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Transformation of *Claviceps purpurea* using a bleomycin resistance gene*

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Summary. To develop a DNA-mediated transformation system for *Claviceps purpurea* a vector was constructed using a bleomycin-resistance gene (*bleo*^{R}) fused in frame to the *Aspergillus nidulans trp* C promoter as a dominant selection marker. The construct was shown to be functional in *Aspergillus nidulans* and *Aspergillus niger* and used to transform a wild strain of *Claviceps purpurea*. Transformants were obtained at low frequencies; they were shown to contain transforming DNA integrated into the chromosomal DNA, probably in multimeric copies and at multiple sites. Combined Southern, Northern and resistance level analysis indicate that the *A. nidulans* promoter is functional in *C. purpurea*.

Introduction

Claviceps purpurea, a phytopathogenic ascomycete, has gained considerable scientific interest because of its biotechnological relevance: since medieval times the parasitic resting structures of this fungus, the sclerotia, were used as drugs; they contain peptide alkaloids, which are still of considerable therapeutic importance (for review see Esser and Düvell 1984). Current strain improvement programs in pharmaceutical industry aim at strains producing high titers of defined ergot alkaloids. In addition, *C. purpurea* is one of the few obligate parasitic fungi which can be easily cultivated in axenic culture, and therefore it is an interesting object to study both host-parasite interaction as well as aspects of secondary metabolism.

Since Claviceps possesses a regular sexual cycle, classical genetic techniques can be applied, though the length of its lifecycle, only part of which can be performed in the laboratory, causes considerable problems (Tudzynski et al. 1982; Esser and Tudzynski 1978).

Therefore it would be desirable to adapt current genetic engineering techniques to this fungus. Transformation of filamentous fungi, considered to be a serious technical problem only a few years ago, recently has become a convenient method also for those organisms devoid of well worked-out genetics. This was due to (a) the more or less universal acceptance of the promoters of "house keeping" genes like the *Neurospora crassa pyr4*, or *A. nidulans arg* B and *trp* C genes among various filamentous fungi (e.g. Ballance and Turner 1985; Parsons et al. 1987; Turgeon et al. 1986) and (b) the availability of dominant selection markers like hygromycinB-resistance (e.g. Rodriguez and Yoder 1987; Wang et al. 1988) and, more recently, bleomycin resistance (e.g. Gatignol et al. 1987; Kolar et al. 1988; Mattern et al. 1988). Bleomycin and phleomycin belong to a relatively novel class of antibiotics. Both in vivo and in vitro they cause scissions in DNA molecules, preferentially at inverted repeat sequences with single stranded DNA and at different unmethylated sites with double stranded DNA. The reaction is mediated by oxygen in the presence of iron salts (Gatignol et al. 1987). Two bleomycin resistance genes have been characterized, from Tn5 (Collins and Hall 1985) and from *Staphylococcus aureus* (Semon et al. 1987). Here we report the construction of a vector expressing the Tn5 bleomycin resistance

