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Transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans*

M. J. Daboussi[1], A. Djeballi[1], C. Gerlinger[1], P. L. Blaiseau[1], I. Bouvier[1], M. Cassan[2], M. H. Lebrun[1], D. Parisot[1], Y. Brygoo[1]

[1]Laboratoire de Cryptogamie et [2]Laboratoire de Génétique des ribosomes, Bât. 400, Université Paris-Sud, F-91405 Orsay Cedex, France

Summary. A gene transfer system originally developed for *Fusarium oxysporum* has been applied to seven species of filamentous fungi of agricultural and industrial importance. This transformation system relies on the selection of mutants deficient in nitrate reductase by positive screening. Such mutants were recovered easily in all the fungi tested – without mutagenic treatments – through their resistance to chlorate. They were transformed by a plasmid vector (pAN301) carrying the *Aspergillus nidulans* wild-type gene (*niaD*). Transformation frequencies ranged from one to ten transformants/μg plasmid DNA. The general properties of the transformants were analyzed. Most of them are mitotically stable, and the integration of the vector into the host genome frequently occurred in a tandem fashion.

Key words: Transformation – Nitrate reductase – Chlorate resistance – Filamentous fungi

Introduction

The development of gene cloning vectors for filamentous fungi now provides the possibility of cloning genes involved in biological processes like the pathogenicity to plants or the production of metabolites and enzymes. However, the successful application of gene cloning technology to fungi of industrial or agricultural importance relies on the development of an efficient transformation system. While transformant strains can be selected on the basis of complementation of the auxotrophic recipient strains by cloned biosynthetic genes, these transformation strategies are often hampered by the lack of required nutritional mutants in fungi that are genetically poorly characterized. This can be circumvented either by the use of dominant selectable markers or by the positive screening of auxotrophic mutants for a defined gene. Recently, reports of filamentous fungal transformation using dominant resistance genes, such as hygromycine (Turgeon et al. 1987; Rodriguez and Yoder 1987; Wang et al. 1988; Kistler and Benny 1988), benomyl resistance (Orbach et al. 1986; Panaccione et al. 1988) or oligomycine resistance (Ward et al. 1986; Bull et al. 1988), have appeared. An alternative approach has been achieved in different filamentous fungi using uracil auxotrophs recovered among 5-fluoro-orotate resistant mutants (Razanampary and Bégueret 1986; van Hartingsveldt et al. 1987; Diez et al. 1987) or using nitrate reductase deficient mutants selected through their resistance to chlorate (Malardier et al., 1989).

The purpose of this study was to determine if the *niaD* transformation system we developed for *Fusarium oxysporum* (Malardier et al., 1989) could be applied to other filamentous fungi that are either pathogenic to plants (*Colletotrichum lindemuthianum, Fusarium oxysporum* f.sp. *lycopersici, Nectria haematococca, Pyricularia oryzae*) or are used for biological control and in microbiological industrial processes (*Aphanocladium album, Beauveria bassiana, Penicillium caseicolum*). We present here the conditions for the isolation of nitrate reductase mutants in seven fungal species by means of positively selecting for chlorate resistance and their successful transformation with the pAN301 plasmid carrying the *Aspergillus nidulans* wild-type gene *niaD*.

Material and methods

Fungal strains and culture media. The following strains were used: *Aphanocladium album,* a mycoparasite of rust (from Dr. Defago, ETH, Zurich, Switzerland); *Beauveria bassiana,* an entomopathogen (from Dr. Riba, INRA, La Minière, France); *Colletotrichum linde-*

