FULL PAPER

Sandra Bartsch · Christine Schimek
Johannes Wöstemeyer

Microprojectile bombardment as a reliable method for transformation of the mucoralean fungus *Absidia glauca*

Abstract Genetic analysis of all *Mucor*-like fungi is severely impaired by the low efficiency of transformation systems and the genetic instability of the introduced plasmid constructs. The transformation efficiency of one of the model systems among mucoralean fungi, *Absidia glauca*, was improved considerably by microprojectile bombardment. For this purpose, a plasmid was constructed conferring (i) neomycin resistance as a selective marker and (ii) fluorescence due to expression of the *gfp* gene from the jellyfish *Aequorea victoria*. Compared with previous techniques, this method offers increased efficiency, with considerably easier handling than procedures based on protoplasts and, therefore, improved reliability. The uninucleate sporangiospores of *A. glauca* can be transformed early during the germination process. At this stage the number of nuclei ranges between 1 and 2. Thus, the abundance of transgenic nuclei in the coenocytic mycelia is high, and fewer problems are encountered with detecting low expression levels of the genes used for selection and monitoring of transformants.

Key words Biolistic transformation · Green fluorescent protein (GFP) · Microprojectile bombardment · Mucoralean fungi · Zygomycetes

Introduction

Mucoralean fungi have proven their value as model organisms for elucidating a variety of fundamental biological phenomena. *Phycomyces blakesleeanus* (Phycomycetaceae) represents the most sensitive blue-light receptor system known in biology. Light-dependent directed growth of sporangiophores in this organism is paradigmatic for many other systems. Light physiology has been studied in detail, and the knowledge on blue-light receptors is increasing (Schmidt and Galland 1999; Yamazaki et al. 1996). Recently, the first steps were made in understanding fungal gravitropism and its relations to photobiology at the biochemical level (Schimek et al. 1999).

Mucor mucedo (Mucoraceae) especially was studied with respect to the sexual recognition system, which is based on the β-carotene derivative trisporic acid (Werkman 1976; van den Ende 1978; Czempinski et al. 1996). The trisporic acid system is believed to represent the general pheromone language of all mucoralean and perhaps of all zygomycetous fungi.

At the molecular level, *Absidia glauca* was studied as a model system for sexual development (Wöstemeyer and Brockhausen-Rohdemann 1987; Wöstemeyer et al. 1990) and for the analysis of parasexual genetic exchange with its mycoparasite, the facultative fusion biotroph *Parasitella parasitica* (Kellner et al. 1993; Vetter et al. 1994; Wöstemeyer et al. 1998). With respect to genetic analysis and manipulation, *A. glauca* offers the most advanced possibilities among zygomycetes. Genome complexity and chromosome sizes are known (Wöstemeyer and Burmester 1986; Kayser and Wöstemeyer 1991), and a variety of vector plasmids for transforming this species have been developed (Burmester et al. 1992; Schilde et al. 2001).

Apart from fundamental research, many *Mucor*-like fungi are biotechnically relevant and are employed for industrial processes such as the production of rennin-like proteases for cheese fermentation and stereoselective hydroxylation of steroid compounds. Fundamental research as well as technical strain improvement of mucoralean fungi are severely impaired by the paucity of genetic manipulation systems. Transformation frequencies are low in comparison with ascomyceteous systems, and essentially only a single genetic reporter system has been developed (Schilde et al. 2001). All techniques based on protoplast manipulations depend on cell wall-lytic enzymes, which work efficiently but are not commercially available and must be prepared from appropriate culture supernatants

S. Bartsch · C. Schimek · J. Wöstemeyer (✉)
Institute of Microbiology und Microbe Genetics, Friedrich-Schiller-University Jena, Neugasse 24, D-07743 Jena, Germany
Tel. +49-3641-949310/1; Fax +49-3641-949312
e-mail: b5wojo@rz-jena.de

(Wöstemeyer et al. 1987; Wöstemeyer and Wöstemeyer 1998).

