resolved through the formation of uninucleate conidiospores. However, in many other fungi, several rounds of streaking for isolation may be necessary to purify a homokaryotic transformant. Apart from the kind of plasmid used, protoplast number and quality also constitute a critical transformation-limiting determinant.

In this article, we describe a novel, simplified, transformation system for *A. nidulans*. This method is based on the use of protoplasts prepared from a specific stage during conidiospore germination. Our method is not only easier and faster but also leads to increased transformation frequencies.

2. Materials and methods

2.1. Growth conditions, strains and plasmids

Minimal media (MM) and complete media (CM) for *A. nidulans* have been described previously (Tilburn et al., 1983; Cove, 1966). Several different strains were used for transformations in this work: *argB2 uapA24 uapC201/401 azgA4 yA2*, *argB2 pabaA1 pantoB100*, *uapA24 uapC201/401 azgA4 pabaA1* and *pyrG89 pabaA1 pantoB100*. Selection of transformants, in different experiments, was based on complementation of *argB2*, *pyrG89* or *uapA24*. *argB2* is a total loss-of-function mutation in the gene encoding ornithine carbamoyl transferase, an enzyme essential for arginine biosynthesis. *argB2* mutants cannot grow in the absence of arginine from the selective medium. *pyrG89* mutants are blocked in pyrimidine biosynthesis because they lack orotidine-5′-phosphate-decarboxylase and, thus, cannot grow in the absence of uracil or uridine from the selective medium. Mutation *uapA24* is a total loss-of-function missense mutation (A404D). Strains lacking UapA activity cannot take up uric acid or xanthine efficiently, especially at 25 °C, and, thus, they do not grow on these purines as sole nitrogen sources. Mutations *azgA4* and *uapC201/401* are loss-of-function mutations in the adenine–hypoxanthine–guanine transporter and general purine transporter, respectively (Diallinas et al., 1995). *yA2* results in yellow conidiospores. *pantoB100* and *pabaA1* result in D-pantothenic acid and *p*-aminobenzoic acid auxotrophies, respectively. These mutations do not affect arginine, purine or pyrimidine metabolism, and, thus, they do not affect the transformation selection schemes used in this work. Plasmids used for transformations in the course of this work were the following: pFB9, a pUC8 vector carrying the *argB* gene (Upshall et al., 1986). pAN503, a plasmid constructed by cloning the *uapA* gene in *pBluescript* KS+ (Gorfinkiel et al., 1993). pAN510, a plasmid constructed by cloning the *uapA* gene in the *Sal*I site of pFB9 (Diallinas et al.,1998). pFB6 is a plasmid carrying the *pyr4* gene of *N. crassa*, which has previously been shown to complement *pyrG89* in *A. nidulans* (Ballance et al., 1983). pFB9, pAN510 and pFB6 are all integrative plasmids, which give stable transformants through the homologous or heterologous recombination of plasmid and chromo-

somal sequences. Transformants with these plasmids can arise from either single-copy or multiple-copy plasmid integrations. pDGH25 is a version of pFB9 also carrying the 5.0-kb replication-promoting element AMA1 (Chaveroche et al., 2000). Transformants with this plasmid are highly unstable due to mitotic plasmid loss.

2.2. Transformations

Transformation of protoplasts prepared from mycelium was essentially as described by Tilburn et al. (1983). *Aspergillus* strains were grown for 7 h 30 min at 37 °C in MM, supplemented with 5 mM urea as a nitrogen source, necessary vitamins and either 5 mM arginine (*argB2* strains) or 10 mM uracil and uridine (*pyrG89*). Under these conditions, approximately 0.4–0.6 mg of young, modestly branched, mycelium was obtained after inoculation of 800-ml cultures, in 2-l flasks, with 10^{10-11} conidiospores. Mycelium was resuspended in 20 ml of isosmotic buffer (1.2 M $MgSO_4$, 10 mM orthophosphate, pH 5.8; Tilburn et al., 1983), and protoplastation was carried out using cell wall lysing enzymes (Glucanex; Novozymes, Switzerland) at a final concentration of 100 mg per 0.5 mg of mycelium, for 90 min, at 30 °C. A total of 10^7 protoplasts, resuspended in 1 M Sorbitol, 10 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.4, were used for each transformation.

