Heterologous transformation of *Cochliobolus lunatus*

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1. SUMMARY

A DNA mediated transformation system has been developed for the filamentous fungus *Cochliobolus lunatus*. Transformants were obtained by using plasmid pAN 7-1 carrying the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) fused to an *Aspergillus nidulans* promoter. The integration of plasmid pAN 7-1 into the fungal genome altered the ability of this microorganism to transform progesterone.

2. INTRODUCTION

*Cochliobolus lunatus* is a fungus of biotechnological importance. It is able to hydroxylate steroids at different positions [1], with the main reaction at specific position 11β [2,3]. Although it has been used for steroid bioconversions for a long time, very little is known about the genetics of this fungus mainly due to its lack of a sexual cycle.

A basic requirement for studying gene expression at the molecular level is a gene transfer system which includes a reproducible transformation procedure and proper selection markers. Although a transformation system based on resistance to the aminoglycosidic antibiotic hygromycin B has now been described for several fungi, including *Saccharomyces cerevisiae* [4,5], the fungal plant pathogen *Cochliobolus heterotrophus* [6], *Glomerella cingulata* [7], *Fulvia fulva* [8] and *Leptosphaeria maculans* [9], there are few reports about the transformation of biotechnologically important fungi [10,11].

This paper reports the use of plasmid pAN 7-1, described previously by Punt and coworkers [12], for transformation of the biotechnologically important species *Cochliobolus lunatus*.

3. MATERIALS AND METHODS

3.1. Strains and plasmids

*Cochliobolus lunatus* m 118, obtained from the Strain Collection of Friedrich Schiller University, Jena, G.D.R. was used as the recipient in transformation experiments. Media, growth conditions and storage of this strain have been described previously [3]. *Escherichia coli* K-12 JM 109 [13] was

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used for maintenance and propagation of the plasmid.

Plasmid pAN7-1 [12] was kindly provided by Dr. R. van Gorcom from MBL-TNO Rijswijk, the Netherlands.

3.2. Preparation of protoplasts

Protoplasts were prepared from approximately 10 g of wet mycelium. A Trichoderma viride exo-enzyme preparation, produced by using myceliar fragments of Rhizopus nigricans as inducer, was used as lytic enzyme [14]. C. lunatus mycelium was resuspended in 20-40 ml filter-sterilized and osmotically stabilized buffer OSB (0.05 M succinic acid–NaOH, pH 5.0, with 1 M NaCl) to which 3.5 mg/ml lytic enzyme mixture was added. The suspension was gently agitated at 28°C for 2-4 h and filtered through a sintered glass filter No. 1 in order to separate protoplasts from mycelial debris. Protoplasts were collected by centrifugation at 3000 ×g for 5 min, washed once in 10 ml 1 M NaCl, twice in 10 ml SCT buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl₂) and resuspended in SCT at a final concentration of 10⁵ protoplasts/ml.

3.3. Transformation procedure

The transformation procedure followed, with some modifications, the method which has been described previously [6]. In this case protoplasts were mixed with plasmid DNA (50 μg DNA/10⁷ protoplasts) in the presence of 10 mM CaCl₂ and 60% PEG, incubated for 20 min at room temperature and transferred to 10 cm plastic Petri dishes. In order to select hygromycin B resistant transformants, the protoplasts were first plated on non-selective medium (1.5% agar, malt extract 6° Bgl, 1.2 M sorbitol) and incubated for 6 h at 28°C. After this period they were overlayed with 10 ml of 1% agar containing hygromycin B (Boehringer, F.R.G.) at a final concentration of 100 μg/ml.

3.4. DNA isolation and manipulations

DNAs from the wild-type and the transformants of C. lunatus were isolated from lyophilized mycelia as described by Reader and Broda [15]. DNA digestion with BamHI, agarose gel electrophoresis and Southern blot hybridization standard procedures were used [16]. Approximately 10 μg of DNA was loaded per lane of the agarose gel. The hybridization probe was prepared from plasmid pAN 7-1, linearized by restriction with BamHI, by randomly primed incorporation of [³²P]α-dCTP (Multiprime, Amersham). The HybondN² filter was finally washed in 2 × SSC at room temperature.

3.5. The assay of progesterone biotransformation

For the in vivo bioconversion assay 0.7 g (wet weight) of 24-h mycelium was resuspended in 10 ml phosphate buffer pH 5.5 (1 mM Na₃PO₄, 0.2 M EDTA, 0.04 M glutathione-SH). Progesterone dissolved in DMF (0.1 mg/ml final concentration) was added as substrate. Cycloheximide, as an inhibitor of protein biosynthesis in eukayrotes, was added to a final concentration of 0.1 mg/ml prior to the substrate addition in parallel incubations. After a 3-h incubation on a rotatory shaker (180 rpm) at 28°C, the reaction mixtures were extracted with chloroform and extracts chromatographed by TLC on F-254 precoated Silica Gel-60 plates (Merck) in a solvent system of ethylacetate : cyclohexane (2:1, v/v).

The inclusion of progesterone into the different metabolic products was followed by using [1,2,6,7,16,17-³H]progesterone (Amershams) as substrate. The combination of cold (0.5 mg) and ³H-labelled (10 μCi) progesterone was added to 5 ml of buffer solution and bioconverted with 350 mg of the pAN7 transformant T4. A control reaction was also performed with only cold progesterone. After developing the chloroform extracts of the broths by TLC, 5 mm silica gel lanes were scraped, mixed with 3 ml of toluene scintillation liquid and counted on a LKB 1214 Rackbeta Liquid Scintillation Counter. The control sample was scanned with a Camag TLC Scanner II under UV 254 nm.