In this article, we focus on introducing the biolistic method as a reliable tool for transforming *Absidia glauca*. The inherent disadvantages of protoplast transformation, the high number of nuclei leading to heterokaryotic situations with only few transformed nuclei in the coenocytic mycelia, handling problems, and a strong dependency of transformant yield on protoplast regeneration rates are efficiently avoided. The method works reliably and reproducibly.

Materials and methods

Strains and growth conditions

The *Absidia glauca* wild-type (+) strain CBS 100.48 (Fungal Reference Centre Jena FSU-329) was used for all experiments. Spores were harvested from plates after growth for 2 weeks at room temperature on supplemented minimal medium (Wöstemeyer 1985), solidified with 12 g/l agar (Oxoid; Wesel, Germany). Spores were induced for sporulation and synchronized by shaking at 150 rpm for 4 h at 20°C in liquid minimal medium, followed by incubation at 4°C overnight.

Plasmids

The plasmid pe-GFP-RAG was used throughout (Schilde et al. 2001). This plasmid carries three elements important for transformation and genetic establishment: the Tn5-derived neomycin resistance gene, the gene for the green fluorescent protein (GFP), controlled by the promotor of the gene for elongation factor-1α from *Absidia glauca*, and a highly repeated genetic element of *Absidia glauca* (*RAG1*; Wöstemeyer and Burmester 1986).

Transformation protocol

For all experiments, the Biolistic PDS-1000/He Particle Delivery System apparatus was employed (Biorad, Munich, Germany); 0.6 × 10^8 *Absidia glauca* asexual spores were plated on a sterile cellophane membrane on top of osmotically stabilized minimal medium, supplemented with 5 g/l yeast extract and 0.8 M sorbitol. The membrane facilitates removal of the spores after transformation. Bombardment was performed with M5 tungsten particles (Biorad) coated with plasmid DNA following a published protocol (Sanford et al. 1993). The Biolistic Particle Delivery System allows varying several parameters to increase transformation efficiency. We optimized helium pressure, target cell distance, flight distance of the Kapton disk, and the distance between rupture disk and flying disk. Changes of the physical parameters had only minor effects on transformation efficiency (Table 1). Most experiments were performed at 1100 psi helium pressure, 9 cm target cell distance, 10 mm

Table 1. Dependency of biolistic transformation rates of *Absidia glauca* on helium pressure

Helium pressure (psi)	Target cell distance (cm)	Transformation rate[a]
900	6	38.4
900	9	46.1
1100	6	50.3
1100	9	59.5
1300	6	45.8
1300	9	59.5
1550	6	59.5
1550	9	64.1

Germinating sporangiospores were used as targets; the flight distance was 10 mm

[a] Transformation rates are given in transformants/µg DNA and 10^7 target cells after selection on neomycin-containing solid medium

flight distance, and 1/4 in. distance between rupture disk and flying disk.

Regeneration and selection of transformants

Liquid medium, 10 ml osmotically stabilized with 0.8 M sorbitol, was added to the transformed cells and incubated for 2 h to enable regeneration of cell membrane lesions. Spores were washed off the cellophane membrane, diluted, and plated on selective medium containing 200 µg/ml neomycin (Sigma, Tanfkirchen, Germany).

Fluorescence and light microscopy

For microscopy, a Zeiss Axiophot photomicroscope equipped with differential interference contrast was used. Fluorescence was monitored with the Zeiss filter combination BP 485 nm excitation, FT 510 nm dichroic, and 515–585 nm emission.

DNA preparation and Southern blotting

DNA from *Absidia glauca* transformants was isolated by a published procedure (Schilde et al. 2001). Hybridization probes were labeled according to Feinberg and Vogelstein (1984); stringency and processing conditions were as described by Burmester and Czempinski (1994).

