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Agrobacterium tumefaciens-Mediated Transformation of *Mucor circinelloides*

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ABSTRACT. The *Agrobacterium tumefaciens*-mediated transformation of the zygomycetous fungus *Mucor circinelloides* is described. A method was also developed for the hygromycin B-based selection of *Mucor* transformants. Transformation with the hygromycin B phosphotransferase gene of *Escherichia coli* controlled by the heterologous *Aspergillus nidulans trpC* promoter resulted in hygromycin B-resistant clones. The presence of the hygromycin resistance gene in the genome of the transformants was verified by polymerase chain reaction and Southern hybridization: the latter analyses revealed integrations in the host genome at different sites in different transformants. The stability of transformants remained questionable during the latter analyses.

The zygomycetous *Mucor circinelloides* (syn. *M. racemosus*) is used as a model organism in several fields of biological research. Investigations have focused among other things on the dimorphic character of the fungus (Orlowsky 1991; Ruiz-Herrera 1993), the production of extracellular enzymes (Outtrup and Boyce 1990; Godtfredsen 1990), the role played by sex pheromones in *Mucorales* (Gooday 1994), the biosynthesis of carotenoids (Iturriaga *et al*. 2000, 2001). All such studies require an efficient transformation system. The aims of our study were: (*i*) to adapt the *Agrobacterium*-mediated transformation method to *Mucor* and to develop a protocol which can be applied to obtain integrative transformants from both homologous and heterologous transformations, and (*ii*) to elaborate a direct selection method for the transformants which does not require the use of auxotroph markers.

Agrobacterium tumefaciens, a Gram-negative plant pathogenic bacterium, naturally causes crown gall tumors at wound site in dicotyledonous plants. *A. tumefaciens* is able to transfer part of its tumor-inducing plasmid (T-DNA) into infected cells, where it integrates in the host genome and undergoes transcription. T-DNA transfer depends on the expression of the virulence genes, which can be induced by compounds secreted from wounded plant cells, such as acetosyringone (AS) (Kado 1991). *A. tumefaciens*-mediated transformation (ATMT) is a very efficient tool to transfer genes to a wide variety of plants; it is also used for insertional mutagenesis. This method was applied successfully to transforming various fungal species, *e.g.*, *Saccharomyces cerevisiae* (Bundock *et al*. 1995), *Aspergillus awamori*, *Trichoderma reesei* (de Groot *et al*. 1998), *Fusarium oxysporum* (Mullins *et al.* 2001), *F. circinatum* (Covert *et al*. 2001), *Calonectria morganii* (Malonek and Meinhardt 2001), *Verticillium fungicola* (Amey *et al*. 2002), *Agaricus bisporus* (Mikosh *et al.* 2001) and *Monascus purpureus* (Campoy *et al*. 2003). Two reports on ATMT of the zygomycetes *Rhizomucor miehei* (Monfort *et al*. 2003) and *Rhizopus oryzae* (Michielse *et al*. 2004) have also been published. ATMT protocols differ in all fungal species; successful transformation requires the optimization of several factors. Here we describe procedures for the genetic transformation of *Mucor* by using an ATMT system. The conditions for a new selection system based on hygromycin B (HygB)-resistance have also been developed and tested for *Mucor*.

MATERIALS AND METHODS

Strains and growth conditions. Mucor circinelloides ATCC1216b was maintained on malt extract agar (MEA; in %: malt extract 0.5, yeast extract 0.5, glucose 0.5, agar 1.5).

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Growth inhibition of *M. circinelloides* was tested by plating 10^4 sporangiospores on a series of yeast extract–glucose agar (YEG; in %: glucose 1, yeast extract 0.5, agar 1.5; pH 6.5) plates supplemented with HygB at concentration of 0, 50, 100, 150 and 200 g/mL. For the selection of transformants, *M. circinelloides* was grown at 25 °C on YEG with 100 g/mL Rose Bengal, 3 g/mL dichloran, 100 g/mL HygB and 300 g/mL mefoxin to inhibit the growth of *A. tumefaciens*. *A. tumefaciens* strain AGL1 (Lazo *et al*. 1991) harboring the pTiBo542 T helper plasmid (Hood *et al*. 1986) was used in the transformation experiments. *A. tumefaciens* was grown on LB medium (in %: sodium chloride 1, tryptone 1, yeast extract 0.5, agar 1.5) with 50 g/mL kanamycin to maintain the plasmid pBHt2.