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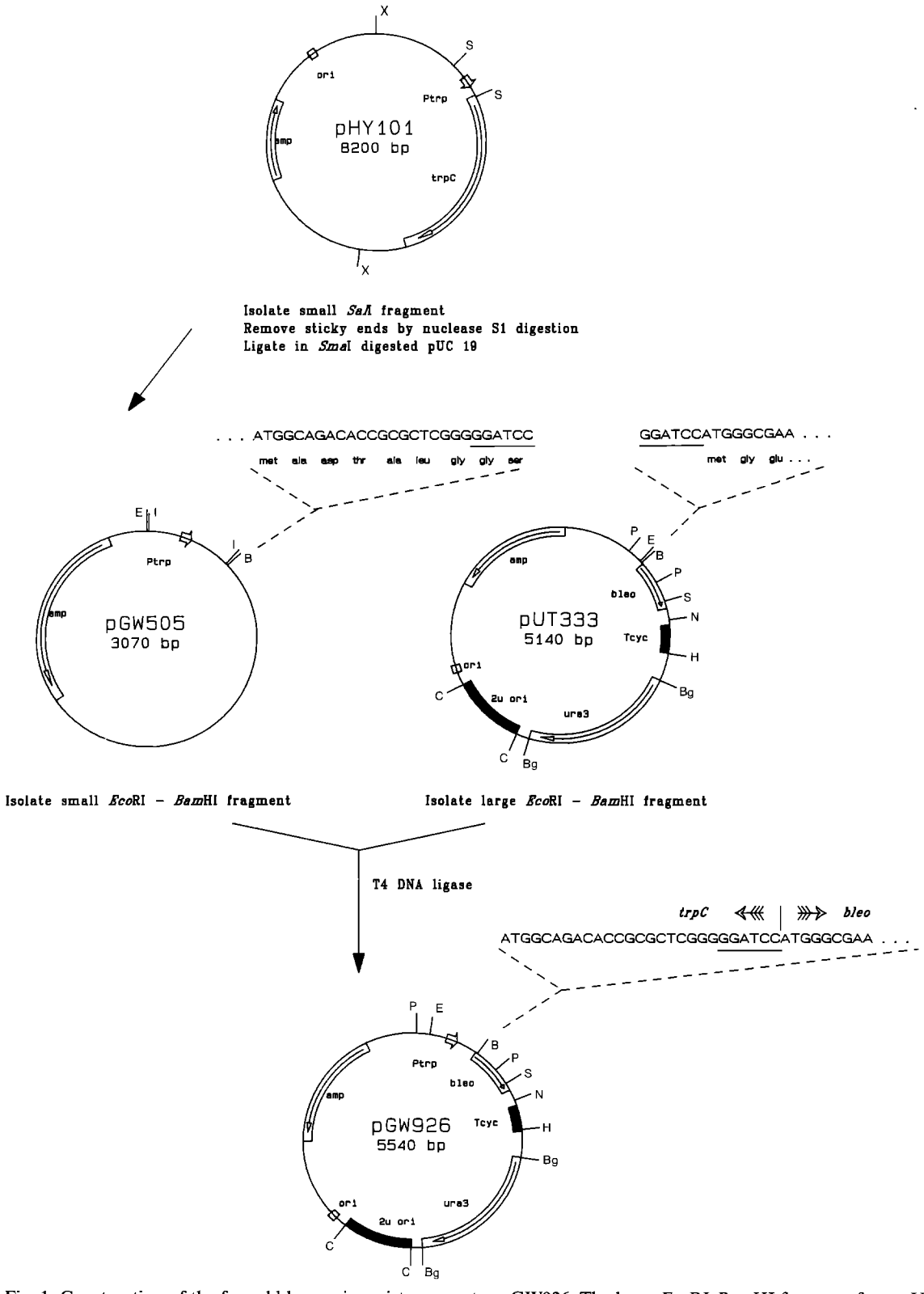

Fig. 1. Construction of the fungal bleomycin resistance vector pGW926. The large *Eco*RI-*Bam*HI fragment from pUT333 containing the coding region of the bleomycin resistance gene, the *CYC*1 terminator from *Saccharomyces cerevisiae* and additional vector sequences, was isolated and ligated to a subcloned fragment containing the promoter of the *A. nidulans trp*C gene. Relevant abbreviations: *Ptrp* promoter of the *A. nidulans trp*C gene (Mullaney et al. 1985); *Tcyc* transcription terminator of the *S. cerevisiae* iso-1-cytochrome c gene; *bleo* coding region of the bleomycin (phleomycin) resistance gene (Gatignol et al. 1987). The DNA sequence partially depicted shows the junction between the first nine amino acids of the *trp*C gene and the complete bleomycin resistance coding region, the *Bam*HI restriction site is underlined. Restriction sites: *B Bam*HI; *Bg Bgl*II; *C Cla*I; *E Eco*RI; *H Hin*dIII; *N Nar*I; *P Pvu*II; *S Sal*I; *X Xho*I

gene under the control of the *A. nidulans trp* C promoter, the development of an efficient protoplast formation and regeneration procedure for *C. purpurea* and the successful use of these procedures to transform *C. purpurea* at low frequencies.

