

DEVELOPMENT OF A TRANSFORMATION SYSTEM FOR *PENICILLIUM BREVICOMPACTUM* BASED ON THE *FUSARIUM OXYSPORUM* NITRATE REDUCTASE GENE

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ABSTRACT

Penicillium brevicompactum is a filamentous fungus that presents a potential for industrial use due its efficient pectinase production. A heterologous transformation system was developed for *P. brevicompactum* based on the complementation of a nitrate reductase mutant. Nitrate reductase mutants were obtained by resistance to chlorate in a rate of 23.24% when compared to other mutations that lead to the chlorate resistance. One mutant named 4457-18X was chosen for the transformation experiments with the pNH24 vector containing de *Fusarium oxysporum* nitrate reductase gene. A frequency of approximately 3 transformants/μg DNA was obtained using the circular vector pNH24. This frequency was multiplied about 10 fold using the linearized vector with the *Xba* I restriction enzyme. Southern analysis of the transformants showed a tendency of the linearized vector to diminish the number of integrations compared to the use of the circular vector. The integration was random and stable in the analyzed transformants. The establishment of a transformation system for *P. brevicompactum* is fundamental for genetic manipulation of this microorganism.

Key words: nitrate reductase, *Penicillium brevicompactum*, heterologous transformation

INTRODUCTION

Pectinolytic enzymes catalyze the degradation of the pectin present in the plant cell wall. Among these enzymes polygalacturonase preferentially hydrolyses pectic acids and pectin liase catalyses cleavage of the α-D-(1,4) glycosidic bounds of pectin by the beta-elimination mechanism. After analyzing the pectinase production of 10 species of the *Penicillium* genus, Pereira *et al.* (17) reported that *Penicillium brevicompactum* was the best pectin liase producer and presented a considerable polygalacturonase activity. Considering the important role that these enzymes have in some biotechnological process [for a review see Kashyap *et al.* (10)] this fungus became a promising organism for industrial use. For these applications, this species must be genetically

improved to obtain an enhanced enzyme production and consequently a higher yield at an accessible cost.

Among the types of transformation systems used for filamentous fungi, the complementation of the nitrate reductase (*niaD*⁻) mutants offers a series of advantages. The spontaneous mutants can be easily obtained by positive selection by chlorate resistance and, since no mutagenic agents are used, the possibility of secondary mutations that would affect important genes is reduced. Besides, these mutants present a single desirable phenotype (inability to use nitrate as sole nitrogen source), being this phenotype not essential and not affecting growth or important metabolic pathways (28).

Heterologous and homologous transformation systems based on the complementation of the *niaD*⁻ mutants have already been described for some filamentous fungi: *Aspergillus*

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oryzae (15,28), *Fusarium oxysporum* (5,13), *Gibberella fujikuroi* (23,27), *Penicillium chrysogenum* (7,35), and *P. griseoroseum* (16,19), among others. In the heterologous transformation systems, the transformation frequency was lesser than 20 transformants/ μg DNA. The optimization of the heterologous transformation protocol for *F. oxysporum* reached a frequency of 100 to 200 transformants/ μg DNA (11). One aspect that leads to an increase in the transformation frequency is the linearization of the vector. For several species, this linearization influenced the transformation frequency positively, as for *Ustilago maydis* with a 20-fold increase (34), *A. niger* and *P. griseoroseum* with a 2-fold increase (25,29). On the other hand, certain species, such as *Coprinus cinereus*, have not presented this effect (8).

One of the applications of a heterologous transformation system is the use as selection marker in cotransformation experiments to improve enzyme production, as for *A. oryzae* that presented up to 3.2-fold increases in polygalacturonase production when transformed with a *P. janthinellum* polygalacturonase gene, where the transformants had been selected by the complementation of a *niaD* mutation (9), and to *A. awamori*, where nearly 90% of the transformants containing polygalacturonase encoding gene copies of *A. niger* presented a two to six-fold increase of this enzyme production (21). If this heterologous system shows random integrations, it can also be used for insertional mutagenesis experiments and future cloning of important genes in this fungus.

Thus, the goal of this work was to develop a heterologous transformation system for *P. brevicompactum* based on the complementation of the nitrate reductase gene that would allow the genetic manipulation of this species.