Results and discussion

pe-GFP-RAG (Fig. 1; Schilde et al. 2001), which carries a selective neomycin resistance gene and the *gfp* reporter gene, offers the opportunity to analyze transformants with a combination of primary selection for resistance, followed by screening for green fluorescence. By this two-step procedure, a general problem in all transformation protocols for mucoralean fungi, the appearance of false positives, is efficiently minimized. All transformants showed sectoring of GFP fluorescence, although the transformation was per-

formed at the 1–2 nuclei stage. This segregation behavior is expected because of autonomous replication and, therefore, mitotic instability of the vector plasmid. Despite many attempts in different organismic systems, integrative transformation of mucoralean fungi is extremely difficult to achieve and is obtained only after prolonged selection for
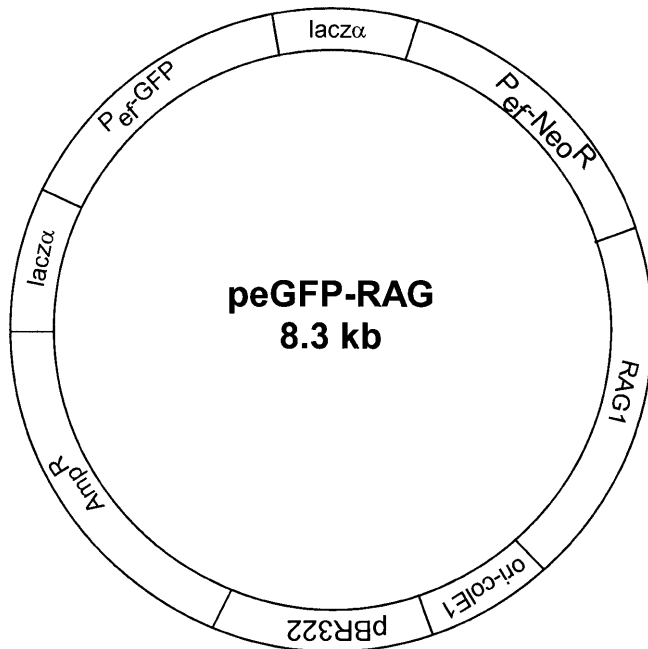

Fig. 1. Schematic map of the Neo^R− *gfp* plasmid *peGFP-RAG*. P_{ef}-*GFP*, *gfp* gene under the control of the *Absidia glauca* elongation factor EF1 promoter, cloned into a laczα cassette; P_{ef}-*NeoR*, Tn5-derived neomycine resistance gene, controlled by the same promoter; *RAG1*, a homologous highly repetitive DNA element

stability over several sporulation cycles and with very low efficiency (Arnau et al. 1991; Arnau and Stromann 1993; Wada et al. 1996). Sectoring in the growing mycelium occurs apparently randomly. In 80% of the transformants, however, sporangia exhibit enhanced fluorescence of the columella (Fig. 2). Presently, we do not know if the higher concentrations of GFP are due to increased protein biosynthesis in this region, or if the eEF1α promoter, which is normally regarded as being constitutive, is preferentially active in the columellae.

The novel transformation procedure improves the experimental protocol considerably. After exposing the spores to DNA-coated tungsten particles, the mixture of transformed and wild-type cells can easily be washed off and diluted to cell densities appropriate for plating under selective conditions. As cell or, according to former protocols, protoplast densities influence the relative amounts of false positives considerably, this point is very important. In comparison with protoplasts, transformed outgrowing spores have a much lower tendency to form aggregates, thus also improving the relative amount of true transformants. Biolistic transformation conditions are optimal if the germinated spores cover the cellophane membrane in a monocellular layer without too much empty space and are diluted afterward to a degree such that transformed derivatives are not able to provide cross-resistance to nontransformed individuals in their neighborhood.