Offprint requests to: M. J. Daboussi

muthianum, a pathogen of French bean (from Dr. Fouilloux, INRA, Versailles, France); *Fusarium oxysporum* f.sp. *lycopersisci,* a pathogen of tomato (from Dr. Louvet, INRA, Dijon, France); *Nectria haematococca,* a soil saprophyte (Daboussi-Bareyre 1980); *Penicillium caseicolum,* a fungus used in the cheese industry (from Laboratoires G. Roger, La Ferté sous Jouarre, France); *Pyricularia oryzae,* a pathogen of rice (from Dr. Notteghem, IRAT, Montpellier, France). *A. album, B. bassiana, F. oxysporum* f.sp. and *N. haematococca* were maintained on potato dextrose agar supplemented with 1 g/l yeast extract (MC1). *P. caseicolum* was grown on medium B (De Falandre et al. 1987) containing 1 g/l yeast extract (MC2). *C. lindemuthianum* was grown on malt agar medium (MC3) and *P. oryzae* on rice agar medium (20 g/l paddy rice, 2 g/l yeast extract, 15 g/l agar, 2 g/l streptomycin: MC4). The following minimal media were used: MM1 (Daboussi-Bareyre 1980) for *A. album, B. brassiana, F. oxysporum* f.sp. and *N. haematococca; MM2 (Medium B in Tanaka 1965) for C. lindemuthianum* and *P. oryzae; MM3 (Bouvier 1967) for P. caseicolum.* Chlorate-resistant mutants were selected for on the appropriate MM (without the original nitrogen source) supplemented with various concentrations of $KClO_3$ and nitrogen sources, depending on the fungus used (see Table 1). The chlorate-resistant mutants were assigned to different classes on the basis of their growth on MM containing one of the four following nitrogen sources: 23 m*M* nitrate, 10 m*M* nitrite (3 m*M* nitrite for *C. lindemuthianum* and *P. oryzae*), 0.7 m*M* hypoxanthine (5 m*M* hypoxanthine for *C. lindemuthianum* and *P. oryzae*), or 5 m*M* ammonium. Minimal media were solidified with 1.5% purified agar (Bacto-agar, Difco) to minimize the growth of the *nia* mutants. All cultures were maintained in the dark at 26°C with the exception of *P. caseicolum* and *C. lindemuthianum,* which were maintained at 23°C.

Preparation and transformation of the protoplasts. The protoplasts were prepared as previously described for *Fusarium oxysporum* f.sp. *melonis* (Malardier et al., 1989), with the following modifications. For *F. oxysporum* f.sp. *lycopersici, N. haematococca* and *P. caseicolum,* Petri dishes containing 25 ml of a solid medium covered with a cellophane disk were inoculated with 10^6–10^7 spores and incubated for 20 h at 26°C (*F. oxysporum* f.sp. and *N. haematococca*) or 36 h at 23°C (*P. caseicolum*). For the remaining four species, 10^6–10^7 spores were added to 100 ml of a complete liquid medium in Roux flasks (MM2 supplemented with 5 g/l yeast extract for *C. lindemuthianum* and *P. oryzae*; MC1 for *A. album* and *B. bassiana*) and incubated at 26°C for 36 h, with the exception of *A. album,* which was incubated for 24 h. Mycelia were collected on a nylon muslin, washed and suspended (1 g wet weight/25 ml) in a lysis buffer (0.6 *M* KCl, 0.1 *M* sodium phosphate, pH 5.8, 50 mg/ml Glucanex (Novo Ferment, Basel, Switzerland); for *C. lindemuthianum,* 1.5 *M* $MgSO_4$ was used instead of KCl. The protoplasts were incubated for 2–3 h at 26°C with gentle shaking, then separated from the conidia and mycelial debris by filtration through a sterile nylon muslin or sintered glass filter (porosity: 50 µm) and finally collected by centrifugation at 3,000 *g* for 10 min at room temperature; *C. lindemuthianum* protoplasts were collected by buoyant density centrifugation on 1.5 *M* $MgSO_4$. The protoplasts were resuspended in 2 ml 0.6 *M* KCl and purified by centrifugation on a cushion of 30% sucrose for 10 min at 3,000 *g*. The protoplast band at the interface was then removed and diluted with 1 vol. MS buffer (1 *M* sorbitol, 10 m*M* MOPS, pH 6.3). The protoplasts were harvested by centrifugation, washed twice and resuspended in 200 µl MSC (10 m*M* $CaCl_2$ in MS). Two different transformation procedures were used: the first (TP1) is that described by Malardier et al. (in press), except that the volume of the plasmid suspension was reduced to 10 µl for *C. lindemuthianum* and *P. oryzae*; the second (TP2) was derived from the method of Tilburn et al. (1983). Generally, 10^7 protoplasts were mixed with 5 or 10 µg of plasmids pAN301 or pFB39.