MATERIALS AND METHODS

Strain and media

The *Penicillium brevicompactum* strain was obtained from the Fundação Tropical de Tecnologia e Pesquisas André Tosello (Campinas/SP, Brazil) under the registration number CCT 4457. Minimum medium described by Pontecorvo *et al.* (18), complete medium according to Pontecorvo *et al.* (18) modified by Azevedo and Costa (2) and potato dextrose agar (PDA) with addition of 2.0 g peptone, 1.5 g hydrolyzed casein, 2.0 g yeast extract, and 1.0 mL vitamin solution (0.2 mg biotin, 10.0 mg p-aminobenzoic acid, 50.0 mg pyridoxine, 50.0 mg thiamine, 100.0 mg nicotinic acid, 100.0 mg riboflavin, distilled water for 100 mL) in 1000 mL of distilled water were used.

Transformation vector

Plasmid pNH24 was described by Diolez *et al.* (5) and has an insert of about 4.0 kb containing the *Fusarium oxysporum* nitrate reductase gene and unique sites for the enzymes *Xba* I and *Eco* RI.

Selection of chlorate resistant mutants

The selection of spontaneous mutants for the nitrate reductase gene (*niaD*) was based on chlorate resistance as described by Unkles *et al.* (28). Approximately 1×10^6 conidia of the *P. brevicompactum* wild strain were plated in minimum medium containing sodium chlorate (470 mM) and glutamine (10 mM) as sole nitrogen source and incubated at 25°C for 10 days. The chlorate resistant colonies were characterized on the basis of simple growth tests in minimum medium containing 10 mM of the following nitrogen sources: NaNO_3 , NaNO_2 , $(\text{NH}_3)_2\text{SO}_4$, glutamate, and hypoxanthine (4). The colonies that presented growth in nitrite, hypoxanthine, glutamate and ammonium, but did not grow in NaNO_3 , were considered *niaD* mutants.

Protoplast production and transformation

Protoplasts of *P. brevicompactum* were obtained as described by Varavallo (32). *P. brevicompactum* conidia were inoculated on Petri dishes with PDA medium, covered with cellophane paper, and incubated at 25°C for 20 to 24 hours. The mycelium was removed and washed twice in osmotic stabilizer 0.8 M NaCl, prepared in 100 mM sodium and potassium phosphate buffer (pH 5.8). Approximately 300 mg of mycelium were incubated for 3 hours under agitation (80 rpm) at 30°C in 5.0 mL of the osmotic stabilizer containing $15 \text{ mg}\cdot\text{mL}^{-1}$ of Glucanex enzyme (Novo Nordisk Ferment Ltd., Dittingen, Switzerland). The protoplasts were separated from the undigested mycelium by filtration, and washed twice in osmotic stabilizer STC (1.0 M Sorbitol, 50 mM CaCl_2 , 100 mM Tris-HCl) by centrifugation at 3000 g during 15 minutes at 4°C. Finally, the pellet was resuspended in a certain STC volume for a final concentration of 1×10^7 protoplasts/0.1 mL.

The transformation was based on the method of Yelton *et al.* (33) and Balance and Turner (3). Approximately 2×10^7 protoplasts were mixed with 5.0 or 10.0 mg of plasmid pNH24 (circular or linearized with *Xba* I restriction enzyme) and 50 mL of 25% polyethylene glycol 6000 (PEG) and 50 mM CaCl_2 in STC, homogenized and incubated on ice for 20 minutes. After this, 500 mL of the same PEG/ CaCl_2 solution were added and the mixture kept at room temperature during 20 minutes, then plated in minimum medium containing nitrate as sole nitrogen source and 0.8 M KCl as osmotic stabilizer. This was the best osmotic stabilizer to the *P. brevicompactum* protoplasts described by Varavallo (32). The negative control was realized with protoplasts that had been treated with PEG/ CaCl_2 solution, but without DNA. Controls of protoplasts regeneration were realized. All Petri dishes were incubated at 25°C.

