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Development of an homologous transformation system for *Acremonium chrysogenum* based on the *β*-tubulin gene

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Abstract. The *β*-tubulin gene was isolated from the filamentous fungus *Acremonium chrysogenum* using a heterologous gene probe to screen an *A. chrysogenum* lambda library. Sequencing of the *A. chrysogenum* gene revealed a mosaic gene which contains five exons and four intervening sequences. The exons encode for a polypeptide of 447 amino-acid residues which showed a high degree of similarity when compared with amino-acid sequences from *β*-tubulins of other eukaryotes. The introns are characterized by typical consensus sequences found in intervening sequences from other filamentous fungi. In-vitro mutagenesis of codon 167 of the *β*-tubulin gene resulted in the substitution of a phenylalanine by a tyrosine in the corresponding polypeptide sequence. The mutated gene was used successfully in the transformation and co-transformation of *A. chrysogenum* to benomyl resistance. The molecular analysis of transformants provided evidence that they contain the mutated *β*-tubulin gene in addition to the wild-type gene, as was proved by Southern-hybridization analysis and direct sequencing of PCR amplification products.

Key words: *Acremonium chrysogenum* – *β*-tubulin gene – Homologous transformation system – Benomyl resistance

Introduction

Tubulin genes encode a superfamily of proteins which are part of the cell-cycle regulatory apparatus in all eukaryotes. Mutations in particular tubulin genes can influence microtubular formation or can affect sensitivity to tubulin inhibitors. Benomyl, a potent fungicide, acts specifically on *β*-tubulin. The first mutant *β*-tubulin gene was isolated from *Aspergillus nidulans* strains with resistance against benomyl (May et al. 1985). Subsequently, *β*-tubulin mutants showing benomyl resistance have been identified in a broad range of filamentous fungi as well as in yeasts (for review see Osmani and Oakley 1991). In several system, the mutated *β*-tubulin genes have been used for the homologous or heterologous transformation of filamentous fungi (for review see Finkelstein 1992). An homologous transformation system was recently developed for *Acremonium chrysogenum*, an industrially-important fungus, using the nitrate reductase-encoding *niaD* gene (Whitehead et al. 1990). However, transformation can only be performed when the appropriate *niaD* mutants are available.

In the present paper we describe the isolation of the wild-type *β*-tubulin gene from *A. chrysogenum* and its in-vitro mutagenesis. The mutated gene is used as an homologous marker gene to transform *A. chrysogenum* to benomyl resistance. Thus, a system is available using the dominant resistance gene, which can be applied not only to wild-type strains from type-culture collections but also to highly-developed industrial *Acremonium* strains. Moreover, the use of an homologous gene can be considered as safe in view of the strict working regulations for recombinant gene technology employed in different countries.

Materials and methods

Microorganisms and growth conditions. For DNA preparation and transformation *A. chrysogenum* (ATCC 14553) was cultivated for 36–72 h in CCM (CCM: 0.3% sucrose, 0.05% NaCl 0.05% K_2HPO_4, 0.05% $MgSO_4 \times 7H_2O$, 0.001% $FeSO_4$, 0.5% tryptic soy broth, 0.1% yeast extract, 0.1% meat extract, 1.5% dextrin, pH 7.0) at 27 °C. Cloning experiments were performed with *E. coli* XL1 (Bullock et al. 1987) which was cultivated in LB (LB: 1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) containing 5 µg/ml of tetracycline at 37 °C.