Preparation of protoplasts from conidiospores was as follows. One liter of MM containing 5 mM urea as a N source, and supplemented with arginine or uracil and uridine depending on the strain used, was inoculated with approximately 10^{10-11} conidiospores and incubated at 37 °C with 140 rpm. Prior to inoculation, conidiospores collected from Petri dish were separated from mycelium by filtration through sterile blutex. After 3 h of incubation, aliquots of 100 ml (10^{9-10} conidiospores) were rapidly removed at 15-min intervals, until 5 h and 15 min of germination. Non-germinated (3 h to 4 h 30 min) or germinated (4 h 45 min to 5 h 15 min) conidiospores were collected with centrifugation at 4000 rpm for 10 min and resuspended in 10 ml of the standard isosmotic buffer (Tilburn et al., 1983). In different experiments, Glucanex was added at concentrations of 50–350 mg per aliquot, and was allowed to act, at 30 °C, for 30–150 min. Best transformation efficiencies were obtained with protoplasts obtained with 100 mg of Glucanex after 60–90 min of incubation. The efficiency of protoplastation was established by counting and comparing viable conidiospores under isosmotic or non-isosmotic conditions. Protoplastation efficiency, under conditions established as best for transformation, reached 50%. Protoplastation could also be viewed directly under a phase-contrast optical microscope. Non-protoplasted conidiospores were darker and smaller than protoplasts. Conidiospore protoplasts were washed twice in 1 M Sorbitol, 10 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.4, resuspended in the same buffer at a concentration 10^8 conidiospores/200 μl and distributed in sterile Eppendorfs. Each Eppendorf was used for a single transformation experiment with 0.1–1 μg of plasmid, as described in a standard protocol (Tilburn et al., 1983). In some experiments, after the final wash, protoplasts were resuspended in 1 ml non-selective medium (MM supplemented for all auxotrophies or CM) and incubated for 2 h, at 37 °C, before plating on selective medium.

3. Results

3.1. Rationale of our approach

In the course of transporter-mediated amino acid or purine uptake studies with germinating conidiospores, we have observed increased non-saturable ‘‘background’’ uptake rates, compatible with diffusion of radiolabelled solutes across the plasma membrane, prior to germ tube emergence. Increased diffusion of solutes is compatible with increased plasma membrane permeability. In addition, a recent study has shown that conidiospores just prior to germ tube emergence form protoplasts more easily than mature hyphae (Jung et al., 2000), an observation that supports the notion that cell walls of conidiospores ready for germination are also looser than cell walls of mycelia. Considering the above observation, we thought whether we could profit from these transient developmental changes in the cell wall and plasma membrane of *A. nidulans* and increase the efficiency by which exogenous DNA enters the cytoplasm. For that, we performed and compared a series of transformations, using different plasmids, with protoplasts prepared from different stages of conidiospore germination and from young mycelium.

Transformations were carried out using selection schemes based on either the complementation of arginine auxotrophy (*argB2*), pyrimidine auxotrophy (*pyrG89*) or the inability to take up uric acid as a nitrogen source (*uapA24*). Five plasmids, described below and in Section 2, were used. Complementation of an *argB2* strain for growth in the absence of arginine was carried out with both integrative (pFB9 or pAN510) and replicative plasmids (pDHG25). Complementation of a *pyrG89* mutant was carried out with integrative plasmid pFB6. Complementation of a *uapA24* strain was carried out using integrative plasmid pAN503. Plasmids pFB9, pFB6 and pAN510 have been reported to have similar transformation efficiencies, ranging from 40 to 500 transformants per microgram of DNA per 10^{6-7} protoplasts (Fincham, 1989; Ballance et al., 1983; Upshall et al., 1986; unpublished results). pAN503 has a rather lower efficiency of transformation (10–40 transformants per microgram of DNA; Gorfinkiel et al., 1993). Replicative plasmid pDHG25 has been reported to give up to 100,000 transformants per microgram of DNA per 10^{6-7} protoplasts (Chaveroche et al., 2000). However, it should be stressed that this very high frequency has been calculated from experiments using 10–100 ng of DNA per 10^{6-7} protoplasts and that the correlation between the amount of DNA added and transformation frequency is linear for up to 100–200 ng of DNA. In our hands, in standard transformations using plasmid concentrations of 0.1–1.0 μg of DNA, pDHG25 gives 1000–10,000 transformants per microgram of plasmid. It is also of significance that pDHG25 transformants are mitotically unstable, being lost from 65–99.9% of asexual progeny.

