Transformation of *Mucor miehei* results in plasmid deletion and phenotypic instability

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Abstract

*Mucor miehei* transformants resistant to geneticin have been obtained by treatment of protoplasts with different plasmids and by *Agrobacterium*-mediated DNA transfer. All transformants exhibited a very unstable phenotype whose maintenance required continuous selective pressure. Molecular analysis of transformants showed that the plasmid DNA was extensively modified and maintained in very low amount. Our results indicate that *M. miehei* reluctance to transformation is due to different causes, including the coenocytic nature of its mycelium and the existence of specific mechanisms for the detection and elimination of foreign DNA.

Keywords: Aspartic protease; Fungal transformation geneticin resistance; *Mucor circinelloides*; *Mucor miehei*

1. Introduction

*Mucor* is the representative genus of filamentous fungi classified within the order *Mucorales*, which in turn is the main group of *Zygomycetes*. From a biotechnological point of view, *Mucor miehei* is the most relevant species of the genus due to its capability of producing enzymes of industrial importance, in particular aspartic protease used as a substitutive of chymosin in cheese making [1] and lipase. The genes encoding these enzymes have been characterized [2,3] and the three-dimensional structures of both protease and lipase have been determined [4–6]. In general, fungal species of biotechnological importance are fairly well known genetically and have been extensively manipulated. Among filamentous fungi, the best-known examples, albeit taxonomically distant form *Mucor*, are *Aspergillus*, *Penicillium* and *Trichoderma*. In contrast, *M. miehei* remains poorly characterized. Whereas the genetic transformation of another *Mucor* species, *Mucor circinelloides*, has been well documented [7–14], transformation of *M. miehei* has not been reported up to the present. In this paper we report the results of our attempts to transform this fungus by different procedures. This is a continuation of previous work [15] aimed to gain knowledge about *M. miehei*, make it more amenable to genetic manipulation and improve its capability of producing enzymes of industrial interest.

2. Materials and methods

2.1. Microbial strains and plasmids

*M. miehei* ATCC 26282 was used as the recipient for transformation. *Agrobacterium tumefaciens* strains LBA4404 [16], GV3101, GV3101pgic, EHA105 and EHA105pgic [17,18] were used as T-DNA donors in the *Agrobacterium*-mediated transformation.

Plasmids pBluescript [19–20] and pUC18 [21] were used for the construction of pAM1, pAM2, pAM3 and pAM4 plasmids. These four plasmids contain the kanamycin resistance gene *aphl* from the *Escherichia coli* transposon Tn903 [22], under control of the promoter and terminator of the TEF gene of *Ashbya gossypii* extracted from plasmid pFA6KanMX4 [23]. Plasmids pAM1 and pAM3 contain an autonomous replication sequence from *Phycomyces blakesleeanus* (ARSP) extracted from plasmid pJL
Plasmids pAM2 and pMA4 contain a 1.6-kb fragment from *M. miehei* rDNA corresponding to the region between 5S and 18S subunits [15]. This fragment was amplified by polymerase chain reaction (PCR) from *M. miehei* DNA by using oligonucleotides M236 and M237 as primers. Plasmids pAM3 and pAM4 contain *M. miehei MMP1* gene encoding aspartic protease. The gene was amplified with oligonucleotides M253 and M143, which correspond to sequences located upstream and downstream of the translated frame, respectively. For *A. tumefaciens*-mediated transformation, plasmids pBin19 [25] and pGreen0000 [26] were used.

### 2.2. Media and culture conditions

Media and growth conditions for *M. miehei* were those described by van Heeswijck and Roncero [7]. *M. miehei* transformants were selected on plates of minimal medium containing 550 μg ml⁻¹ of the kanamycin analogue G-418 (geneticin) sulfate (Gibco BRL). *A. tumefaciens* cultivation and induction was done following standard procedures [27,28]. The antibiotic concentration initially employed for transformation was several times higher than the minimal inhibitory concentration. Such a high concentration was chosen to minimize risks of selecting false positive clones that might arise as a consequence of high density plating or mutation. A lower amount of the antibiotic was found to be effective in subsequent experiments.

### 2.3. DNA techniques

For the purification of genomic DNA from *M. miehei* spores were inoculated in liquid complete medium and grown for 24 h. When the DNA was prepared from transformants, the medium was supplemented with G-418 sulfate (100 μg ml⁻¹). Mycelium was harvested by filtration from 200 ml cultures (yielding ca. 4 g wet weight), washed with water, frozen in liquid nitrogen, ground with a mortar and the DNA was extracted by the Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biotech). DNA sequences were amplified by PCR as previously described [15]. Oligonucleotides used as primers are listed in Table 1. In some cases, the sequence of the primer was modified with respect to its template by including a site for a restriction endonuclease that facilitated subsequent cloning of the amplified fragment. Other molecular biology techniques were carried out following standard protocols [29].