Construction of recombinant plasmids pCN1 and pCN3. The construction of a lambda library from *A. chrysogenum* was described by Kück et al. (1989). Using recombinant plasmid p*β*5 which contains the *β*-tubulin gene from *A. nidulans* (May et al. 1985) as a probe, lambda clone L-T6 was isolated by plaque filter hybridization techniques (Benton and Davis 1977). A 4.7-kb *Eco*RI/*Xba*I fragment

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harbouring the β-tubulin gene of *A. chrysogenum* was isolated from lambda clone L-T6 and ligated into pBluescriptIIKS+ (Stratagene, Heidelberg, Germany) to give plasmid pCN1 (see Fig. 1). pCN3 was constructed by ligation of the following fragments: (1) a 5.0-kb *BalI*/*SphI* fragment from plasmid pCN1 consisting of pBluescriptIIKS+ and the 3′ end of the β-tubulin gene; (2) a 2.0-kb *BalI* fragment derived from plasmid pCN1, covering the 5′ region of the β-tubulin gene; (3) a 155-bp *BalI*/*SphI* PCR fragment which was generated as described in the "in-vitro mutagenesis" section.

DNA sequencing. DNA sequencing was carried out with the T7-Polymerase™-Sequencing kit and the Deaza G/A T7 Sequencing™ Mixes (Pharmacia, Freiburg, Germany). Sequencing products were separated by electrophoresis on 6% acrylamide buffer-gradient gels containing 7 M urea (Biggin et al. 1983). A set of sequencing primers (Table 1) was synthesized according to the β-cyanoethyl phosphoamidit method (Sinha et al. 1984) with an Applied Biosystems 318A DNA synthesizer (Applied Biosystems, Weiterstadt, Germany), followed by HPLC purification as described previously (Kück et al. 1987).

Table 1. Sequences of oligonucleotides used in this investigation. Oligonucleotide no. 375 was used for in-vitro mutagenesis, oligonucleotide nos. 375 and 376 for the PCR amplification experiment (see the Materials and methods section). Oligonucleotides nos. 341 and 376 were used for direct sequencing of PCR-amplified DNA fragments. All other oligonucleotides were used as primers for the chain-termination sequencing

Oligonucleotide no.	Sequence
340	5′ CCGTTGAAGTAGACGCTCATGC 3′
341	5′ GCCAAGGGCCACTACACTGAGGGT 3′
359	5′ CAGGAATGTTGTTGCTCGACGC 3′
360	5′ TACGACATCTGCATGCGTACC 3′
373	5′ TGACCTGCTCTGCCATCTTG 3′
374	5′ GGAGTAGGTGGCCATCATGC 3′
375	5′ GATGGCCACCTACTCCGTCG 3′
376	5′ GGGTACGCATGCAGATGTCG 3′
377	5′ TGAGGGCATGGACGAGATGG 3′
378	5′ TGATGGAGAAGAGTGTGTG 3′
433	5′ CCACCACACACTCTTCTCC 3′
434	5′ CCATCTCGTCCATGCCCTCA 3′
439	5′ ATCATCAGCCAGATTGG 3′
440	5′ GGAACAAGACAATAGAGC 3′
441	5′ CGTTCTATTTATTTCAATTCG 3′

In-vitro mutagenesis and PCR amplification. The site-specific substitution of a single nucleotide in the sequence of the β-tubulin gene was accomplished by the amplification of a 155-bp fragment using oligonucleotides 375 and 376 (Table 1). Oligonucleotide 375 harbors a nucleotide substitution in codon 167 of the β-tubulin gene (see Fig. 6). The 50-μl PCR reaction mixture contained 10 ng of plasmid DNA, 0.3 μg of each oligonucleotide primer, and 0.33 mM dNTPs in 1 × Replitherm buffer. The amplification was started by adding two units of Replitherm (Biozym, Hameln, Germany) and continued over 40 cycles of 1 min at 92 °C, 2 min at 60 °C, and 0.5 min at 72 °C each (modified from Saiki et al. (1985).

Transformation and co-transformation of A. chrysogenum. The procedure described by Skatrud et al. (1987) and modified by Walz (1992) was used. For transformation, 10^9 protoplasts/ml were mixed with 20 μg of DNA and spread on CCM plates. After 24 h, germinating protoplasts were overlayed with 5 ml of top agar, containing at least 15 μg of Benomyl/ml for selection.