Materials and methods

Strains and growth conditions. Claviceps purpurea wild isolate T5 (Düvell et al. 1988) was used as recipient strain. Mycelia were grown at 30°C in a complete medium according to Mantle and Nisbeth (1975) or B2-medium (Esser and Tudzynski 1978). *A. nidulans* strain WG096 (*yA2, pabaA1*) and the wild type *A. niger* strain N400 were used as recipients to test the functionality of the bleomycin vector construct by transformation and expression in Aspergilli. Standard media were used for the growth of the Aspergillus species (Pontecorvo et al. 1953; Cove 1966).

Vectors. Plasmid pGW926 containing the Tn5 bleomycin resistance gene under the control of the *A. nidulans trp* C promoter was constructed as detailed in the legend of Fig. 1. Plasmids pHY101 and pUT333 were kindly provided by Dr. W. Timberlake and Dr. G. Tiraby, respectively. pUC19 DNA was used as a control for transformation.

Standard recombinant DNA procedures. (Preparation of plasmid DNA from *E. coli*, Southern/Northern hybridization, labeling of DNA) were performed according to Maniatis et al. (1982), with modifications as described (Tudzynski and Esser 1986; Düvell et al. 1988).

Preparation of total DNA and RNA from Claviceps. 50 ml liquid cultures (B2 medium with 50 g Sucrose/l), inoculated with sporulating mycelial pads, were incubated for 40 h (DNA) or 65 h (RNA) on a rotary shaker (150 rpm, 28°C). Mycelia was harvested by filtering over miracloth and washed with destilled water. DNA was prepared according to Schechtmann (1987) with the following modifications: DEPC (Diethylpyrocarbonate) was omitted; the lysis period was prolonged to 30 min and proteinase K was added to a final concentration of 0.2 mg/ml. For preparation of RNA the protocol of Hoge (1982) was used.
Separation of RNA on formaldehyde-agarose gels was performed according to Maniatis et al. (1982).

Transformation procedures. Transformation of the Aspergillus species was performed as described earlier (Wernars 1986; Goosen et al. 1987). The cellophane method used for the preparation of protoplasts from *A. nidulans* (Ballance and Turner 1985) and *A. niger* (Goosen et al., unpublished data) was adapted for Claviceps. Fresh medium plates were covered with sterile cellophane sheets and 10^5 *C. purpurea* conidiospores were spread over the surface. After incubation at 28°C for 2–3 days, sheets were transferred to 10 ml 0.85 M sorbitol – 0.2 M potassium maleate (pH 5.8) — 50 mM $CaCl_2$, containing 5 mg/ml filter sterilized Novozym 234, and left in this solution for 90 min at 30°C with slight agitation. Protoplasts were separated from cell debris by filtration over glass wool, washed twice in 0.85 M sorbitol-10 mM Tris-HCl (pH 7.5)-50 mM $CaCl_2$. Portions of 200 µl (10^8 protoplasts/ml) were mixed in a polypropylene tube with typically 20 µg of undigested DNA. 50 µl 25% (w/v) polyethyleneglycol in 10 mM Tris-HCl (pH 7.5)-50 mM $CaCl_2$ was added, and the mixture was incubated on ice for 20 min. Another 2 ml of PEG solution was added and the mixture was incubated at 20°C for 5 min and diluted with 4 ml STC. Of the transformation mixture 1 ml was — together with 4 ml 1.2% topagar (44°C) — spread over selective plates (B2 pH 8, including 20% Sucrose). Top- and bottomagar both contained the amounts of phleomycin indicated in the text (typically 50 µg/ml). Plates were incubated at 28°C for 8 to 10 days before bleomycin resistant colonies appeared.

Reagents and enzymes. Metal-free Bleomycin B2 was a gift from Dr. T. Takita (Japan), copper-containing phleomycin was purchased from Cayla (Toulouse, France). Novozym 234 was obtained from Novo Biolabs (Denmark), PEG 6000 from Merck (Darmstadt, FRG), Hybond N from Amersham, Gene Screen from Dupont. All enzymes used were either from Bethesda Research Laboratories, Boehringer, Pharmacia or Promega and used as recommended by the manufacturers.