### 2.4. Transformation

Transformation of *M. miehei* protoplasts with plasmids was carried out as described for *M. circinelloides* [7]. Protoplasts were prepared according to the procedure described by van Heeswijck [30]. *Agrobacterium*-mediated T-DNA transfer experiments were performed as described by Bundock et al. [28] with the modifications of Groot et al. [31].

### 2.5. Milk-clotting assay

The medium used for protease production is a paste made of wheat bran and wheat flour prepared as described by Thakur et al. [32]. The medium was inoculated with about 10⁴ spores g⁻¹ and incubated at 37°C for 4 days. The enzyme was extracted from the culture with water (5 ml g⁻¹) and milk-clotting activity was measured as described by Thakur et al. [32]. Activity is expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 ml of a solution containing 0.1 g skim milk powder and 1.5 mg calcium chloride, at 35°C, in 40 min.

### 3. Results

#### 3.1. Plasmid-mediated transformation

Plasmids pAM1 to pAM4 (Fig. 1) were constructed to try different transformation strategies for *M. miehei*. Plasmids pAM1 and pAM3 were designed to test autonomous replication, as they contain the ARSP sequence from *P. blakesleeanus* [24]. Plasmids pAM2 and pAM4 were designed to promote their integration into the rDNA locus of the *M. miehei* genome, by homologous recombination. They lack the ARSP element and carry a 1.6-kb fragment from the region of *M. miehei* ribosomal DNA situated between the 5S and 18S subunits [15]. Plasmids pAM3 and pAM4, in addition to aforementioned elements, contain the *MMP1* gene encoding *M. miehei* aspartyl protease [2]. *M. miehei* protoplasts were treated with pJL2, pFA6-KanMX4, and pAM1-pAM4 plasmids and transformants were selected in medium containing geneticin. Fig. 2 shows the number of transformants recovered for each plasmid as a function of the amount of DNA used in the transformation. About 10 transformants per μg DNA were obtained using pJL2 and a similar number was obtained...
with pAM1 and pAM3. Transformation with pFA6-KanMX4 also gave colonies with resistance to kanamycin but the frequency of transformation was lower, about two transformants per \( \mu g \) of DNA. Results for plasmids pAM2 and pAM4 were better than those of pFA6-KanMX4, but lower than those of pJL2. The fact that the frequency of transformation obtained with plasmids harboring \( P. \) blakesleeanus ARSP was consistently higher than that obtained with the other plasmids suggests that this sequence is functional in \( M. \) miehei.

Production of aspartic protease by \( M. \) miehei clones transformed by plasmids pAM3 and pAM4, which carried the \( MMP1 \) gene, was assayed by measuring the milk-clotting activity of crude preparations of secreted protein produced by these clones. Transformants that had been subjected to several cycles on selective medium were used to assure a plasmid content as high as possible. Enzyme preparations were obtained as described in Section 2. No significant variation of enzyme activity was observed when the amounts of enzyme produced by the wild-type strain and pAM3 and pAM4 transformants were compared. Similar mean values of enzyme activity, about 4000 units per ml of crude enzyme preparation, were obtained after analyzing several clones from the wild-type and each type of transformant.

3.2. Agrobacterium-mediated transformation

\( A. \) tumefaciens has been successfully used as a mediator for the transformation of different fungal species: \( S. \) cerevisiae, \( A. \) awamori, \( A. \) niger, \( C. \) gloeosporioides, \( F. \) venenatum, \( T. \) reesei, \( N. \) crassa and \( A. \) bisporus, with genes included in tumor-inducing plasmids [28,31]. We have tested this procedure to transform \( M. \) miehei. We constructed plasmid pAM5 by cloning the coding region from the \( E. \) coli transposon Tn903 kanamycin resistance gene between the left border (LB) and right border (RB) repeats (T-DNA) of vector pUC18. The positions of different oligonucleotides (M239, M240, etc., see Table 1) are indicated.

Fig. 1. Maps of plasmids used for protoplasts transformation. A: pJL2 (6.2 kb); B: pFA6kanMX4 (5.0 kb); C: pAM1 (4.8 kb); D: pAM2 (5.6 kb); E: pAM3 (6.4 kb); F: pAM4 (7.2 kb). Relevant genetic elements are shown. An 1.5-kb fragment with the promoter (P) and terminator (T) of the TEF gene of A. gossypii flanking the aphJ (kanamycin resistance) gene is present in most plasmids. The presence of the ARSP from \( P. \) blakesleeanus (0.9 kb), the ribosomal DNA from \( M. \) miehei (Mm rDNA) (1.6 kb), and the aspartic protease gene from \( M. \) miehei (MMP1) (1.4 kb) are indicated. All six plasmids carry an ampicillin resistance gene and an \( E. \) coli replication origin taken from the pUC18 vector. The positions of different oligonucleotides (M239, M240, etc., see Table 1) are indicated.