3.2. Transformation frequencies are dependent on the germination stage of conidiospores

The vegetative mycelium of *A. nidulans* is composed of multinucleate cells partitioned at uniform intervals by septa. Mycelium development is initiated by the germination of the asexual uninucleate conidiospores. Resting conidiospores are arrested in the G1 phase of the cell cycle and undergo the first nuclear division as the spore swells and breaks dormancy, and a second nuclear division concomitant with germ tube emergence. First and second nuclear divisions occur approximately after 3–4 and 5–6 h of germination, respectively, in MM at 37 °C (see Fig. 1). In rich or complete media, this occurs at 2 and 3 h 30 min, respectively (unpublished observations). Very few studies have addressed gene expression during conidiospore germination, but it seems that conidiospores committed to germination, after the first nuclear division, are metabolically similar to young mycelium (Tazebay et al., 1997; S. Amillis et al., submitted).

We have allowed conidiospores of *argB2*, *pyrG89* or *uapA24* strains to germinate in appropriately supplemented MM for a period of 3 h 15 min to 5 h 15 min (see Section 2). Under these conditions, germ tube emergence was microscopically observed in samples germinated for at least 4 h 30 min (see Fig. 1). Samples were collected at 15-min intervals and used to prepare protoplasts as described in Section 2. *argB2* protoplasts were transformed with plasmids carrying an *argB*$^{+}$ allele (pFB9, pAN510 or pDHG25), *pyrG89* protoplasts with plasmid pFB6 carrying the *N. crassa pyr4*$^{+}$ gene, and *uapA24* protoplasts with plasmid pAN503. In these experiments, 0.5 μg of integrative plasmids pFB9, pAN510, pAN503 or pFB6, or 0.1 μg of replicative plasmid pDHG25, was used per transformation (see below for the rationale of using these concentrations). Transformants were visible after 2 days of incubation at 37 °C for *argB2* or *pyrG89* complementation or after 4 days of incubation at 25 °C for *uapA24* complementation. Fig. 1 shows average transformation frequencies of three independent experiments using pFB6, pAN510, pDHG25 or of two experiments using pAN503. Results with pFB9 were more or less identical to those obtained with pAN510 (not shown). Significantly higher frequencies were obtained, with all plasmids used and with protoplasts from a very specific germination point, which is 4 h 30 min after initial inoculation. Highest transformation frequencies coincided with germ tube emergence and tend to decrease in young developing mycelium (5 h 15 min).

3.3. Transformation frequencies with conidiospores are higher than those with mycelium

Results in Fig. 1 strongly suggested that transformation using germinating conidiospores might be more efficient than transformation using mycelium. We directly compared the relative transformation