Binary Ti vector. pBHt2, a derivative of the plasmid pCAMBIA (*Cambia*, Australia; constructed by Mullins *et al*. 2001), carries the hygromycin B phosphotransferase gene (*hph*); under the control of the *Aspergillus nidulans trpC* promoter, on the 3′ end of the *hph* gene, it was ligated to the cauliflower mosaic virus poly(A) signal. The plasmid also contains the kanamycin-resistance gene as a selection marker of *Agrobacterium*.

Transformation (ATMT) was done according to de Groot *et al*. (1998) with some modifications. *M. circinelloides* was cultivated on MEA plates for 7 d at 25 C and sporangiospores were collected by washing the plates with sterile distilled water. The *A. tumefaciens* culture was prepared according to Bundock and Hooykaas (1996). *A. tumefaciens* and *M. circinelloides* were co-cultivated in Petri dishes (diameter 50 mm) as follows: 25 L of the *A. tumefaciens* culture (A_{660} = 0.6) was mixed with an equal volume of a sporangiospore suspension (10^5 sporangiospores per mL) and the mixture was plated onto sterile cellophane sheets placed on the induction medium (IM) (Bundock and Hooykaas 1996) both in the presence (IM + AS) and absence (IM AS) of 200 mol/L acetosyringone (AS). After a 6-d incubation at 15 C, the cellophane sheets were transferred to selection medium and incubated for 5 d at 25 C. Putative transformants were then transferred to fresh selection medium plates and monosporangial cultures (from single colony isolations) were established.

Analysis of transformants. To extract the genomic DNA, transformants of *M. circinelloides* were grown in YEG medium containing 100 g/mL Rose Bengal, 3 g/mL dichloran and 100 g/mL HygB for 4 d at 25 C. Mycelia were harvested and ground to a fine powder in liquid nitrogen in a mortar. DNA purification was done according to Leach *et al*. (1986). The *hph* gene in putative transformants was detected by PCR using the primers NYhph1 (5′-CCT TCT AGA ATG CCT GAA CTC ACC GCG-3′) and NYhph2 (5′-CCT GGA TCC CTA TTC CTT TGC CCT CGG-3′). Each 25 L of reaction mixture contained 2 U Taq polymerase (Dupla-A-Taq; *Zenon*, Hungary), 2.5 L Dupla-A-Taq buffer, 2.5 mmol/L $MgCl_2$, 400 mol/L each of dATP, dCTP, dGTP and dTTP (*Fermentas*), 600 nmol/L of primers and 20 ng of genomic DNA. Amplifications were done in a PTC-100-60 DNA thermocycler (*MJ Research*) programmed for an initial denaturing step of 3 min at 95 C, followed by 35 cycles of 1-min denaturation (95 C), 2-min annealing (56 C) and 2-min polymerization (72 C). To examine the integration of the T-DNA, genomic DNA from the putative transformants was digested with *Nco*I; restriction fragments were separated by electrophoresis and blotted onto nylon membrane (*Fluka*) (Sambrook *et al.* 1989). Pre-hybridization, hybridization and washes of the membrane were carried out at 68 C. The 1.0-kb *hph* fragment was labeled with the PCR DIG Labeling Mix (*Roche Diagnostics*) according to the instructions of the manufacturer and detected with the chemiluminescent substrate CDP-Star (*Roche Diagnostics*).

Mitotic stability of transformants. Eight randomly selected fungal transformants were cultured on YEG agar without HygB, Rose Bengal and dichloran at 25 C for 7 d. Sporangiospores from single colonies were transferred successively to fresh YEG plates. After 5 transfers, transformants were inoculated onto YEG plates with 100 g/mL HygB, 100 g/mL Rose Bengal and 3 g/mL dichloran. In parallel, transformants were also subcultured several times under selective conditions.