Analysis of A. chrysogenum transformants. Genomic DNA from *A. chrysogenum* strains was isolated as described by Kück et al. (1989) and modified by Walz and Kück (1993). Electrophoretically-separated DNA was denatured, neutralized, and blotted onto nitrocellulose filters or Hybond-N nylon membranes (Southern 1975). DNA filters were pre-hybridized in 5 × SSPE, 0.2% SDS, 100 μg/ml of herring sperm-DNA, 50% formamide, pH 7.0–7.5 at 37 °C for 1–2 h, followed by the addition of the radioactively labelled hybridization probe. After 16 h incubation at 37 °C, filters were washed in 5 × SSPE, 0.2% SDS at 50–65 °C, dried, and exposed to X-ray films for several hours.

Direct sequencing of PCR fragments. Fragments of the β-tubulin gene from the wild-type strain and from transformants were amplified with oligonucleotides 341 and 376, using 5 μg of genomic DNA. The products were purified using the Quiagen-PCR purification kit (Diagen, Düsseldorf, Germany), followed by ethanol precipitation. Direct sequencing was done with oligonucleotide 341 as a primer in a chain-termination sequencing reaction (Sanger et al. 1977) with the following modifications: after a heat denaturation step (3 min, 90 °C) a contemporary hybridization and labelling reaction was performed.

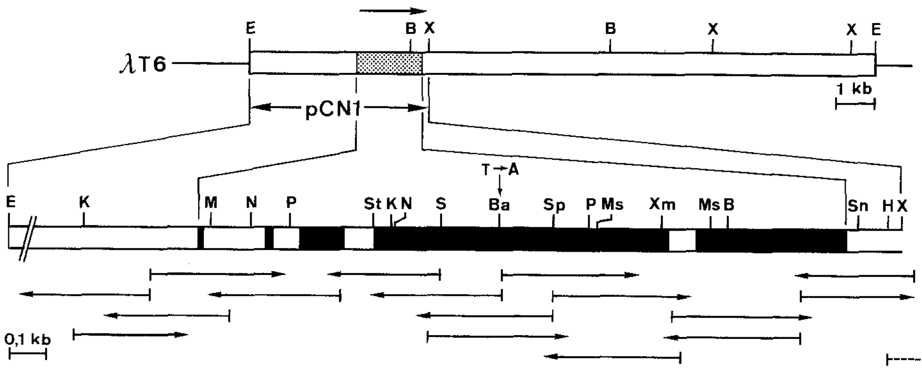

Fig. 1. Restriction map of recombinant lambda clone L-T6 containing the β-tubulin gene (*shaded box*) from *A. chrysogenum*. The *arrow* indicates the direction of transcription. pCN1 marks a 4.7-kb *Eco*RI-*Xba*I restriction fragment which was cloned into vector pBluescriptIIKS+ for DNA sequencing. For details of construction see text. The organization of the β-tubulin (lower map) is given as was deduced from the DNA sequence. Locations of exons (*black*) and introns (*white boxes*) are indicated. Abbreviations: *B*, *Bam*HI; *Ba*, *BalI*; *E*, *Eco*RI; *H*, *Hin*fIII; *K*, *KpnI*; *M*, *MluI*; *Ms*, *MstI*; *N*, *NcoI*; *P*, *PvuII*; *S*, *SalI*; *Sn*, *SnaI*; *Sp*, *SphI*; *St*, *StuI*, *X*, *XbaI*; *Xm*, *Xma*III

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   1 CGCAATTTGTCCCACATCATCAGCCCATTGGTCATCACCCCCCCTCAGGTACCAACCAGTGAACACCTTACTGTAAAGGCTCC   83