Another major advantage of biolistic transformation is that the target cells can be processed at a developmental stage with a defined number of nuclei. We transformed spores at the 1–2 nuclei stage, whereas protoplasts of our former procedures contained typically between 10 and 20 nuclei. Consequently, biolistic transformants have a lower degree of heterokaryosis and are easier to select and to
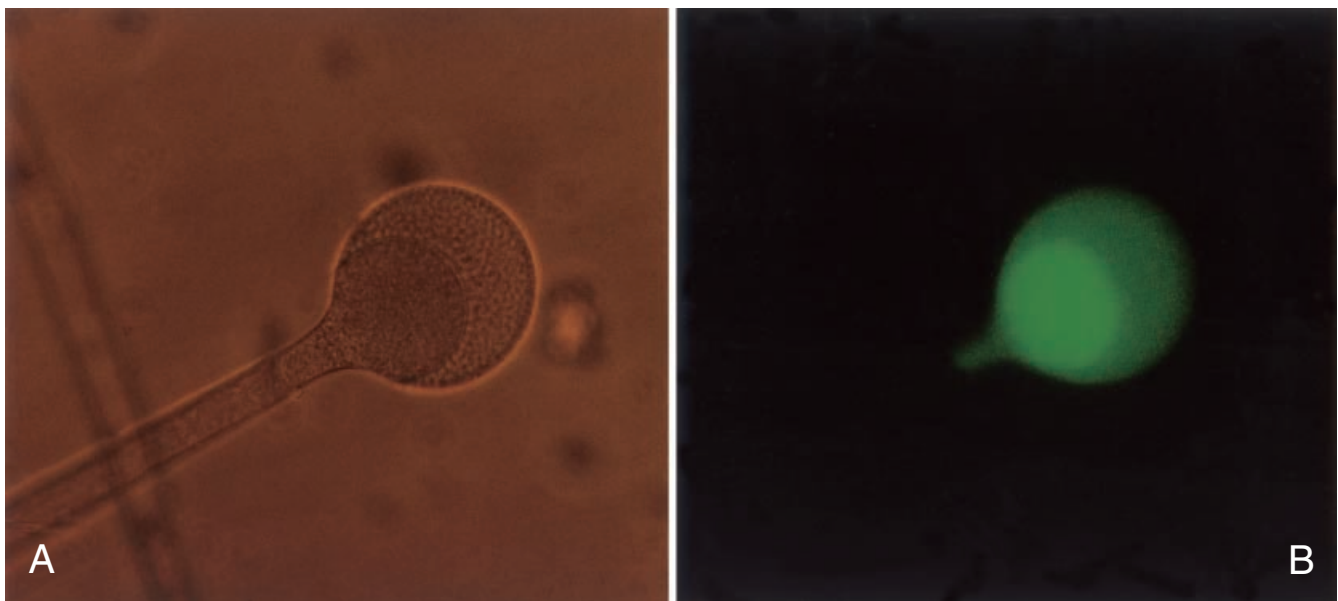

Fig. 2. Green fluorescent protein (GFP) expression in sporangia of *Absidia glauca* (14-day-old culture). **A** Light microscopy. **B** Fluorescence microscopy

Fig. 3. Southern blot analysis of selected *Absidia glauca* derivatives, transformed with peGFP-RAG. Bulk DNA was restricted with *Sal*I and hybridized with the bacterial part of the vector after transfer to nitrocellulose membranes. *Lanes 1–17*, different independent transformants; *lane 18*, wild type; *M*, 1-kb ladder (MBI Fermentas)
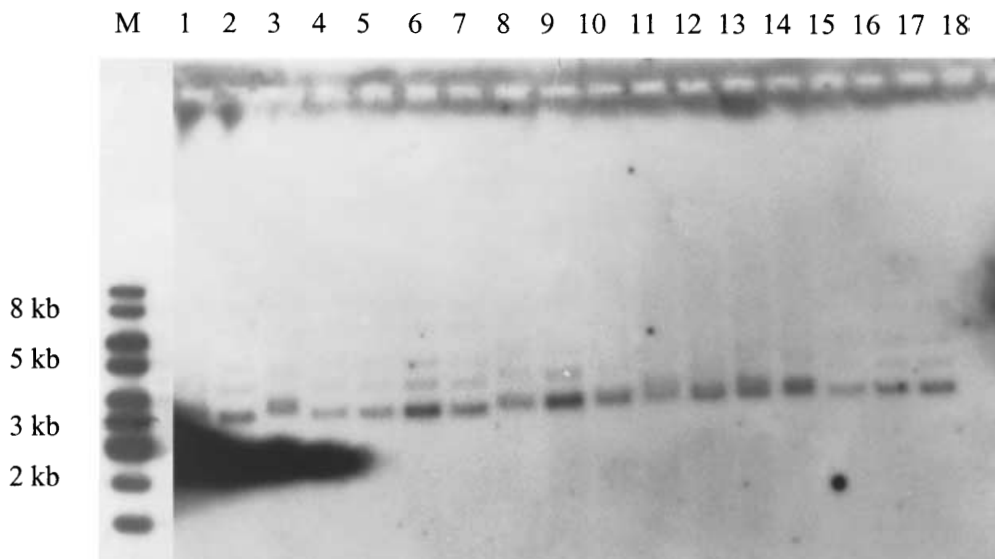
maintain. We checked 120 individual and independent transformants at the DNA level by Southern hybridization with radioactively labeled bacterial parts of the vector as probe (Fig. 3). About 70% were true transformants and showed the expected plasmid bands in addition to being neomycin resistant and fluorescent. Among these transformants, 80% showed enhanced fluorescence of the columella (see Fig. 2). The remaining 30% were false positives that somehow had adapted to the neomycin-containing media; they were not fluorescent and were negative in Southern experiments. In this respect, procedures depending on protoplasts are much less efficient. Especially if larger protoplasts with many nuclei are used, the proportion of true transformants can be lower than 10%.