Fig. 2. Transformation frequency of \( M. \) miehei protoplasts as a function of the amount of plasmid DNA used.

Fig. 3. Plasmid used for Agrobacterium-mediated transformation of \( M. \) miehei. Plasmid pBin19 is a standard vector used for the transformation of \( A. \) tumefaciens, described by Bevan [25]. Plasmid pAM5 was constructed by inserting the aphJ (kanamycin resistance) gene within the transference region of an \( A. \) tumefaciens vector.
also used plasmid pBin19, which contains a kanamycin resistance gene (\textit{nptIII}) within the T-DNA region [25]. Plasmids pAM5 and pBin19 were transferred by the freeze–thaw method [33] into different \textit{A. tumefaciens} strains. Incubation of \textit{M. miehei} germinated spores with the transformed \textit{A. tumefaciens} led to the formation of geneticin resistant colonies. All tested \textit{Agrobacterium} strains treated either with pAM5 or pBin19 gave rise to kanamycin resistant \textit{M. miehei} transformants at frequencies ranging between a minimum of about 50 (strain GV3101) and about a maximum of about 800 (strain LBA4404) transformants per 10^7 spores.

### 3.3. Plasmid stability

It is known that transformation of 	extit{Zygomycetes} is difficult and often gives rise to unstable clones. The coenocytic nature of the mycelium makes transformation more complicated and likely affects stability [8,9,11,34–36]. In our case, mitotic instability of the kanamycin resistant phenotype and plasmid loss became all too evident. Speres of the transformant clones obtained with the different plasmids were grown in non-selective medium and sporulated. The resulting spores were plated on plates of both selective and non-selective medium. The mitotic stability of transformants was determined as the percentage of spores able to yield colonies on geneticin-containing plates. The values obtained were similar for the different plasmids, about 0.1%. In an attempt to select for stable transformants, strains harboring plasmids pJL2, pAM1 and pAM3 were subjected to consecutive cycles of sporulation in selective medium. After each cycle, spores were also plated in non-selective medium to determine plasmid stability. Results are shown in Fig. 4A. Stability improved after a few cycles, reaching a maximum of 35% for plasmid pJL2, and then became steady. In the case of pFA6KanMX4, pAM2 and pAM4, which lack the ARSP and were expected to undergo chromosome integration, a slightly different scheme was followed. Rounds of sporulation on selective medium were alternated with others on non-selective medium. Fig. 4B shows the evolution of the stability of these transformants. A similar scheme was followed with \textit{Agrobacterium} T-DNA transformants. Results are shown in Fig. 4C. In all cases, removal of the selective pressure led to a quick loss of the transformed phenotype.

### Fig. 4. Phenotypic stability of \textit{M. miehei} transformants. Stability was measured as the percentage of colonies grown on plates of non-selective medium which retained the capability of growing on selective medium. A: Analysis of transformants obtained with plasmids containing \textit{P. blakesleeanus} ARSP element, subjected to consecutive cycles of sporulation in selective medium. B: Analysis of transformants obtained with plasmids lacking ARSP. Rounds of sporulation on selective medium were alternated with others on non-selective medium. C: Analysis of transformants obtained by \textit{Agrobacterium}-mediated DNA transfer. Rounds of sporulation on selective medium were alternated with others on non-selective medium.

### Fig. 5. PCR amplification of plasmid sequences from the DNA of \textit{M. miehei} transformants. A: Lane 1, DNA size markers. All other lanes correspond to the result of PCR reactions carried out with oligonucleotides M239 and M240 as primers and the following DNAs as templates: lane 2, plasmid pJL2; lane 3, \textit{M. miehei} (untransformed) DNA. Lanes 4–8, \textit{M. miehei} transformed by the following plasmids: lane 4, pJL2; lane 5, pAM1; lane 6, pAM2; lane 7, pAM3; lane 8, pAM4. B: Lane 1, DNA size markers. Oligonucleotide pairs used as primers in the different reactions were: lanes 2, 4 and 6, M239 and SM240; lanes 3, 5 and 7, M239 and M328. DNA templates were: lanes 2 and 3, plasmid pAM3; lanes 4 and 5, untransformed \textit{M. miehei}; lanes 6 and 7, \textit{M. miehei} transformed with plasmid pAM3. C: Lane 1, DNA size markers. Oligonucleotide pairs used as primers in the different reactions were: lanes 2, 5 and 8, M239 and M240; lanes 3, 6, 9, M239 and M143; lanes 4, 7 and 10, M239 and M327. DNA templates were: lanes 2–4, plasmid pAM4; lanes 5–7, untransformed \textit{M. miehei}; lanes 8–10, \textit{M. miehei} transformed with plasmid pAM4.
3.4. Molecular analysis of transformants