RESULTS

Optimization of selection conditions for transformants of M. circinelloides. *M. circinelloides*, like other members of the order *Mucorales*, exhibits an intensive radial growth and a very low sensitivity to HygB. (pH 3.0–3.5 in the selection media for *Mucor* is generally recommended to achieve compact colony growth; however, the sensitivity of cells to HygB is pH dependent.) The greatest sensitivity of *Mucor* was detected at pH 6. To make transformants selectable in the presence of this compound, Rose Bengal and dichloran were used to suppress the hyphal growth and increase the sensitivity of the fungus. Even at 200 g/mL HygB was not able to inhibit the fungal growth; it was however completely blocked at ≤50 g/mL HygB in the presence of dichloran and Rose Bengal (Table I). Therefore, 100 g/mL HygB was used in further studies to increase the stringency of the selection conditions (Fig. 1).

Transformation. A. tumefaciens cells carrying pBHt2 were co-cultivated with spores of *M. circinelloides* on cellophane membranes laid on the surface of the IM. Application of the transparent cellophane discs (with the colored media applied) resulted in easier monitoring and collection of putative transformants than in the case of nitrocellulose membranes. Further, cellophane membranes are much less costly.

During the initial transformation studies, the incubation temperature was shown to be the crucial factor in the co-cultivation. At 25 °C, *M. circinelloides* grew significantly faster than *A. tumefaciens*, completely covering the IM plates after 2 d of incubation. To balance the bacterial and fungal growth it was necessary to decrease the incubation temperature to 15 °C. As in ATMT experiments on other fungi, the presence of AS in the IM was essential for the appearance of *Mucor* transformants; in the absence of AS, no transformants were formed. The length of the co-cultivation period was also demonstrated to be critical for transformants; HygB-resistant colonies were obtained only after a >4-d incubation on IM plates. An exposure time of 6 d on IM + AS was therefore used in the routine transformation experiments at 15 °C. Resistant fungal clones were observed after 5–6 d of incubation at 25 °C. The results of some successful transformation experiments are in Table II.

Table I. Growth inhibition tests on YEG medium for *M. circinelloides*[a]

Dichloran[b]	Rose Bengal[b]	Hygromycin B[b]	Growth
0	0	200	+
3	100	0	+
3	100	25	+
3	100	50	
3	100	100	

[a]10^4 spores were inoculated onto the plates in each experiment.
[b]In g/mL.

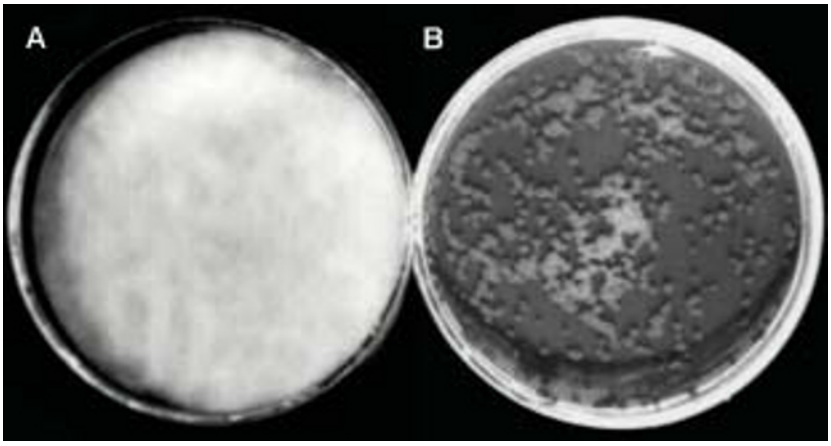

Fig. 1. Effects of Rose Bengal and dichloran on the radial growth of *M. circinelloides*; colony formation on YEG medium (**A**) and YEG medium supplemented with 100 g/mL Rose Bengal and 3 g/mL dichloran (**B**); 2×10^3 spores were plated onto both media.

Analysis of transformants. The PCR reaction with *hph* specific primers yielded the expected amplification product in each transformant analyzed (Fig. 2). Southern blotting of genomic DNA extracted from several transformants hybridized with the entire PCR-labeled *hph* sequence also confirmed that each clone contained the resistance gene and thus was indeed transformed. Hybridization analysis of the digested genomic DNA isolated from the untransformed strain and 3 randomly chosen transformants suggested the single copy integration of the transforming sequence in the *Mucor* genome (Fig. 3). In the three transformants analyzed the *hph* gene integrated at different sites.

Single-spore isolates of the transformants were transferred to nonselective medium (YEG without HygB) in order to investigate the mitotic stability of the introduced gene. After 5 cycles of cultivation on YEG, transformants were plated on a medium with HygB. All examined transformants retained their resistance, *hph* gene was detectable by PCR.