  84 CGCTGTCAGGCCCAGCAGCAGCTCCAGCACTCACCGCAAGAGGCCGGGGTCCCGTGGCGGTGAGGACGGGACCAATTTCCATC  166

 167 CAGCACCTCACCCACCTCTCCCCCAGCAAAAATCCAACCATCCACCACACACTCTTCTCCATCACTCACACCCGACCACCCCC  249

 250 CATCTCCACGGAACTCAAGCTCTGTTGCTCAGCACAGCTCTCGAGGCCCTCGCTCTAGCTACCGATCACGTCCTTTTCCCTTC  332

 333 AATAATCCTCAAA ATG CGT GAG ATT gtgagtcctccaacggacgcccaacgcgtccctcgcggtgcccctgatttacc  410
                   MET Arg Glu Ile
                     1
 411 ccgccgacgggcgtcgagcaacaacattcctgcccgacacactacccacagttcgagatggatcaagaacgtgctgaccatgg  493

 494 accttttttttgttcttgtgatatag GTT CAC CTC CAG ACC GGC CAG TGC gtaagttacttctttccggacacg  567
                                Val His Leu Gln Thr Gly Gln Cys
                                                     10
 568 tttgcggtgtgggaggacacagctgatggacgtgatggaatag GGC AAC CAG ATT GGT GCT GCT TTC TGG CAG  640
                                                 Gly Asn Gln Ile Gly Ala Ala Phe Trp Gln
                                                                              20
 641 ACC ATC TCT GGC GAG CAT GGC CTC GAC AGC AAC GGT GTC TAC AAC GGC AGC TCT GAG CTC CAA  703
     Thr Ile Ser Gly Glu His Gly Leu Asp Ser Asn Gly Val Tyr Asn Gly Ser Ser Glu Leu Gln
                                  30                                      40
 704 CTC GAG CGC ATG AGC GTC TAC TTC AAC GAG gtacgtggatgaactagctacaactgtactgctcgagcagtca  776
     Leu Glu Arg MET Ser Val Tyr Phe Asn Glu
                              50
 777 atgctagtgtgggtcctaacaagctgtgcag GCC TCT GGC AAC AAG TAT GTC CCT CGC GCC GTC CTC GTC  846
                                     Ala Ser Gly Asn Lys Tyr Val Pro Arg Ala Val Leu Val
                                                              60
 847 GAT CTT GAG CCC GGT ACC ATG GAC GCT GTT CGT GCG GGT CCT TTC GGC CAG CTC TTC CGC CCC  909
     Asp Leu Glu Pro Gly Thr MET Asp Ala Val Arg Ala Gly Pro Phe Gly Gln Leu Phe Arg Pro
                  70                                      80
 910 GAC AAC TTC GTC TTC GGC CAG TCC GGT GCT GGC AAC AAC TGG GCC AAG GGC CAC TAC ACT GAG  972
     Asp Asn Phe Val Phe Gly Gln Ser Gly Ala Gly Asn Asn Trp Ala Lys Gly His Tyr Thr Glu
              90                                     100
 973 GGT GCC GAG CTC GTC GAC AAC GTC CTC GAT GTC GTC CGC CGC GAG GCC GAG GGC TGC GAC TGC 1035
     Gly Ala Glu Leu Val Asp Asn Val Leu Asp Val Val Arg Arg Glu Ala Glu Gly Cys Asp Cys
         110                                     120
1036 CTC CAG GGC TTC CAG ATC ACC CAC TCC CTG GGT GGT GGC ACT GGT GCC GGT ATG GGC ACC CTG 1098
     Leu Gln Gly Phe Gln Ile Thr His Ser Leu Gly Gly Gly Thr Gly Ala Gly MET Gly Thr Leu
     130                                     140                                     150
1099 CTC ATC TCC AAG ATC CGC GAG GAG TTC CCC GAC CGC ATG ATG GCC ACC TTC TCC GTC GTC CCC 1161
     Leu Ile Ser Lys Ile Arg Glu Glu Phe Pro Asp Arg MET MET Ala Thr Phe Ser Val Val Pro
                                         160                                     170
1162 TCC CCC AAG GTC TCC GAT ACC GTC GTC GAG CCC TAC AAC GCC ACC CTC TCC GTG CAC CAG CTC 1224
     Ser Pro Lys Val Ser Asp Thr Val Val Glu Pro Tyr Asn Ala Thr Leu Ser Val His Gln Leu
                                     180                                     190
1225 GTT GAG CAC TCC GAC GAG ACC TTC TGT ATC GAC AAC GAG GCC CTC TAC GAC ATC TGC ATG CGT 1287