Results and discussion

Construction of a functional fungal bleomycin vector

The prokaryotic resistance determinant of Tn5 as present in pUT333 (G. Tiraby, unpublished) was fused to the *A. nidulans trp* C promoter (Mullaney et al. 1985) in such a way that an in frame fusion with the complete bleomycin coding region should result (for details see Fig. 1). The functionality of this construct (pGW 926) was tested by transforming protoplasts from both *A. nidulans* and *A. niger* with 5 µg pGW 926. Transformants were selected on minimal medium (pH 8.0) containing various amounts of bleomycin at 30°C (*A. niger*) or 37°C (*A. nidulans*). The results are summarized in Table 1. Especially with *A. niger* omis-

Table 1. Functional test of the bleomycin construct in *Aspergillus*
2×10^7 fungal protoplasts were incubated with 5 µg pGW926 and plated on minimal medium containing various concentrations of bleomycin. The agar overlay used for spreading the protoplasts after transformation contained either the same concentration of bleomycin or no drug at all. Bleomycin resistant colonies were counted from 6 plates and are given as transformants/µg vector DNA

Selection conditions	Concentration Bleomycin (µg/ml)	Number of $bleo^R$ transformants/µg DNA	
		A. nidulans	*A. niger*
No Bleomycin In topagar	2	42	31
	5	25	14
	10	7	2
Bleomycin in topagar	2	36	10
	5	18	1.5
	10	10	0.5

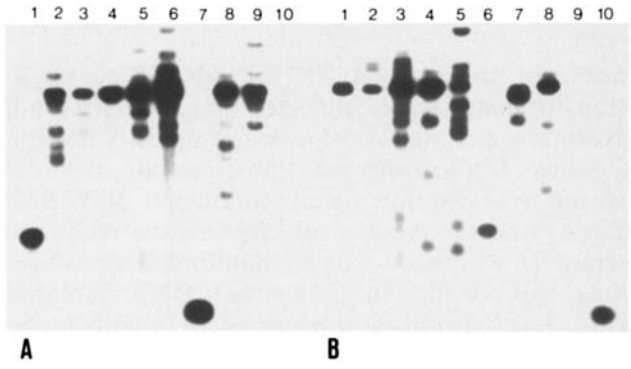

Fig. 2. Southern hybridisation analysis of Aspergillus transformants. Panel **A:** DNA from *A. nidulans bleo*^{R} transformants, digested with *Hind*III. Lanes *1* and *7* contain *Eco*RI-*Hind*III and *Eco*RI-*Sal*I digested pUT333 DNA, giving rise to hybridisation signals at 800 bp and 360 bp respectively. Lane *10* contains DNA from untransformed *A. nidulans*. Panel **B:** DNA from *A. niger bleo*^{R} transformants, digested with *Hind*III. Lanes *6* and *10* contain the 800 bp and 360 bp markers from pUT333. Lane *9* contains DNA from untransformed *A. niger*. Blots were hybridized with the ^{32}P-labeled *Eco*RI-*Sal*I fragment from the *bleo*^{R} gene

sion of the antibiotic in the topagar overlay resulted in increased transformation frequencies. Similar experiments using selection on complete media resulted in an approximately five-fold decrease in transformation frequencies (data not shown). Bleomycin resistant transformants from both species were subjected to Southern blotting analysis (Fig. 2). In all transformants analysed transformation had occurred by integration of vector sequences into the genome, a phenomenon now invariably reported in fungal transformation. Most transformants contain multiple copies of the bleomycin resistance determinant, often in a tandemly repeated fashion. There is a general correlation between the number of integrated copies and the level of the bleomycin resistance expressed (not shown). These results confirmed not only the functionality of the construct, they also indicated that indeed the *A. nidulans trp* C promoter was functional in *A. niger*.