DNA isolation and manipulation. Roux flasks containing 100 ml of liquid medium (MC1 for all species except *C. lindemuthianum* and *P. oryzae* (MM2-yeast extract) were inoculated with 10^6–10^7 spores. After 48–60 h of growth, the mycelium was collected by filtration on a sterile sintered glass filter, washed with sterile distilled water and ground to a fine powder in liquid nitrogen. The powder was suspended in the extraction buffer (50 m*M* Tris HCl, 50 m*M* EDTA, 150 m*M* NaCl, 2% (w/v) Sarcosyl, pH 9) at a final concentration of 0.5–1 g/ml. The suspension was then cooled in ice and treated with an equal volume of phenol saturated with 1.5 *M* Tris HCl, pH 8.8. The supernatant was centrifuged twice at 3,000 *g* for 15 min, mixed with 1 vol. isopropranol and left in place for 30 min. The precipitate was collected by centrifugation at 3,000 *g* for 15 min. The DNA pellet was resuspended in TE buffer overnight at 4°C (TE: 10 m*M* Tris HCl, 1 m*M* EDTA, pH 8) and eventually centrifuged at 10,000 *g* for 1 h to precipitate the residual polysaccharides. The DNA was then purified by isopicnic centrifugation on cesium chloride. Restriction enzyme digestions of fungal genomic DNA and plasmids, transfer of DNA to Hybond nylon filters (Amersham) and labeling of plasmids by nick translation were performed according to the manufacturers procedures and standard protocols (Maniatis 1982).

Results

Isolation of nitrate reductase deficient strains

Since nitrate reductase deficient mutants can be selected on the basis of their resistance to chlorate, we first tested each of the seven fungal species studied for their sensitivity to chlorate. Each strain was grown on minimal medium supplemented with various nitrogen sources and different chlorate concentrations (0.1–0.5 *M*). These experiments showed that chlorate toxicity varied both with the fungal species tested and the nitrogen source used. The conditions which led to the strongest inhibition of growth in each strain are presented in Table 1. As *A. album, N. haematococca* and *P. oryzae* were only weakly sensitive to chlorate – their growth was slightly inhibited at 0.5 *M* chlorate – the detection of chlorate resistant mutants in these fungi was not easy. Despite this drawback, chlorate resistant mutants were isolated using morphological differences associated with this resistance, such as the formation of sectors or colonies with more aerial mycelium or faster growth on the selection media. For the other fungi, the resistant colonies could easily be identified by their fast growth. The selected chlorate resistant mutants were transferred to MM containing nitrate as the sole nitrogen source. Those that grew with thin mycelia were considered *nit* mutants and subsequently characterized by being grown on minimal medium amended with different nitrogen sources (Cove 1976). These nutritional tests showed that mutants able to grow on all nitrogen sources except nitrate could be recovered from all of the fungi tested. They presumably resulted from a mutation in a nitrate reductase structural gene. These *nia* mutants were found frequently among the

Table 1. Selection of the recipient strains and estimation of the transformation frequency

Organisms	Selection of recipient strains			Transformation
	Selection media		Ratio of *nia/nit*	Number of transformants
	KClO3 (m*M*)	Nitrogen source		
Colletotrichum lindemuthianum	200	Glutamic acid	43/58	24
Fusarium oxysporum	200	Urea	44/60	38
Beauveria bassiana	500	Hypoxanthine	25/60	20
Penicillium caseicolum	500	Glutamic acid	4/ 4	11
Aphanocladium album	500	Urea	3/22	30
Nectria haematococca	500	Urea	6/16	12
Pyricularia oryzae	500	Hypoxanthine	29/59	22

Transformants were obtained by treating 10^7 protoplasts with 5 µg pAN301 using TP1 protocol. The only exception to this was the *Aphanocladium* transformants, which were obtained using the TP2 protocol

nitrate non-utilizing mutants for all species tested except *A. album* and *N. haematococca* (Table 1). Other distinct nitrate non-utilizing strains showed phenotypes identical to the *Aspergillus nidulans cnx* (cofactor) or *nirA* (regulatory) mutations. The reversion frequency of selected *nia* mutants was lower than 10^{-6} viable spores, which meant that transformation experiments could be carried out.