Table II. Hygromycin B-resistant (HygR, CFU) *M. circinelloides* clones after ATMT experiments[a]

Number of spores	Incubation on IM, d	HygR, CFU
8000	5	3
	6	31
2000	4	1
	5	3
	6	5

[a]For details *see Materials and Methods*.

However, a gradual decrease of the resistance to HygB was observed during the successive transfers onto both selective and nonselective media; growth of the transformants on selective media was slower and the introduced gene could be detected with more difficulty. After 7 transfers, the *hph* marker could be detected with PCR but not with Southern hybridization due to the smaller amount of the integrated DNA.

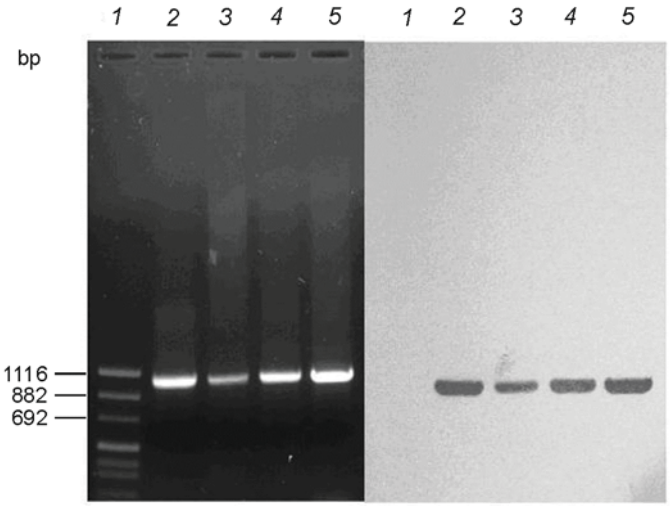

Fig. 2. Amplification pattern obtained with *hph* specific primers from *Mucor* transformants after agarose gel electrophoresis(*left part*). *1* – size standards (pUC Mix Marker; *MBI Fermentas*), *2* – pBHt2 plasmid, *3*–*5* – transformants M20/A1, M20/A2 and M20/A3, respectively; the size of the detected amplification product is 1020 bp; Southern hybridization of the amplicons with digoxigenine-labeled *hph* probe is shown on the *right part*.

Performed TAIL-PCR analysis (to investigate the chromosomal DNA flanking the T-DNA insert) did not yield specific PCR products and, therefore, the location of the integration could not be determined.

DISCUSSION

With the described *Agrobacterium*-mediated method, we succeeded in generating HygB-resistant transformants in *M. circinelloides*. The transformants contained the transferred T-DNA in 1 copy integrated into the fungal genome. Southern analysis revealed that integration occurred at different chromosomal sites in each transformant. This is the third report on the application of ATMT in a zygomycetous fungus.

To date, transformation systems based on the complementation of leucine, uracil and methionine auxotroph markers have been developed for *Mucor* (Roncero *et al.* 1989; Anaya and Roncero 1991; Benito *et al.* 1992; Velayos *et al.* 1998). However, the availability of other selection markers is limited (Velayos *et al.* 1998; Wolf and Arnau 2002) and this transformation system has the drawback that a stable mutant with the required deficiency has to be isolated from each strain to be transformed. The use of inhibitors such as HygB would make possible the direct transformation of wild-type isolates. On this basis, the efficient transformation of several fungal species in various taxonomic positions was achieved. However, HygB (and other antibiotics) inhibits the growth of numerous zygomycetous fungi, such as *Mucor*, only in high concentration (*e.g.*, *M. circinelloides* can grow even in the presence of 200 g/mL HygB). The described approach overcomes this problem through a controlled increase of the sensitivity of the fungus for the selective drug. Media with dichloran and Rose Bengal have been used for the enumeration and isolation of fungi (especially those belonging to *Zygomycetes*) from foods and silage (King *et al.* 1979; Hocking 1981; Skaar and Stenwig 1996). Our experiments revealed that a combination of these compounds not only

Fig. 3. Southern analysis of transformants obtained by ATMT of *M. circinelloides*; DNA samples were digested with *Nco*I, which single cuts the *hph* gene; digoxigenin-labeled *hph* gene was used as hybridization probe. *1* – pBHt2 plasmid, *2* – wild type strain of *M. circinelloides*, *3*–*5* – transformants M20/A1, M20/A2 and M20/A3, respectively.

reduces the colony size and suppresses the production of aerial hyphae in *Mucor* but also increases the sensitivity of the fungus to HygB (Table I).