Preparation of protoplasts from Claviceps and regeneration

Preparation of protoplasts from *C. purpurea* using relatively old mycelial material has been described by several groups (e.g. Stahl et al. 1977; Keller et al. 1980). As reported by Villanueva (1971) for several fungal systems, regeneration and viability of protoplasts decrease with age of the mycelium resulting in low recoveries and poor regeneration. By adapting the protocol used for the preparation of protoplasts from Aspergilli optimal yields and high values for viability and regeneration could be obtained for *C. purpurea*. Especially, the use of very young membrane-grown mycelium turned out to be an important pre-requisite. Using the cellophane method (Ballance and Turner 1985) it was possible to obtain 5×10^7–5×10^8 protoplasts from one sheet on B2-medium (pH 5.2) with regeneration values up to 75%; with the liquid precultures these values were more variable and in general about tenfold lower due to the age of the mycelium. Some components in the protoplasting buffer were shown to be very important. Addition of potassium maleate (pH 5.8) instead of MES-buffer (pH 5.8) had a positive effect on the formation of protoplasts and their regeneration. Omitting the $CaCl_2$ had a strong negative effect on protoplast formation. Regeneration was positively influenced by the concentration of PEG (optimal between 25% and 40%). The pH of the regeneration medium was very important: at pH 5.2 regeneration was optimal, at pH 8.0 a drastic reduction occurred (about 70% versus 5%).

Transformation of Claviceps and selection for phleomycin resistance

The minimum inhibitory concentration of different batches of phleomycin for *C. purpurea* conidiospores in B2 medium (pH 8.0) was determined to be 40 μg/ml. The inhibitory concentration of two different batches of bleomycin B2 was quite different, the reason why all transformation and selection experiments were performed with phleomycin. For both drugs the sensitivity of conidiospores could be increased by low osmolality and high pH (pH = 8.0). Since the sensitivity of the conidiospores and the regenerating protoplasts of *C. purpurea* was found to be pH-dependent, a compromise had to be found for the selection of drug resistant transformants with respect to the stabilising osmoticum and the pH for regeneration and selection. Using a pH value of 8.0 a drastic decrease in the regeneration rate had to be accepted, using a pH value of 5.2 we had to accept the decrease in sensitivity. Further points with respect to sensitivity were a) the carbon source used for growth of the cells (the better the growth, the lower the sensitivity), b) the amount of protoplasts plated on the selection plates (growth of 5×10^5 protoplasts per dish was completely inhibited at 25–40 μg/phleomycin/ml at pH 8.0), c)

Table 2. Data obtained from transformation of Claviceps purpurea using the *bleo*^{R} vector pGW926
No phleomycin resistant colonies were observed with pUC 19 DNA used as control DNA in each experiment. Details see Materials and methods

Exp. No.	Amount of pGW926 DNA (μg)	Regeneration rate (%)	Phleomycin concentration (μg/ml)	Transformants obtained	Code
1	20	5	40	6	T5-01-T5-06
2	20	8	50	4	T5-07-T5-10
3	40	3	50	4	T5-11-T5-14
4	36	10	50	3	T5-15-T5-17

the moment of selection (delaying the selection by leaving phleomycin out of the toplayer decreased the sensitivity).

Taking these points into account and using such carefully prepared protoplasts from membrane grown mycelia transformation with plasmid pGW926 was successful (Table 2): in several independent experiments phleomycin resistant colonies were obtained, be it with low rates (probably due to the selection problems).

Fate of the transforming DNA

Some of the phleomycin resistant *C. purpurea* transformants were subjected to Southern and Northern analysis. As shown exemplarily in Fig. 3, total DNA from all transformants showed strong hybridisation signals to labeled pGW 926 DNA, whereas the control DNA of the recipient strain T5 obviously had no significant homology with this vector. In undigested DNA samples (Fig. 3A) hybridisation occurred only with high-molecular weight material, strongly suggesting absence of autonomously replicating vector molecules. This also indicated that the yeast 2 μm replication origin present on the vector does not function in Claviceps (similar results were also obtained for the Aspergillus species), and that transformation probably occurred by integration of the transforming vector into the genome.