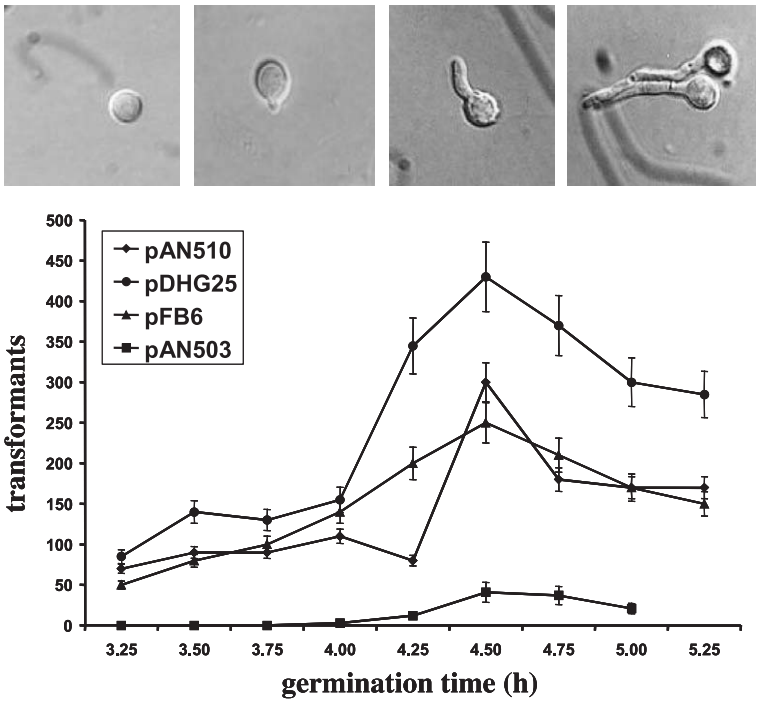

Fig. 1. Transformation efficiency obtained with protoplasts prepared from various stages of conidiospore germination. Experiments were carried out as described in Section 2. Results shown are averages of at least three independent experiments with standard deviations less than 30%. Plasmids used were the following: integrative plasmids pAN510 (*argB*$^+$), pAN503 (*uapA*$^+$) and pFB6 (*pyr4*$^+$) and replicative plasmid pDHG25 (*argB*$^+$). Concentrations used were 500 ng for the integrative plasmids and 100 ng for the replicative plasmid.

efficiencies of different plasmids using protoplasts isolated from conidiospores at the time of germ tube emergence and protoplasts obtained, using standard protocols, from mycelium. Conidiospores germinating for 4 h 30 min and mycelium developed after 7 h 30 min were collected from two cultures, one of an *argB2* strain, the other of a *pyrG89* strain. Different transformations were carried out with pAN510, pDHG25 and pFB6, at plasmid concentrations ranging from 10 to 2000 ng. Results presenting two independent experiments are shown in Table 1. In all cases using integrative plasmids pAN510 and pFB6, conidiospore transformation was more efficient than mycelium transformation. Increases in individual experiments as high as 12-fold were obtained. An average increase of 8-fold in transformation efficiency was obtained with conidiospores transformed with plasmid pAN510, and of 2.5-fold increase with pFB6. No difference was observed in transformation efficiencies with replicative plasmid pDHG25.

Transformation efficiencies with integrative plasmids increase linearly from 20 ng to 2 μg. Transformation efficiency with replicative plasmid pDHG25 increases linearly from 10 to 200 ng. These results were obtained with 10^8 conidiospore protoplasts or 10^7 mycelium protoplasts per transformation. Lower transformation frequencies were obtained in experiments using 10^7 conidiospore protoplasts or 10^6 mycelium protoplasts.

3.4. Homologous versus heterologous integration in conidiospore and mycelium transformations

We have analyzed 50 transformants obtained with conidiospores and 50 transformants obtained with mycelium, by Southern blots. Transformants analyzed were obtained in several independent experiments using plasmid pAN510. Genomic DNA extracted from these transformants was digested with *Eco*RI, a restriction endonuclease that does not cut within the *uapA* or *argB* sequences or vector sequences of

Table 1
Comparison of transformation efficiencies obtained with protoplasts isolated from conidiospores and from mycelium using different amounts of plasmid DNA

Nanograms of plasmid DNA/ transformation reaction	Number of transformants					
	Conidiospores			Mycelium		
	pAN510	pFB6	pDHG25	pAN510	pFB6	pDHG25
10	nd	nd	13–20	nd	nd	2–12
20	0–4	3–10	26–42	nd	nd	35–44
50	11–14	15–19	244–269	nd	nd	215–245
100	52–70	30–54	451–557	6–10	15–19	498–541
200	106–119	56–96	1002–1321	nd	nd	nd
500	187–214	136–148	>1000	nd	nd	nd
1000	492–513	163–171	nd	42–102	50–123	>1000
2000	>1000	211–242	nd	nd	nd	nd