     Val Glu His Ser Asp Glu Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr Asp Ile Cys MET Arg
                                 200                                     210
1288 ACC CTC AAG CTG TCT AAC CCC TCC TAC GGC GAC CTG AAC TAC CTC GTC TCC GCT GTC ATG TCT 1350
     Thr Leu Lys Leu Ser Asn Pro Ser Tyr Gly Asp Leu Asn Tyr Leu Val Ser Ala Val MET Ser
                             220                                     230
1351 GGT GTC ACC ACC TGC CTC CGC TTC CCC GGT CAG CTG AAC TCT GAC CTG CGC AAG CTG GCT GTC 1413
     Gly Val Thr Thr Cys Leu Arg Phe Pro Gly Gln Leu Asn Ser Asp Leu Arg Lys Leu Ala Val
                         240                                     250
1414 AAC ATG GTT CCC TTC CCT CGT CTG CAC TTC TTC ATG GTC GGC TTC GCC CCC CTG ACC AGC CGT 1476
     Asn MET Val Pro Phe Pro Arg Leu His Phe Phe MET Val Gly Phe Ala Pro Leu Thr Ser Arg
                     260                                     270
1477 GGT GCC CAC TCC TTC CGC GCC GTC AGC GTC CCC GAG CTC ACC CAG CAG ATG TTC GAC CCC AAG 1539
     Gly Ala His Ser Phe Arg Ala Val Ser Val Pro Glu Leu Thr Gln Gln MET Phe Asp Pro Lys
                 280                                     290
1540 AAC ATG ATG GCT GCC TCC GAC TTC CGC AAC GGC CGC TAC CTG ACC TGC TCT GCC ATC TT  gta 1601
     Asn MET MET Ala Ala Ser Asp Phe Arg Asn Gly Arg Tyr Leu Thr Cys Ser Ala Ile Ph
             300                                     310
1602 agttataaagatacgccgagctctattgtcttgttccatctgaagctaacatggaaacag  C CGT GGC AAG GTC GCC 1677
                                                                   e Arg Gly Lys Val Ala
                                                                             320
1678 ATG AAG GAG GTC GAG GAC CAG ATG CGC AAC GTC CAG AGC AAG AAC TCG TCC TAC TTC GTC GAG 1740
     MET Lys Glu Val Glu Asp Gln MET Arg Asn Val Gln Ser Lys Asn Ser Ser Tyr Phe Val Glu
                                 330                                     340
1741 TGG ATC CCC AAC AAC ATC CAG ACC GCT CTC TGC GCC ATT CCT CCC CGT GGC CTC AAG ATG TCC 1803
     Trp Ile Pro Asn Asn Ile Gln Thr Ala Leu Cys Ala Ile Pro Pro Arg Gly Leu Lys MET Ser
                             350                                     360
1804 TCC ACC TTC ATC GGC AAC TCC ACC TCC ATC CAG GAG CTG TTC AAG CGT GTC GGT GAG CAG TTC 1866
     Ser Thr Phe Ile Gly Asn Ser Thr Ser Ile Gln Glu Leu Phe Lys Arg Val Gly Glu Gln Phe
                         370                                     380
1867 ACT GCC ATG TTC CGT CGC AAG GCT TTC CTG CAT TGG TAC ACT GGT GAG GGC ATG GAC GAG ATG 1929
     Thr Ala MET Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly Glu Gly MET Asp Glu MET
                     390                                     400
1930 GAG TTT ACC GAG GCC GAG TCC AAC ATG AAC GAC CTC GTC TCC GAG TAC CAG CAG TAC CAG GAT 1992
     Glu Phe Thr Glu Ala Glu Ser Asn MET Asn Asp Leu Val Ser Glu Tyr Gln Gln Tyr Gln Asp
                 410                                     420
1993 GCT GGC ATC GAC GAG GAG GAG GAG GAA TAC GAG GAG GAG CTC CCC CTC GAG GGT GAG GAA TAA 2055
     Ala Gly Ile Asp Glu Glu Glu Glu Glu Tyr Glu Glu Glu Leu Pro Leu Glu Gly Glu Glu TER
             430                                     440                         447
2056 AAAAAAAAGCTCGCCACCAGAGGCTTGCCGTATACACGGTCCGGCGCGTCGTTCCCGCCAACTGTGGTAACCTTTTGAAGTTT 2138