Digestion of transformants' DNA was performed with *Pvu* II, which yields two fragments of vector pGW926 (see Fig. 1), 0.8 and 4.8 kb respectively. As may be seen from Fig. 3B, almost all transformants tested contain both original vector bands (with the exception of T 5-07 which shows only the 0.8 kb fragment), indicating integration of multiple tandem vector copies. The intensities of these hybridizing fragments differ

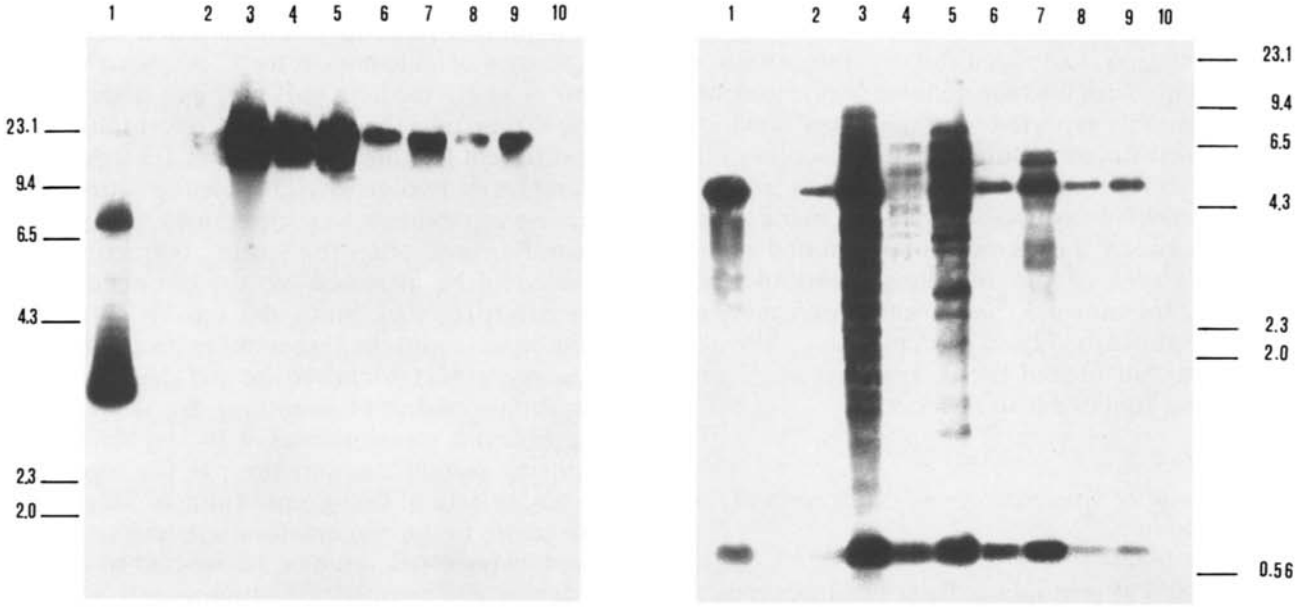

Fig. 3A, B. Southern hybridization analysis of Claviceps transformants. Panel **A:** Undigested DNA from transformants T5-10, T5-08, T5-07, T5-05, T5-04, T5-13, T5-12, T5-11 (lanes *2–9*); pGW926 DNA (lane *1*), and recipient strain T5 (lane *10*). Localisation of molecular weight marker (lambda DNA digested with *Hind*III) is indicated. Panel **B:** *Pvu*II digested DNA from transformants, relative orientation as in panel **A.** Vector pGW926 (lane *1*) gives rise to hybridisation signals of 4.8 and 0.8 kb. Blots were hybridized with ^{32}P-labeled pGW926 DNA

considerably, e.g. between T5-10 and T5-08, in spite of comparable DNA concentrations on the gel, pointing to gross differences in the number of integrated copies. The various minor hybridization signals obtained could represent different border fragments or smaller integrated vector fragments, indicating multiple integration sites.

This is not unexpected, since vector pGW926 contains no region with high homology to Claviceps DNA. The yeast *Ura*3 gene obviously is not homologous enough to the corresponding Claviceps gene to give a major hybridization signal.