Transformation experiments

In a typical experiment approximately 10^7 protoplasts were mixed with 5–10 µg of plasmids pAN301 or pFB39 following one of the two transformation protocols mentioned above. The protoplasts were plated on a medium containing nitrate as the sole nitrogen source (MM). The controls, protoplasts incubated with the plasmid vector pFB39, produced no transformants. When we treated the protoplasts with the pAN301 plasmid containing the *niaD* gene of *A. nidulans*, however, growing colonies with aerial mycelium were obtained for all the fungi tested 7–14 days after plating: 5 µg pAN301 produced transformation frequencies that ranged from 1 to 10 transformants/µg DNA (Table 1). The transformants were purified from single conidia transferred to minimal medium. Stable transformants were recovered for all the organisms.

Molecular analysis of the transformants

For each fungus, genomic DNA was isolated from the *nia* recipient strain and from three randomly chosen stable transformants. When undigested DNA from the transformants was probed with labeled pAN301 plasmid, a strong hybridizing band associated with high molecular weight DNA (> 30 kb) was apparent; no signal was detected when DNA from the recipient strains was probed in the same way. These data strongly suggest that the transforming DNA was integrated into the host genome. The hybridization of the labeled pAN301 plasmid to the transformant DNA digested with EcoRI could be classified into two groups. The first pattern consisted of four strongly hybridizing bands (8.5, 3.2, 2.7 and 1.3 kb) that corresponded in size to the pAN301 EcoRI fragments and two other fainter bands. This type of pattern could be best explained by the tandem integration of multiple copies of the plasmid at one site. In some transformants numerous fainter bands were discerned, suggesting different integration sites and/or internal rearrangements into the integrated copies. The second type of pattern that we observed had only three fragments in common with the EcoRI-digested pAN301 and two additional fainter bands. This pattern could be explained by the integration of only one copy of the pAN301 plasmid into the host genome and was detected in only some of the *F. oxysporum* f.sp., *P. oryzae* and *C. lindemuthianum* transformants. In each of the other fungal species, the three transformants analyzed showed that multiple copies of the plasmid integrated into the host genome.

Discussion and conclusion

With the exception of *N. haematococca* (Daboussi and Parisot 1981; Tegtmeier and van Etten 1982) and *P. oryzae* (Crawford et al. 1986), the fungal species used in this study are not genetically well-characterized. Despite this drawback, we were able to transform these fungal species using the methodology developed for *F. oxysporum*, which is also a genetically uncharacterized

species. We are therefore able to conclude that this transformation system may be applied to any filamentous fungi even those in which it is often difficult to screen for auxotrophic mutants.

The successful application of such system relies on the recovery of mutants impaired in a nitrate reductase structural gene – without the use of a mutagen – through positively screening for resistance to chlorate. This selection system appears to be possible for many fungi (Cove 1976; Tomsett and Garrett 1980; Klittich and Leslie 1988; Newton and Caten 1988). Even though the sensitivity to chlorate of some of the fungal species was low, we were able to select *nia* mutants. All of these *nia* mutants could be transformed with a functional copy of the *A. nidulans* gene, thus illustrating the permissive expression of this gene in taxonomically diverse fungi. While transformation frequency is low in comparison to that obtained in some *A. nidulans* and *N. crassa* systems, it is similar to that obtained in many other filamentous fungi. Some of the fungi studied here have been recently transformed using drug resistance markers – *C. lindemuthianum* (Rodriguez and Yoder 1987; Panaccione et al. 1988), *N. haematococca* (Turgeon et al. 1987), *F. oxysporum f. sp.* (Kistler and Benny 1988) – or with genes conferring the ability to utilize novel sustrates, such as acetamidase in *C. lindemuthianum* (Rodriguez and Yoder 1987), or complementing auxotrophic mutants in *P. oryzae* (Parsons et al. 1987). The transformation efficiencies reported in these systems are in the same range (0.2–35 transformants/μg DNA) as those obtained in our system (1–10 transformants/μg DNA). If such molecular biology techniques as the screening of gene libraries by the complementation of mutants and the disruption or replacement of genes are to be applied practically, transformation frequencies must be improved. This can be achieved either by the development of the homologous system (van Hartingsvelt et al. 1987) or by the analysis of the factors involved in the transformation process (Huiet and Case 1985; Specht et al. 1988).