Mucor transformation protocols were traditionally based on PEG-mediated methodology and necessitated protoplast formation from hyphae. The current lack of suitable commercially available protoplast-forming enzymes can hinder the application of these methods (Jung *et al*. 2000). The *Agrobacterium*-mediated transformation of *Mucor* can solve this problem by using germinating spores and hyphae without protoplast formation. On the other hand, only protoplasts were shown to be applicable for the transformation of *R. oryzae* with *A. tumefaciens*, indicating the different cell-wall composition among zygomycetes (Michielse *et al*. 2004).

The PEG-mediated transformation or electroporation of *Mucor* allows the high-frequency transformations of plasmids, which almost exclusively replicate autonomously in the transformants (van Heeswijck and Roncero 1984; Anaya and Roncero 1991; Iturriaga *et al*. 1992; Benito *et al*. 1995; Velayos *et al.* 1998). Integrative transformation can be forced in these systems, but with significantly lower efficiency (Arnau *et al*. 1991; Arnau and Stroman 1993; Wolff and Arnau 2002). In fact, only a limited number of reports have been published on heterologous gene expression in *Mucor* (Dickinson *et al*. 1987; Iturriaga *et al*. 1992; Ruiz-Hidalgo *et al*. 1999; Wolff and Arnau 2002). In contrast, *Agrobacterium* transfers a particular DNA fragment (T-DNA) to the host cell, which integrates into the nuclear genome. Our study demonstrated a random single-copy integration of the bacterial *hph* gene into the *M. circinelloides* genome. Similar random T-DNA integration events have also been described in yeasts and higher fungi (de Groot *et al.* 1998; Bundock *et al*. 2002), while in the case of the zygomycete *R. oryzae*, integrations at the same locus were observed in the tested transformants (Michielse *et al*. 2004). Unfortunately, our efforts to determine the regions flanking the integration site have not been successful.

Although integration of the *hph* gene into the *Mucor* genome was demonstrated, all tested transformants lost the resistant phenotype during successive cultivation cycles (after ≈7–9 cycles). A successive decrease of the HygB resistance was observed along with the loss of the introduced *hph* marker. Mitotic instability was observed also in *R. miehei* transformants obtained with ATMT where bacterial kanamycin resistance gene served as selection marker (Monfort *et al*. 2003). However, these transformants rapidly lost the T-DNA and the integration event could not be demonstrated. In contrast, Michielse *et al*. (2004) successfully used ATMT to obtain stable and integrative transformants from *R. oryzae* with the *R. niveus pyr4* marker, which complements the uracil auxotrophy. Based on these results, they suggested that ATMT of *Zygomycetes* can only be used for the introduction of endogenous DNA. As the molecular background of an exogenous DNA processing mechanism (if any) is unknown, further rigorous studies are required to confirm or contradict this statement. Our results support the possibility of a specific genome defense mechanism in zygomycetous fungi eliminating exogenous DNA *via* rearrangements and deletions as postulated in some of the previous studies (Obraztsova *et al*. 2003; Monfort *et al.* 2003; Michielse *et al*. 2004). The different fate of the introduced transforming DNA in *Mucor*, *Rhizomucor* and *Rhizopus* implies variations in the efficiency of this mechanism in these related species.

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REFERENCES

AMEY R.C., ATHEY-POLLARD A., BURNS C., MILLS P.R., BAILEY A., FOSTER G.D.: PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. *Mycol.Res*. **106**, 4–11 (2002).

ANAYA N., RONCERO M.I.G.: Transformation of a methionine auxotrophic mutant of *Mucor circinelloides* by direct cloning of the corresponding wild-type gene. *Mol.Gen.Genet*. **230**, 449–455 (1991).

ARNAU J., STROMAN P.: Gene replacement and ectopic integration in the zygomycete *Mucor circinelloides*. *Mol.Gen.Genet*. **23**, 542–546 (1993).