As a first attempt to detect plasmid DNA in the transformants, we tried Southern analysis. Experiments were carried out using different probes and DNA preparations from different transformants. All of them failed to show hybridization. In some experiments, using a $^{32}$P-labelled probe of the aphI gene obtained by amplification with oligos M239 and M240, and DNA from transformants with highest stability, hybridization signals could be observed, although this result was not reproducible with other transformants or DNA preparations. As an alternative procedure to detect the presence of plasmid DNA in the transformants, we used PCR amplification. The positions of the different oligonucleotides used as primers in this analysis are shown in Fig. 1 and their sequences in Table 1. Fig. 5A shows the result of analyzing the presence of the aphI gene in the DNA of transformants obtained with the different plasmids. Primers used, M239 and M240, were expected to yield a 0.4-kb fragment, according to the sequence of the gene [23]. A band corresponding to this size resulted from the amplification of DNA from all transformants, but not from the untransformed strain. Fig. 5B shows the results of the amplification of DNA from a transformant obtained with plasmid pAM3. Amplification with primers M329 and M328 yields a fragment smaller than the expected size of 3.0 kb, which is recovered when plasmid pAM3 is used as the template. Fig. 5C shows the results of the amplification of DNA from a transformant obtained with plasmid pAM4. Amplification with primers M329 and M143 yields a fragment of 3.7 kb, which is the same size of the fragment resulting from amplification of plasmid pAM4 DNA. However, priming the transformant DNA with oligonucleotides M329 and M327 did not produce any amplification, suggesting that a modified version of the plasmid is present in the transformant.

4. Discussion

Our results show that transformation of M. miehei is feasible, but maintenance of the transformed phenotype is only possible under continuous selective pressure. Despite phenotypic instability, our results show that kanamycin-resistant colonies are transformants, and not the result of mutation or tolerance to the antibiotic. This was checked by negative controls included in each experiment, in which protoplasts that had not been treated with transformant DNA did not grow at all on the selective plates. Additionally, PCR analysis consistently detected the presence of foreign DNA in the transformants. Protoplasts are reproducibly transformed by using a relatively large amount of plasmid DNA. As protoplasts are fairly permeable to DNA, the need for an elevated amount of plasmid can be attributed to the action of defensive molecular mechanisms that prevent the establishment of foreign DNA. A separate effect is the high instability of transformed clones once the heterologous DNA has been expressed. In this respect, M. miehei has shown to be less amenable to transformation than other wild-type organisms. For instance, industrial yeasts usually require high amounts of DNA for their transformation, but once the transformation barrier has been overcome, it is possible to maintain and express the foreign DNA quite stably [37]. These observations suggest the existence of specific mechanisms that detect the presence of foreign DNA and determine its deletion. Interestingly, a higher frequency of transformation is consistently obtained with plasmids harboring the ARSP element suggesting that, although very unstable, these plasmids have the capability of autonomous replication in M. miehei. Plasmids lacking the ARSP element are also capable of transformation, although at lower efficiency, which might reflect the need of genomic integration.

Although the presence of heterologous DNA in M. miehei transformants has been shown by PCR amplification, analysis of transformants indicated that the plasmid DNA was modified and present in very low amount, what made Southern analysis impossible. These results could be explained by the coenocytic nature of the fungus: the presence of the gene conferring resistance to geneticin in a small fraction of the nuclei may be sufficient to confer resistance to the antibiotic.

Results obtained with M. miehei can be better understood by comparison with those derived from two taxonomically related species which have been extensively studied: M. circinelloides and P. blakesleeanus. Transformation systems for M. circinelloides are well developed. Transformants can be obtained with either integrative or self-replicating plasmids [8–10,13,14]. However, P. blakesleeanus has proved to be reluctant to transformation. Although transformation of P. blakesleeanus has been reported [24,34], available procedures are of no practical use because of the extreme instability of transformants [38], which behave in this respect very much like M. miehei. Procedures to improve the transformability of M. miehei must be devised if this species is going to be fruitful biotechnologically. The use of homologous promoters and ARS, to be characterized, could help to this purpose. Mutants less reactive to foreign DNA could be obtained. Because of the coenocytic nature of the fungus, another important objective would be to select against untransformed nuclei, which could be achieved by using appropriate genetic markers.

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