Protoplasts form conidiospores (germination time: 4 h 30 min) and from mycelium (germination time: 7h 30 min) were transformed with integrative plasmids pAN510 (*argB* complementation) or pFB6 (*pyrG* complementation) or with replicative plasmid pDHG25 (*argB* complementation). Results shown represent two independent experiments. nd = not determined.

pAN510. Southern analysis was performed with both *uapA* and *argB* specific probes obtained by PCR (not shown). This allows the identification of three kinds of recombination events leading to transformants arising from the homologous integration of pAN510 in either the *uapA* or the *argB* genomic loci, or by heterologous integration in unknown loci. Transformants obtained with mycelium were 58% the result of homologous recombination (29 transformants) and 36% the result of heterologous recombination (18 transformants). Of the strains analyzed, 6% did not show any evidence for plasmid integration and should, thus, have arisen by gene conversion of the *argB2* mutation. These frequencies were in agreement with results obtained in the course of many years in our laboratory. Transformants obtained with conidiospores were 18% the result of homologous recombination (9 transformants) and 78% the result of heterologous recombination (39 transformants). Two strains were compatible with gene conversion at the *argB2* locus. In both cases, homologous integrations occurred approximately 50% in the *argB* locus and 50% in the *uapA* locus. Thus, it seems that the method described herein favors heterologous integration events. Whether this is a general effect or specifically due to the plasmid used (pAN510) was not further investigated. Finally, transformations using conidiospores or mycelium showed similar relative frequencies of single versus multiple integrations events (approximately 50%) or *argB2* gene conversion frequencies (4–6%).

3.5. In search of other factors affecting transformation frequencies

Cell wall lysis and protoplastation is accomplished using commercially available mixtures of enzymes (cellulase, protease, chitinase, b-glucuronidase) such as Novozyme 234 (InterSpex Products), Lysing Enzymes (Sigma) or Glucanex (Novozymes). However, particular lots of these enzyme mixtures give different protoplastation efficiencies. A recent study has shown that β-D-glucanase (InterSpex Products), combined with yeast lytic enzyme from *Arthrobacter luteus* (ICN Biomedicals), as a substitute for the above enzyme mixtures, is a good alternative for efficient protoplastation (Jung et al., 2000). In addition, and to our knowledge, no published study has systematically examined neither the significance of adding different concentrations of cell wall lysing enzymes nor the effect of different protoplastation periods on the efficiency of transformation. We have examined the effect of these two factors on transformation frequencies using Glucanex and the method described herein. Table 2 summarizes our results and shows that both factors significantly affect transformation efficiency. Best results were obtained with 100 mg of Glucanex for 60–90 min. Higher amounts of Glucanex or longer incubation periods might give more protoplasts, as judged microscopically, but it seems that in those cases, most of the protoplasts were not viable (results not shown).

Table 2
The effect of cell wall lysing enzyme (Glucanex) concentration and incubation time on conidiospore transformation efficiency

Effect of concentration		Effect of incubation time	
Glucanex concentration (mg/ml)	Number of transformants/ 200 ng pAN510	Time of incubation with Glucanex (min)	Number of transformants/ 200 ng pAN510
50	22	30	5
100	60	60	34
150	17	90	35
200	6	120	7
250	2	150	9
300	7		
350	5		

Experiments were carried out as described in Section 2. Results shown are averages of two independent experiments with standard deviations less than 20%. The effect of Glucanex concentration was tested in incubations of 90 min. The effect of incubation time was tested in the presence of 100 mg/ml of Glucanex.

A recent study has examined the role of dithiothreitol (DTT) on *A. nidulans* transformation and reported that 1 mM DTT increased transformation efficiency 2- to 13-fold (Dawe et al., 2000). We have examined the role of DTT on our method and found no effect on transformation frequencies. Whether this is due to the different isosmotic buffers or different recipient strains used, the different developmental stage from which protoplasts were prepared, or to the fact that in the previous study, protoplasts were stored overnight at 4 °C, was not investigated.