ARNAU J., JEPSEN L.P., STROMAN P.: Integrative transformation by homologous recombination in the zygomycete *Mucor circinelloides*. *Mol.Gen.Genet*. **225**, 193–198 (1991).

BENITO E.P., DÍAZ-MINGUEZ J.M., ITURRIAGA E.A., CAMPUZANO V., ESLAVA A.P.: Cloning and sequence analysis of the *Mucor circinelloides pyrG* gene encoding orotidine-5′-monophosphate decarboxylase: use of *pyrG* for homologous transformation. *Gene* **225**, 59–67 (1992).

BENITO E.P., CAMPUZANO V., LÓPEZ-MATAS M.A., DE VICENTE J.I., ESLAVA A.P.: Isolation, characterization and transformation by autonomous replication of *Mucor circinelloides* OMPdecase-deficient mutants. *Mol.Gen.Genet*. **248**, 126–135 (1995).

BUNDOCK P., HOOYKAAS P.J.J.: Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. *Proc.Nat.Acad.Sci.USA* **93**, 15272–15275 (1996).

BUNDOCK P., DENDULKRAS A., BEIJERSBERGEN A., HOOYKAAS P.J.J.: Transkingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J.* **14**, 3206–3214 (1995).

CAMPOY S., PEREZ F., MARTIN J.F., GUTIERREZ S., LIRAS P.: Stable transformants of the azophilone pigment-producing *Monascus purpureus* obtained by protoplast transformation and *Agrobacterium*-mediated DNA transfer. *Curr.Genet.* **43**, 447–452 (2003).

COVERT S.F., KAPOOR P., LEE M., BRILEY A., NAIRN C.J.: *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. *Mycol.Res*. **105**, 259–264 (2001).

DICKINSON L., HARBOE M., VAN HEESWIJK R., STROMAN P., JEPSEN L.P.: Expression of active *Mucor miehei* aspartic protease in *Mucor circinelloides*. *Carlsberg Res.Commun*. **52**, 243–252 (1987).

GODTFREDSEN S.E.: Microbial lipases, pp. 255–274 in W.M. Fogarty, C.T. Kelly (Eds): *Microbial Enzymes and Biotechnology*, 2nd ed. Elsevier, London 1990.

GOODAY G.W.: Hormones in mycelial fungi, pp. 401–411 in J.G.H. Wessels, F. Meinhardt (Eds): *The Mycota, Vol. 1.* Springer-Verlag, Berlin 1994.

DE GROOT M.J.A., BUNDOCK P., HOOYKAAS P.J.J., BEIJERSBERGEN A.G.M.: *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnol.* **16**, 839–842 (1998).

VAN HEESWIJCK R., RONCERO M.I.G.: High frequency transformation of *Mucor* with recombinant plasmid DNA. *Carlsberg Res.Commun.* **49**, 691–702 (1984).

HOCKING A.D.: Improved media for enumeration of fungi in foods. *CSIRO Food Res.Quart.* **41**, 7–11 (1981).

HOOD E.E., HELMER G.L., FRALEY R.T., CHILTON M.D.: The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in the region pTiBo542 outside the T-DNA. *J.Bacteriol*. **168**, 1291–1301 (1986).

ITURRIAGA E.A., DÍAZ-MINGUEZ J.M., BENITO E.P., ALVAREZ M.I., ESLAVA A.P.: Heterologous transformation of *Mucor circinelloides* with the *Phycomyces blakesleeanus leu1* gene. *Curr.Genet*. **21**, 215–223 (1992).

ITURRIAGA E.A., VELAYOS A., ESLAVA A.P.: Structure and function of the genes involved in the biosynthesis of carotenoids in the *Mucorales*. *Biotechnol.Bioproc.Eng*. **5**, 263–274 (2000).

ITURRIAGA E.A., VELAYOS A., ESLAVA A.P., ALVAREZ M.I.: The genetics and molecular biology of carotenoid biosynthesis in *Mucor*. *Rec.Res.Dev.Genet*. **1**, 79–92 (2001).

JUNG M.K., OVECHKINA Y., PRIGOZHINA N., OAKLEY C.E., OAKLEY B.R.: The use of -D-glucanase as a substitute for Novozym 234 in immunofluorescence and protoplasting. *Fungal Genet.Newslett*. **47**, 65–66 (2000).