Mitotic stability

Mitotic stability of the *niaD*⁺ transformants was examined by growth on a non-selective medium (complete medium). Cultures were sequentially transferred to nonselective medium

five times and cultured five days each time. Cultures were then transferred back to selective medium (minimum medium with nitrate as sole nitrogen source) to determine whether the selective marker had been lost.

Southern-blot analysis

For this analysis, the total DNA of three transformants obtained with the circular vector and three transformants obtained with the *Xba* I linearized vector was extracted according to the protocol of Specht *et al.* (26). Approximately, 3.0 µg of total DNA of each strain was used in the cleavage reactions with the *Eco* RI restriction enzyme. Electrophoresis and DNA transference to the nylon membrane (Stratagene) were carried out according to standard protocols (22). Plasmid pNH24 was used as probe. DNA probe labeling, hybridization and signal detection were performed with the “Dig High Prime Labeling and Detection Starter Kit II” (Roche) following the instructions of the manufacturer.

RESULTS AND DISCUSSION

Selection of nitrate reductase mutants

After 10 days of growth at 25°C, 43 *P. brevicompactum* chlorate resistant mutants were selected (Table 1). Mutants with the chlorate resistance phenotype can be obtained by mutation in different genes (4), like the nitrate permease gene (*crnA*), the genes required for the biosynthesis of a molybdenum cofactor (*cnxA-J*), the specific and general regulators genes of the nitrate assimilation (*nirA* and *areA*) and in the proper nitrate reductase (*niaD*) gene. Independent of which gene is affected, mutation will prevent the cell from reducing chlorate to chlorite. These mutations can be easily differentiated on the basis of growth tests. Despite the phenotype of not growing in nitrate and nitrite could also stand in connection with the mutation of the nitrite

reductase gene (*niiA*), there is no theoretical relation between the mutation in this gene and chlorate resistance.

Nitrate reductase mutants were obtained in a rate of 23.24% when compared to other mutations that lead to the chlorate resistance. Since the isolated mutants were spontaneous and because mutation is a random event, the number of isolated *niaD*⁻ mutants for each species will predictably not follow a pattern, as reported for *P. griseoroseum*, where 8.33% of the chlorate resistant mutants were *niaD*⁻ mutants (19), while in *P. canescens* the isolated *niaD*⁻ mutants made up 49.0% (1), and 73.68% in *P. chrysogenum* (7).

After the isolation of *niaD*⁻ mutants, the recipient strain for transformation procedures must be chosen. All the 10 *niaD*⁻ mutants were tested for possible revertants and the reversion frequency was < 1 × 10⁻⁶ in all of them. The mutant designated 4457-18X was chosen for the transformation experiments since it presented the lowest residual growth in minimum medium containing nitrate as sole nitrogen source.

Transformation

Plasmid pNH24, which contains the *F. oxysporum niaD* gene, was used to transform the mutant 4457-18X. After two days at 25°C, the transformants colonies were observed in selective medium containing nitrate as sole nitrogen source. This is a notably reduced time for the appearance of transformants. In comparison, the *P. griseoroseum* homologous *niaD*⁺ transformants were observed after five days (16), and for *P. chrysogenum*, 40% of the transformants appeared after seven days and the rest after the forty day (35). This growth speed seems to be an inherent characteristic of the *P. brevicompactum* and is of interest, since it diminishes the time required to obtain the desired transformants. All selected transformants transferred to a new selective medium were able to grow, without the presence of abortive transformants. Transformants were obtained in all tested treatments (Table 2).

The PEG treatment reduced the protoplast regeneration frequency by 9%, a similar rate reported for *Metarhizium flavoviride* with a 10% reduction of the regeneration rate (6) and for the CG423 strain of *M. flavoviride* with a 10 to 25% reduced protoplast viability (30).

The transformation frequency with the circular vector was around 3 transformants/µg DNA. This frequency is superior to the one obtained for *G. fujikuroi* of 1 to 2 transformants/µg DNA, using the *A. niger niaD* gene (23). However the frequency obtained in this study is lower than the one reported for *P. griseoroseum* of 13 transformants/µg DNA (25) or that reported by Ribeiro (20) of 19 transformants/µg DNA for *P. expansum* and reported by Levis *et al.* (12) of 10 transformants/

Table 1. Growth of the *P. brevicompactum* chlorate mutants in minimum medium containing different nitrogen sources.