2139 GCAGCCTGTTGCGTTCTATTTATTTCAATTCGGGGTTGTGGAGGTAATTGTGAGAATGGGGTTCTAGA 2206
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Fig. 2. Nucleotide sequence and the deduced amino-acid sequence of the *A. chrysogenum* β-tubulin gene. Intron sequences are given in *lower case letters* and characteristic consensus sequences are *underlined.* The sequence is available in the EMBL data library under accession no. X 72789

Results and discussion

Isolation and DNA sequencing of the β-tubulin gene from A. chrysogenum

In order to isolate the β-tubulin gene from *A. chrysogenum*, a genomic library was screened with a heterologous probe (pβ5), containing the β-tubulin gene from *A. nidulans* (May et al. 1985). Screening of 5×10^3 recombinant clones from the lambda library revealed two clones with identical restriction maps, showing homology to the *A. nidulans* β-tubulin gene. For further molecular investigations one clone with the designation L-T6 was hybridized with the heterologous probe to localize the *A.*

```
           ↓                                                ↓
IVS1: 5' - GTGAGT ... GCTGACC ... 27      ... TAG - 3'
IVS2:    - GTAAGT ... GCTGATG ... 12      ... TAG -
IVS3:    - GTACGT ... GCTAGTG ... 19      ... CAG -
IVS4:    - GTAAGT ... GCTAACA ...  6      ... CAG -

Consensus-sequence

N.c.:    - GTACGT ... GCTGACT ...  7-18 ... CAG -
               A      A  A  A
A.n.:    - TGTAAGT... CTAAC    ......... TTACAGC -
           G  G         G               CCC

S.c.:    - GTATGT ... TACTAAC ... 18-53 ... TAG -
                                             C
h.e.:    - GTAAGT ........................ CAG -
```

Fig. 3. Comparison of sequences for 5′ and 3′ splice sites and the internal splice signal of the *A. chrysogenum* β-tubulin gene with intron consensus sequences from other fungi. *Vertical arrows* indicate exon-intron splice sites. Abbreviations: *IVS*, intervening sequence; *An*, *Aspergillus nidulans*; *Nc*, *Neurospora crassa*; *Sc*, *Saccharomyces cerevisiae*; *he*, higher eukaryotes

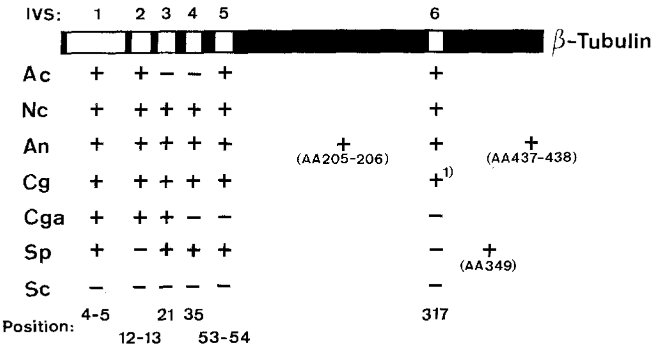

Fig. 4. Comparison of the organization of β-tubulin genes from different fungi. +/− indicates the presence/absence of introns in the corresponding β-tubulin gene. Modified and extended according to Orbach et al. (1986). Abbreviations: *Ac*, *Acremonium chrysogenum*; *Cg*, *Colletotrichum graminicola* (Panaccione and Hanau 1990); *Cga*, *Colletotrichum graminicola* f. sp. *aeschynomene* (Buhr and Dickman 1993); *Sp*, *Schizosaccharomyces pombe*; for all other abbreviations see legend of Fig. 3