4. RESULTS AND DISCUSSION

Transformation to drug resistance is particularly well suited for organisms which are genetically undeveloped, such as C. lunatus. Wild-type cells can be used as recipients and specific muta-
Fig. 1. Plasmid transformants of *C. lunatus*, growing on HmB agar. (A, B, C) Real transformants. (D) Abortive colonies.

Fig. 2. Hybridisation pattern of *Bam*HI restricted DNAs from pAN 7-1 transformants of *C. lunatus*. T1–T15, different transformants: C.I., the native strain. 10 μg of particular DNA was restricted with 30 U *Bam*HI at 37°C for 3 h. Agarose gel concentration was 0.8%. 50 ng of hybridisation probe (ccc form of plasmid pAN 7-1, linearized with *Bam*HI) was labelled with [³²P]α-dCTP (Multiprime, Amersham).
tions are not needed for transformation and selection. The only requirement is that the organism to be transformed is sensitive to the chosen antibiotic.

To test sensitivity to hygromycin B, both hyphae and protoplasts of *C. lunatus* were inoculated into the minimal medium [3]. Since many ions inactivate the antibiotic [16], 1.2 M sorbitol was used as an osmotic stabilizer. Even 10 μg HmB/ml clearly reduced the growth of both hyphae and protoplasts and 50 μg HmB/ml completely inhibited the growth and regeneration. After incubation of *C. lunatus* protoplasts with pAN7-1 plasmid DNA, hygromycin resistant colonies appeared after 6–10 days. In various and repeated experiments the transformation frequency was found to be 0.15–0.29 transformants per μg of DNA. The colonies were large and were able to grow on selective agar plates with up to 1000 μg HmB/ml (Fig. 1A–C). Together with these typical transformant colonies a number of smaller and weakly growing abortive colonies were obtained as well (Fig. 1D).

Transformation with pAN 7-1 dramatically altered the life cycle of the fungus. In comparison to the nonsporulating wild type, which lacks a sexual cycle, all the transformants were found to sporulate. The frequency of transformation achieved

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**Fig. 3.** Bioconversion of progesterone by *C. lunatus* and its pAN 7-1 transformants and the effect of cycloheximide. 1, progesterone (P); 2, bioconversion with the native strain; 3, 11β-OH-P; 4, 11α-OH-P; 5, 6β-OH-P; 6, 8, 10, bioconversion with different plasmid transformants; 7, 9, 11, the same as 6, 8, 10, but with addition of 0.1 mg/ml cycloheximide.
was comparable with the results obtained for taxonomically related fungi such as Cochliobolus heterotrophus [6] or Curvularia lunata [11], but rather low when compared with other systems [8,9,12,17]. In all systems described, the transformation was integrative. Since the expression of the hygromycin phosphotransferase gene in plasmid pAN 7-1 is controlled by the Aspergillus nidulans gdp and trpC expression signals, the low frequency of transformation may in future be improved by the inclusion of homologous promoter sequences into the transforming plasmid, as demonstrated for Aspergillus niger [18].

After subculture of plasmid transformants under nonselective conditions for at least 6 passages over three months none of the 16 tested transformants lost their ability to grow on the medium containing the antibiotic. This observation indicates that information encoded in the plasmid was firmly integrated in the genome of C. lunatus. For studying the incorporation of plasmid pAN 7-1 into the genome, DNA from 15 transformants and the native strain was isolated, totally restricted with BamHI, which has one restriction site within pAN 7-1, and subjected to Southern blot analysis. Fig. 2 shows the complex hybridization pattern, which opens the question whether this pattern resulted from multiple integration events at different genomic sites, or from a single site integration involving recombinant vector sequences, as also discussed in the literature [20]. From the fact that after hybridization the native strain C. lunatus showed some homology with the heterologous vector plasmid pAN 7-1, it is more likely that integration of the plasmid into the genome is site-specific. This proposal is supported by the finding that in comparison to the native strain all pAN 7-1 transformants showed an equivalently altered pathway of progesterone bioconversion. The main reaction of the native strain is the bioconversion of precursor steroids into biologically active compounds via hydroxylation at the 11β position [1]. In the bioconversion of progesterone, side products at 11α and 6β positions are also present. Integration of the plasmid into the genome interrupted this function and two new products, named X and Y, were observed (Fig. 3). Under conditions of prolonged fermentation a third product, named Z, was also detected. Since cycloheximide as inhibitor of protein biosynthesis in eukaryotes inhibited progesterone bioconversion in only one-half of the plasmid transformants, it could be concluded that two types of transformants must exist: the first class, in which the synthesis of enzymes included in the steroid bioconversion is inducible, and the second, where a constitutive mode of enzyme synthesis could be found.

By the biotransformation of [1,2,6,7,16,17-3H]progesterone (Fig. 4) it was demonstrated that the new substances were in fact derivatives of progesterone. The structural studies of the new metabolites are under investigation.

In our opinion, the transformation system described provides good prospects for molecular analysis of the gene structure and gene expression of steroid transforming enzymes in the fungus Cochliobulus lunatus.

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