The general properties of these transformants are the same as those described for other fungi. The transforming vector was integrated into the host genome in one or multiple copies. The multiple copies could be integrated at one locus in a tandem repeated fashion or located at different sites in the genome.

An interesting characteristic of our system is the dual property of the *nia* selectable marker, i.e., the inability to utilize nitrate and a resistance to chlorate. This latter character was used to check the stability of the transformants (Malardier et al., in press). It may also be possible to use it to directly select for the introduction of a mutated allele into the *nia* gene.

We have shown that the *nia* transformation system is simple and rapid, and believe that it should prove useful in fungal species in which a genetic transformation system is not yet established. Furthermore, it can be used as a second selectable marker to test for co-transformation efficiency in organisms yet to be transformed.

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References

Bouvier J (1967) CR Acad Sci Ser D 265:1305–1308
Bull JH, Smith DJ, Turner G (1988) Curr Genet 13:377–382
Cove DJ (1976) Heredity 36:191–203
Crawford MS, Chumley FG, Weaver CG, Valent DB (1986) Genetics 114:1111–1129
Daboussi-Bareyre MJ (1980) J Gen Microbiol 116:425–433
Daboussi-Bareyre MJ, Parisot D (1981) In: Nelson PE, Toussoun TA, Cook RJ (eds) *Fusarium:* diseases, biology and taxonomy. Pennsylvania State University Press, pp 306–317
De Falandre A, Bouvier-Fourcade I, Seng JM, Leroux P (1987) Appl Environ Microbiol 53:1500–1503
Diez B, Alvarez E, Cantoral JM, Barredo JL, Martin JF (1987) Curr Genet 12:277–282
Huiet L, Case M (1985) In: Bennett JW, Lasure LL (eds) Gene manipulation in fungi. Academic Press, New York, pp 229–244
Kistler HC, Benny UK (1988) Curr Genet 13:145–149
Klittich CJR, Leslie JF (1988) Genetics 118:417–423
Malardier L, Daboussi MJ, Julien J, Roussel F, Scazzocchio C, Brygoo Y (1989) Gene 78:147–156
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Newton AC, Caten CE (1988) Trans Brit Mycol Soc 90:199–207
Orbach MJ, Porro EB, Yanofsky C (1986) Mol Cell Biol 6:2452–2461
Panaccione DG, McKiernan M, Hanau RM (1988) Mol Plant Microbe Interact 1:113–120
Parsons KA, Chumley FG, Valent B (1987) Proc Natl Acad Sci USA 84:4161–4165
Razanamparany C, Bégueret J (1986) Curr Genet 10:811–817
Rodriguez RJ, Yoder OC (1987) Gene 54:73–81
Specht CA, Munnoz-Rivas A, Novotny CP, Ullrich RC (1988) Exp Mycol 12:357–366
Tanaka S (1965) In: The rice blast disease. The Johns Hopkins Press, Baltimore, pp 23–33
Tegtmeier KJ, van Etten HD (1982) Phytopathology 72:604–607
Tilburn J, Scazzocchio C, Taylor GG, Zabicky-Zissman JH, Lockington RA, Davies RW (1983) Gene 26:205–221
Tomsett AB, Garrett RH (1980) Genetics 95:649–660
Turgeon BG, Garber RC, Yoder OC (1987) Mol Cell Biol 7:3297–3305
van Hartingsvelt W, Mattern IE, van Zeijl CMJ, Pouwels PH, van den Hondel CAMJJ (1987) Mol Gen Genet 206:71–75
Wang J, Holden DW, Leong SA (1988) Proc Natl Acad Sci USA 85:865–869
Ward M, Wilkinson B, Turner G (1986) Mol Gen Genet 202:265–270

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