KADO C.I.: Molecular mechanisms of crown gall tumorigenesis. *Crit.Rev.Plant Sci*. **10**, 1–32 (1991).

KING A.D. Jr., HOCKING A.D., PITT J.I.: Dichloran–Rose Bengal medium for enumeration and isolation of molds from foods. *Appl. Environ.Microbiol*. **37**, 959–964 (1979).

LAZO G.R., STEIN P.A., LUDWIG R.A.: A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology* **9**, 963–967 (1991).

LEACH J., FINKELSTEIN D.B., RAMBOSEK J.A.: Rapid miniprep of DNA from filamentous fungi. *Fungal Genet.Newslett.* **33**, 32–33 (1991).

MALONEK S., MEINHARDT F.: *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*. *Curr.Genet.* **40**, 152–155 (2001).

MICHIELSE C.B., SALIM K., RAGAS P., RAM A.F.J., KUDLA B., JARRY B., PUNT P.J., VAN DEN HONDEL C.A.M.J.J.: Development of a system for integrative and stable transformation of the zygomycete *Rhizopus oryzae* by *Agrobacterium*-mediated DNA transfer. *Mol.Genet.Genomics* **271**, 499–510 (2004).

MIKOSH T.S.P., LAVRIJSSEN B., SONNENBERG A.S.M., VAN GRIENSVEN L.J.L.D.: Transformation of the cultivated mushroom *Agaricus bisporus* (LANGE) using T-DNA from *Agrobacterium tumefaciens*. *Curr.Genet.* **39**, 35–39 (2001).

MONFORT A., CORDERO L., MAICAS S., POLAINA J.: Transformation of *Mucor miehei* results in plasmid deletion and phenotypic instability. *FEMS Microbiol.Lett.* **224**, 101–106 (2003).

MULLINS E.D., CHEN X., ROMAINE P., RAINA R., GEISER D.M., KANG S.: *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology* **91**, 173–180 (2001).

OBRAZTSOVA I.N., PRADOS N., HOLZMANN K., AVALOS J., CERDÁ-OLMEDO E.: Genetic damage following introduction of DNA in *Phycomyces*. *Fungal Genet.Biol.* **41**, 168–180 (2003).

ORLOWSKY M.: *Mucor* dimorphism. *Microbiol.Rev.* **55**, 234–258 (1991).

OUTTRUP H., BOYCE C.O.L.: Microbial proteinases and biotechnology, pp. 227–254 in W.M. Fogarty, C.T. Kelly (Eds): *Microbial Enzymes and Biotechnology*, 2nd ed. Elsevier, London 1990.

RONCERO M.I.G., JEPSEN L.P., STROMAN P., VAN HEESWIJCK R.: Characterization of a *leuA* gene and an *ARS* element from *Mucor circinelloides*. *Gene* **84**, 335–343 (1989).

RUIZ-HERRERA J.: Dimorphism in *Mucor* species, pp. 257–265 in H. van den Bossche, F.C. Odds, D. Herridge (Eds): *Dimorphic Fungi in Biology and Medicine*. Plenum Press, New York 1993.

RUIZ-HIDALGO M.J., ESLAVA A.P., ALVAREZ M.I., BENITO E.P.: Heterologous expression of the *Phycomyces blakesleanus* phytoene dehydrogenase gene (*carB*) in *Mucor circinelloides*. *Curr.Microbiol.* **39**, 259–264 (1999).

SAMBROOK J., FRITSCH E.F., MANIATIS T.: *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.

SKAAR I., STENWIG H.: Malt-yeast extract–sucrose agar, a suitable medium for enumeration and isolation of fungi from silage. *Appl. Environ.Microbiol.* **62**, 3614–3619 (1996).

VELAYOS A., ALVAREZ M.I., ESLAVA A.P., ITURRIAGA E.A.: Interallelic complementation at the *pyrF* locus and the homodimeric nature of orotate phosphoribosyltransferase (OPRTase) in *Mucor circinelloides*. *Mol.Gen.Genet.* **260**, 251–260 (1998).

WOLF A.M., ARNAU J.: Cloning of glyceraldehyde-3-phosphate dehydrogenase-encoding genes in *Mucor circinelloides* (syn. *racemosus*) and use of the *gpd1* promoter in recombinant protein production. *Fungal Genet.Biol.* **35**, 21–29 (2002).