chrysogenum gene on a 4.7-kb *Eco*RI/*Xba*I restriction fragment (Fig. 1). After subcloning of the 4.7-kb fragment in bacterial vector pBluescriptIIKS+, resulting in recombinant plasmid pCN1, a region of 2.2 kb was used for sequence determination. The resulting DNA sequence and the deduced amino-acid sequence are shown in Fig. 2. The predicted amino-acid coding sequence is interrupted by four intronic sequences, which are characterized by typical consensus sequences found in the introns of other filamentous fungi (Fig. 3). The introns, varying in size from 63 to 152 bp, are characterized by a GT and AT dinucleotide at their 5′ and 3′ termini, respectively. In addition, an internal intron consensus sequence can be identified in all intronic sequences, as indicated in Fig. 3. Remarkably, the intron sites in the *A. chrysogenum* gene are identical with those found in the β-tubulin genes from several other ascomycetes (Fig. 4). Similarly to the other β-tubulin genes compared in this investigation, most introns are located near the 5′ end of the gene. Additionally, as already observed in many other constitutively-expressed genes, β-tubulin genes seem to contain more intronic sequences than differentially-expressed genes.

An open reading frame of 1344 bp which encodes 447 amino-acid residues, can be identified from our sequence analysis. Sequence alignment of the deduced amino-acid sequence shows a significant homology, of between 82% and 96%, with the corresponding amino-acid sequence of β-tubulin genes from other eukaryotes (Fig. 5). This high degree of similarity, however, is not surprising since the tubulins have in general retained a conserved structure in all eukaryotic organisms investigated so far. This also holds true for those amino-acid residues which are considered to be involved in the binding of the fungicide benomyl. The sequence comparison reveals, for example, conserved amino-acid residues at positions, 6, 165 and 198 (Fig. 5). These residues have been found to be mutated in benomyl-resistant strains of *Neurospora crassa* and *A. nidulans*, and it has been suggested that they are important for benomyl-binding (Osmani and Oakley 1991).

In-vitro mutagenesis of the β-tubulin gene

Mutated β-tubulin genes which confer benomyl resistance have been widely used as a dominant selectable marker in transformation experiments (e.g., Orbach et al. 1986). These marker genes all carry single mutations in codons for highly conserved amino-acid residues which are involved in benomyl-binding. For example, at amino-acid codon 167 from the *N. crassa* wild-type gene, a phenylalanine to tyrosine substitution confers benomyl resistance.

Using synthetic oligonucleotides for PCR amplification experiments, we have mutated amino-acid codon 167 of the *A. chrysogenum* wild-type gene (Fig. 6). With oligonucleotides 375 and 376, a 155-bp PCR fragment was generated which contains a thymidine to adenosine substitution in codon 167. The PCR fragment is flanked by two different endonuclease restriction recognition sites, namely for *Bal*I and *Sph*I. After restriction with both endonucleases, the PCR fragment was ligated with two restriction fragments containing parts of the wild-type β-tubulin gene and of the bacterial vector pBluescriptIIKS+, as detailed in the Materials and methods section. The resulting plasmid was designated pCN3 and contains the mutated β-tubulin gene. Using the set of oligonucleotide primers shown in Table 1, the complete β-tubulin gene from plasmid pCN3 was sequenced to exclude random mutations which may be generated by PCR amplification or in-vitro recombination.