These data strongly imply that the phleomycin resistance phenotype of these strains is due to integration of vector DNA into chromosomal DNA of the recipient strain, i.e. that they represent true transformants.

Phenotypic expression and stability

Expression of the *bleo*^{R} gene was good as judged from sensitivity studies: all transformants grew vigorously when small mycelial blocks were transferred to fresh selective medium (50 μg/ml phleomycin); of four transformants tested on higher inhibitor concentration, all tolerated up to 600 μg/ml phleomycin. The best growth-rate on this high antibiotic concentration was shown by tansformant T5-08, which according to Southern analysis (see above) has a high copy number of integrated vector DNA. This indicates that also in *C. purpurea* the *trp*C promoter of *A. nidulans* is functionally accepted. Expression of the integrated *bleo*^{R} gene in the transformants was also tested at the RNA level: in total RNA prepared from three transformants a prospective *bleo*^{R} transcript of about 0.7 kb could be detected by Northern hybridization, which did not occur in the untransformed wild strain (see Fig. 4). This value corresponds well with the expected transcript size derived from the vector's map (see Fig. 1). The most prominent hybridization signal was obtained with transformant T5-08. These data again indicate a copy-number correlated expression of the integrated *bleo*^{R} gene. Since the *bleo*^{R} gene product is not yet characterized, a further detailed analysis of *bleo*^{R} expression in transformants is not possible at the moment.

To test the mitotic stability of transformants, 3 different phleomycin resistant colonies were grown on non-selective medium for a series of 4 consecutive conidial transfers; upon transfer back to selective medium they still were phleomycin resistant.

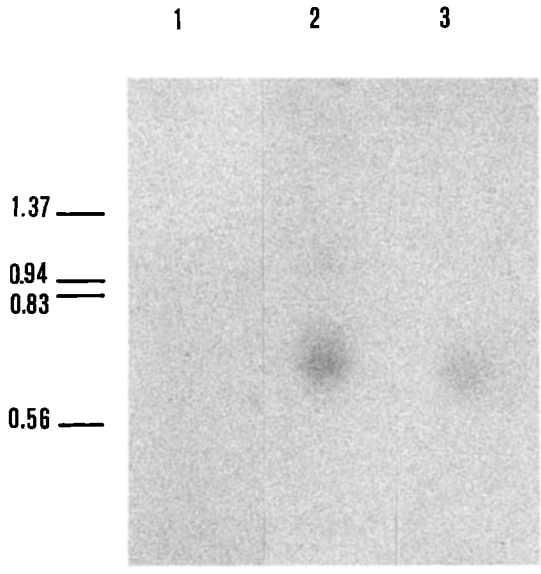

Fig. 4. Northern analysis of *bleo*^{R} transformants of *C. purpurea*. Lane *1:* Untransformed strain T5, lane *2:* T5-08, lane *3:* T5-09. 25 μg total RNA of each strain was separated on a 1% formaldehydagarose gel, transferred to Genescreen membrane and hybridized under stringent conditions to the ^{32}P labelled 0.7 kb *Eco*RI/*Sal*I-fragment of vector pGW926 (see Fig. 1). Relative localization of molecular weight marker (lambda DNA digested with *Eco*RI/*Hind*III) is indicated

In conclusion, it was shown that a wild isolate of *Claviceps purpurea* can be transformed using the heterologous bleomycin resistance system. It should be possible to enhance the transformation rate by the use of a homologous promoter which can be easily screened once a transformation protocol has been established (see e.g. Turgeon et al. 1987). Since the low frequencies of transformation may be due to problems encountered by the selection system (low regeneration rate at selective conditions; difficult timing of selection), a transformation system based on complementation of *pyr* mutants is currently under investigation.

The main advantage of the dominant selection system described here, however, is the possibility to transform any wild or commercial strains of *Claviceps purpurea*.

This opens the way for the application of molecular genetic techniques on strain improvement of Claviceps as well as for investigation of host-parasite interaction.

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