In most experiments, transformation rates between 30 and 70 transformants/μg DNA and per 10^7 sporangiospores were observed. Compared with protoplast procedures, the biolistic approach is only slightly increased with respect to transformant yield. The value of the procedure is primarily based on the ease of the experimental procedure and on its reproducibility. In addition, variations of physical parameters of the biolistic apparatus influence transformation efficiency only marginally (see Table 1), which renders the approach quite robust. It is much more important to define the optimal cell stage, cell density, and regeneration conditions.

Although the biolistic transformation procedure renders molecular manipulation of the model zygomycete *Absidia glauca* considerably easier, it was not possible to increase the relative amount of integrative transformants. We obtained stable transformants that retained the plasmid used for transformation through several successive sporulation cycles (Table 2) but could define no experimental conditions favoring integration. We have no explanation for the mitotic stabilization of the plasmid peGFP-RAG through subsequent sporulation cycles. Southern blots (Fig. 3) provide evidence neither for integration into chromosomal

Table 2. Stability of transformants after several sporulation cycles

Transformant no.	S1	S2	S3
97	2.8	35	89
93	3.7	44	68
114	3.1	30	88
118	2.4	26	87
138	7.4	75	100
142	4.2	48	100
147	nt	55	100
150	nt	31	74

Data reflect the percentage of spores expressing neomycin resistance and green fluoresent protein (GFP) fluorescence after the mitotic sporulation cycles S1–S3
nt, not tested

DNA nor for recombination of stabilizing DNA elements from the nuclear DNA into the plasmid (Burmester et al. 1990). In all cases, exclusively covalently closed circular forms of the plasmid (see Fig. 1) were found (see Fig. 3). The hybridization pattern shows the expected ccc form migrating with the highest mobility and several slower forms that probably have a lower degree of superhelicity.

Exposition of germinating sporangiospores to DNA-damaging treatments, which are believed to induce recombination, did not lead to increased integration levels. UV irradiation to 50% survival or incubation of spores with 2.5 μg/ml phleomycin for 2 h, which also reduced viability to 50%, led to a reduction in transformation rate of approximately 20% and did not stimulate integration of plasmid DNA. The general phenomenon in all zygomycetes analyzed so far, the preferential autonomous replication of plasmids, seems to be independent of the transformation procedure.

References

Arnau J, Stromann P (1993) Gene replacement and ectopic integration in the zygomycete *Mucor circinelloides*. Curr Genet 23:542–546

Arnau J, Jepsen LP, Stromann P (1991) Integrative transformation by homologous recombination in the zygomycete *Mucor circinelloides*. Mol Gen Genet 225:193–198

Burmester A, Czempinski K (1994) Sequence comparison of a segment of the gene for 3-hydroxy-3-methylglutaryl-coenzyme A reductase in zygomycetes. Eur J Biochem 220:403–408

Burmester A, Wöstemeyer A, Wöstemeyer J (1990) Integrative transformation of a zygomycete, *Absidia glauca*, with vectors containing repetitive DNA. Curr Genet 17:155–161