Mutation	Numer of mutantes	Nitrogen source				
		Nitrate	Nitrite	Hypoxanthine	Glutamate	Ammonium
<i>niaD</i>	10 (23.24%)	-	+	+	+	+
<i>crnA</i>	15 (34.89%)	+	+	+	+	+
<i>nirA</i>	13 (30.24%)	-	-	+	+	+
<i>cnxA-J</i>	5 (11.63%)	-	-	-	+	+
Total	43 (100.0%)					

All nitrogen sources were adicioned in a concentration of 10 mM. + indicates normal growth, and – indicates absence of growth. The phenotype symbols used for *A. nidulands* were adapted for *P. brevicompactum*.

Table 2. Transformation of *P. brevicompactum* nitrate reductase mutants with the pNH24 vector containing the *F. oxysporum* nitrate reductase gene.

Vector	From	Amount of DNA (µg)	Number of transformants				Frequency (transformants.µg ⁻¹)
			Experiments			Mean	
			1	2	3		
pNH24	Circular	5	12	13	14	13	2.6
		10	31	32	36	33	3.3
	Linear*	5	39	36	39	38	7.6
		10	355	346	337	346	34.6

*Vector linearized with *Xba* I restriction enzyme.

µg DNA for *Botrytis cinerea*, being all of these also based on the complementation of a *niaD* mutation with the pNH24 vector. Nevertheless, Langin *et al.* (11) presented a transformation frequency of 100 to 200 transformants/µg DNA for a heterologous system for *F. oxysporum*, by simply enhancing some aspects of the transformation protocol.

A number of studies have reported increased transformation frequency when using the vector in linear form. The integration of these linear vectors must occur by a non-homologous recombination mechanism, in which microhomologous regions of the simple strand ends of the vector would act in the integration. Enzymes like the topoisomerase I might be involved in this recombination type in *S. cerevisiae* (36).

For *P. brevicompactum* the use of the linearized pNH24 vector with the *Xba* I restriction enzyme increased around 10 times the transformation frequency when using the concentration of 10 mg DNA. In *U. maydis*, the linearization of the vector pHL1 increased the transformation frequency by about 20 times (34), and studies into other fungi, such as *A. bisporus*, *M. grisea*, and *S. cerevisiae* have reported the same observation (14,24,31). Whitehead *et al.* (35) showed that the use of the linearized vector pSTA10, which carries the *A. niger niaD* gene, increased the transformation frequency from 6.2 to 13.3 transformants/µg DNA. In the homologous transformation system of *A. niger*, the linearization of the vector increased the transformation frequency from 64 to 455 transformants/µg DNA (29).

Transformant analysis

After the monosporic purification, an analysis of the mitotic stability of the transformants was carried out. All analyzed transformants preserved their ability to use nitrate as sole nitrogen source after five transfers in complete medium. This

indicates that the vector pNH24 integration was stable in the transformants. In heterologous transformation systems, vector integration is not always stable. Malardier *et al.* (13) demonstrated that some *F. oxysporum* transformants were instable after integrating the *A. nidulans niaD* gene into the genome.

Hybridization experiments were realized with three transformants originated by the treatment with the circular vector and three by the treatment with the *Xba* I linearized vector. The physical presence of the vector was observed in all these transformants, indicating its integration in the genome (Fig. 1).

Fig. 1 shows that the copies of the pNH24 vector are in different positions, indicating random integration of the vector into the host genome. These random integrations are probably a consequence of the low similarity among the nitrate reductase encoding genes in *P. brevicompactum* and *F. oxysporum*. Sequence analysis of the NIAD protein

of *P. griseoroseum*, carried out by Pereira (16), revealed a similarity of 57% between the nitrate reductase proteins of *P. griseoroseum* and *F. oxysporum*. Besides, Queiroz *et al.* (19) and Soares (25) did not observe hybridization between the total DNA of *P. griseoroseum* and the *F. oxysporum nia* gene in the tested conditions (65°C), indicating the low homology of this gene between these two species.