We also examined the possibility that the introduction of a non-selective incubation step prior to selection of transformants might increase frequencies of transformants. This step might allow time for an ''incoming'' plasmid to be properly expressed. After the last wash, transformed protoplasts were resuspended in either CM or appropriately supplemented MM, for 2 h, harvested by centrifugation and plated on selective media (results not shown).

4. Discussion

The method described for *A. nidulans* transformation provides several advantages when compared to classical methods making use of protoplasts prepared from mycelium. First, it is significantly faster (5 h 30 min from initial inoculation to final plating on selective media, compared to 10–20 h of previous methods) avoiding the inconvenience of longer incubation (8–12 h). Second, it is much easier as it does not require a step for separating protoplasts from non-protoplasted mycelium. Third, and more important, the method described herein gives higher transformation frequencies with all integrative plasmids and strains used. Increased transformation frequencies vary from experiment to experiment and depend on the plasmid and the strain used, but on the average, range from 2- to 8-fold increases, and in individual experiments up to 12-fold, compared to standard methods. In these experiments, we used four different integrative plasmids, one replicative plasmid, three different selection schemes, and several strains with different genetic backgrounds. After numerous transformation experiments, not described herein, carried out in our laboratory for the last 30 months, the method described always showed higher transformation frequencies than those obtained previously using standard methods. The fact that this method does not lead to increased transformation efficiencies with replicative plasmids suggests that increased transformation efficiencies obtained with integrative plasmids, might not be due, as we have hypothesized initially, to increased protoplastation efficiency and increased plasma membrane permeability of conidiospores, but rather, to either more efficient plasmid integration on the genome, or more efficient localization in the nucleus, of the recipient strain.

This work showed systematically that integrative plasmid transformation efficiencies in *A. nidulans* are dependent, not only on the plasmid and the recipient strain used, but also on the developmental stage from which protoplasts were made. Evidently, this stage should be defined for each new recipient strain used. The fact that the replicative plasmid transformation efficiencies do not depend on the developmental stage from which protoplasts were made suggests that either stable integration of the transforming sequences on the genome or trafficking to the nucleus, might be a transformation efficiency-limiting step that is affected by the developmental stage of the recipient cells. Plasmid sizes cannot be responsible for this difference as pDHG25 (replicative) and pAN510 (integrative) are of similar size. Further evidence that increased transformation efficiencies obtained with conidiospores might be due to increased heterologous integration events came from the Southern blot analysis described.

How could the developmental stage of protoplasts affect heterologous integration of plasmids on the genome? Conidiospores used to obtain increased transformation efficiencies are at a stage immediately after the first nuclear division and before the second nuclear division. Previous work in our laboratory has shown that several genes are differentially regulated in germinating conidiospores and mycelium, an indication that the chromatin status in these two distinct developmental stages should differ (Tazebay et al., 1997; S. Amillis et al., submitted). In a relevant study, it has been shown that the efficiency of gene targeting (homologous integration) was unaltered when transcription was induced at different stages in the transformation procedure, and that the most dramatic factor affecting targeting efficiency was the specific locus studied, with targeting of *niaD* (nitrate utilization) being at least fivefold more efficient than *amdS* (acetamide utilization) (Bird and Bradshaw, 1997). This is not in contradiction with our suggestion on the role of chromatin on integration, as our results not only refer to another gene (*argB*) but also suggest that increased transformation efficiency in conidiospores arises from increased heterologous, rather than homologous, integration. The same study has also shown that protoplast regeneration temperature has an effect, with enhanced homologous integration observed at 25 °C compared with 37 °C. We have shown that a non-selective pre-incubation step prior to selection increases, mildly, transformation frequencies, but we have not investigated further whether this affects homologous or heterologous integration events.

This work does not intend to address questions concerning why transformation efficiencies with integrative plasmids are increased in conidiospores or what is the role of the chromatin on plasmid integration. However, it clearly shows that biochemical transformation protocols can further be improved and provides the rationale for exploiting aspects of *A. nidulans* life cycle, which, in combination with specific strains and selection markers, will allow the development of novel highly improved transformation methods. Finally, it could offer a good alternative approach with strains where mycelium is either recalcitrant to protoplastation or protoplasts from mycelium are inefficiently transformed.

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