Burmester A, Wöstemeyer A, Arnau J, Wöstemeyer J (1992) The *SEG1* element: a new DNA region promoting stable mitotic segregation of plasmids in the zygomycete *Absidia glauca*. Mol Gen Genet 235:166–172

Czempinski K, Kruft V, Wöstemeyer J, Burmester A (1996) Purification of 4-hydroxylmethyl-trisporate dehydrogenase from *Mucor mucedo*, an enzyme of the sexual pheromone pathway, and cloning of the corresponding gene. Microbiology 141:2647–2654

Feinberg, AP, Vogelstein B (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267

Kayser T, Wöstemeyer J (1991) Electrophoretic karyotype of the zygomycete *Absidia glauca*: evidence for differences between mating types. Curr Genet 19:279–284

Kellner M, Burmester A, Wöstemeyer A, Wöstemeyer J (1993) Transfer of genetic information from the mycoparasite *Parasitella parasitica* to its host *Absidia glauca*. Curr Genet 23:334–337

Sanford JC, Smith FD, Russel JA (1993) Optimising the biolistic process for different biological applications. Methods Enzymol 217:483–509

Schilde C, Wöstemeyer J, Burmester A (2001) Green fluorescent protein as a reporter for gene expression in the mucoralean fungus *Absidia glauca*. Arch Microbiol 175:1–7

Schimek C, Eibel P, Grolig F, Horie T, Ootaki T, Galland P (1999) Gravitropism in *Phycomyces*: a role for sedimenting protein crystals and floating lipid globules. Planta 210:132–142

Schmidt W, Galland P (1999) Light-induced absorbance changes in *Phycomyces*. Evidence for cryptochrome-associated flavosemiquinones. Planta 208:274–282

van den Ende H (1978) Sexual morphogenesis in the phycomycetes. In: Smith JE, Berry DR (eds) The filamentous fungi, vol III. Arnold, London, pp 257–274

Vetter M, Wöstemeyer J, Burmester A (1994) Characterisation of specific cDNA clones of the zygomycete *Parasitella parasitica*, derived from mRNAs which are regulated by the pheromone trisporic acid. Microbiol Res 149:17–22

Wada M, Beppu T, Horinouchi S (1996) Integrative transformation of the zygomycete *Rhizomucor pusillus* by homologous recombination. Appl Microbiol Biotechnol 45:652–657

Werkman B (1976) Localization and partial characterization of a sex-specific enzyme in homothallic and heterothallic Mucorales. Arch Microbiol 109:209–213

Wöstemeyer A, Wöstemeyer J (1998) Fungal protoplasts: relics or modern objects of molecular research? Microbiol Res 153:97–104

Wöstemeyer A, Teepe H, Wöstemeyer J (1990) Genetic interactions in somatic inter-mating type hybrids of the zygomycete *Absidia glauca*. Curr Genet 17:163–168

Wöstemeyer J (1985) Strain dependant variation in ribosomal DNA arrangement in *Absidia glauca*. Eur J Biochem 146:443–448

Wöstemeyer J, Brockhausen-Rohdemann E (1987) Inter-mating type protoplast fusion in the zygomycete *Absidia glauca*. Curr Genet 12:435–441

Wöstemeyer J, Burmester A (1986) Structural organization of the genome of the zygomycete *Absidia glauca*. Curr Genet 10:903–907

Wöstemeyer J, Burmester A, Weigel C (1987) Neomycin resistance as a dominantly selectable marker for transformation of the zygomycete *Absidia glauca*. Curr Genet 12:625–627

Wöstemeyer J, Wöstemeyer A, Burmester A, Czempinski K (1998) Horizontal gene transfer in the host-parasite system *Absidia glauca–Parasitella parasitica*. In: Syvanen M, Kado CI (eds) Horizontal gene transfer. Chapman and Hall, London, pp 118–127

Yamazaki Y, Kataoka H, Miyazaki A, Watanabe M, Ootaki T (1996) Action spectra for photoinhibition of sexual development in *Phycomyces blakesleeanus*. Phytochem Photobiol 64:387–392