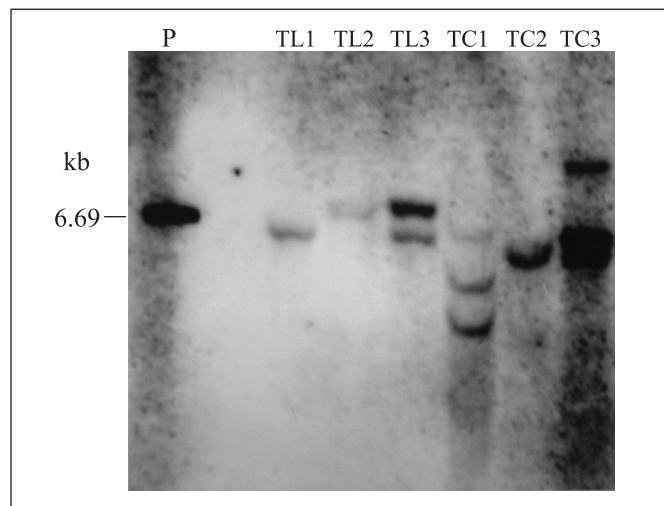


Figure 1. Molecular analysis of *P. brevicompactum* transformants. Three transformants originating in the treatment with the linear vector (TL1, TL2 e TL3) and 3 transformants originating with the circular vector (TC1, TC2 e TC3) were analyzed using the vector pNH24 as probe. (P) indicates the vector pNH24 linearized with *Eco* RI restriction enzyme.

An analysis of the number of vector insertions into the transformant genome showed that there is a tendency of the linearized vector to reduce the number of integrations. Nevertheless, an analysis with a higher number of transformants is necessary to comprove this tendency. One vector copy seems to have been integrated in the transformants TL1, and TL2, while at two to three copies were integrated in the transformants TC1, and TC3. Soares (25) reported that two transformants originated from the transformation of *P. griseoroseum* with the circular plasmid pNH24 showed the integration of only one copy. However, this seems more likely to be an exception, as suggested by Queiroz *et al.* (19).

Unique and random integrations are the main requirements for the insertional mutagenesis technique, since they facilitate the verification of the integration into the gene that is responsible for the mutant phenotype and the future cloning of the interrupted gene. Queiroz *et al.* (19) reported that the greatest part of the pNE24 vector integrations in the *P. griseoroseum* genome occurred in heterologous sites. This characteristic was used by Soares (25) to develop an insertional mutagenesis system for *P. griseoroseum* using the REMI (Restriction Enzyme-Mediated Integration) technique, obtaining morphologic and auxotrophic mutants.

In conclusion, based on the results of this study, vector pNH24 can be used in an insertional mutagenesis system for *P. brevicompactum*, since this vector integrates randomly into the fungus genome and has a low number of copies when linearized.

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RESUMO

Desenvolvimento de um sistema de transformação para *Penicillium brevicompactum* baseado no gene da nitrato redutase de *Fusarium oxysporum*

Penicillium brevicompactum é um fungo filamentoso que apresenta um potencial para a aplicação industrial devido a sua eficiente produção de enzimas do complexo pectinolítico. Neste trabalho foi desenvolvido um sistema de transformação heterólogo para *P. brevicompactum* baseado na complementação de um mutante nitrato redutase. Mutantes nitrato redutase foram obtidos pela resistência ao clorato de sódio em uma taxa de 23,24%. O mutante denominado 4457-18X foi escolhido para os experimentos de transformação com o vetor pNH24, que contém

o gene da nitrato redutase de *Fusarium oxysporum*. Uma freqüência de cerca de 3 transformantes/mg de DNA foi obtida utilizando-se o vetor pNH24 na forma circular e um aumento de cerca de 10 vezes nessa freqüência foi alcançado com a utilização desse vetor linearizado com a enzima de restrição *Xba* I. A análise dos transformantes pela técnica de hibridização revelou uma tendência do vetor linearizado diminuir o número de integrações em relação ao vetor circular. A integração foi aleatória e estável nos transformantes analisados. O estabelecimento de um sistema de transformação para *P. brevicompactum* é essencial para a manipulação genética desse microrganismo.

Palavras-chave: nitrato redutase, *Penicillium brevicompactum*, transformação heteróloga

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