Transformation and co-transformation of A. chrysogenum with the mutated β-tubulin gene

In order to demonstrate that the mutated β-tubulin gene can be used as a dominant selectable marker, plasmid pCN3 was used to transform *A. chrysogenum* to benomyl resistance. Our procedure gave resistant transformants at a frequency of about 1–10 transformants per 10 µg of

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 1 MREIVHLQTGQCGNQIGAAFWQTISGEHGLDSNGVYNGSS-ELQLERMSVYFNEASGNKYVPRAVLVDLEPGTMDAVRAGPFGQLFRPDNFVFGQSGAGNN
 1 M..............................AS.....T.-.......N....................................................
 1 M..............................GS.....T.-D......N..........................C.......E.................
 1 M...I.ISA..Y.........E..C......F..T.H.T.-DI.K..LN.......SG.W...SIN.....W.I....NSAI.N......YI....S...V
 1 M.....I.G.........K..EVV.D...I.PT.T.H.D.-D.....IN.....T.GR......I.M........S..S..Y..I..........T.....
 1 M...L.V.G.......GSK..EV.CD...V.PT.R...D.AD.....IN..Y....GR........M........SI.S..Y..I................
 1 M.....I.A.........K..QT..D...I.PT.S.H.D.-D.....IN..Y..T.........I..........S..S.....I................

101WAKGHYTEGAELVDNVLDVVRREAEGCDCLQGFQITHSLGGGTGAGMGTLLISKIREEFPDRMMATFSVVPSPKVSDTVVEPYNATLSVHQLVEHSDETF
101..............Q...............................................................................N.....
101................V...................................................................................
101..............S.M..I........S...............S......F...K..L..........L....T.........................
101............I.S......K...S........VC........S.............Y.....L.............................NA..CM
102............I.A......K...N........VC........SQ............Y.....L....F........................NA..CM
101..............S......K.S.S........L.........S.............Y...I.N....M........................NT...Y

201CIDNEALYDICMRTLKLSNPSYGDLNYLVSAVMSGVTTCLRFPGQLNSDLRKLAVNMVPFPRLHFFMVGFAPLTSRGAHSFRAVSVPELTQQMFDPKNMM
201..........................H..........VS........................................H....................
201..........................H.........................W..........................Y....................
201...........Q.....NQ.......N...S.......S..Y..............L............Y....AI.SQ...SLT.........EA....
201VL.........F.....TT.TF....H.I......I.C.........A........LI............T......SQQY..LT........W.A....
202VL.........F......T..F....H.I..T.....CS.................LI...................SQQYISLT........W.A....
201...........F.....TT.T.....H....T...............A...................P..........QQY..LT..........S....

301AASDFRNGRYLTCSAIFRGKVAMKEVEDQMRNVQSKNSSYFVEWIPNNIQTALCAIPPRGLKMSSTFIGNSTSIQELFKRVGEQFTAMFRRKAFLHWYTG
301.....................S............N.............V.....S............V....A.......I...................
301.....................S..........I....Q............S...S...........................D.................
301..A.P.......VA.F.....SV.....E.HK......D.........V...V.SVA.Q..D.AA...A.............D..S...K.........S
301C.A.P.H.....A..L...RMST...DE..L...N.............VKSSV.D...K.....A.......A...M....S..................
302C.A.P.H.....A..M...MMST...DE.IL...N.............VKSSV.D...T.I..A...V........M.R..S...F..............
301..C.P.H.....VA.....RMS....DE..L...N.............VK..V.D.........A.......A.......IS..................

401EGMDEMEFTEAESNMNDLVSEYQQYQDAGIDEEEEEYEEELPLEGEE*               100%  -  (1) Acremonium chrysogenum
401.............................V..........A......*                96%  -  (2) Neurospora crassa
401............................S.S.G....A..EIM....*                95%  -  (3) Aspergillus nidulans
401.....L..S.................E.TVEDD..VD.NGDFGAPQNQDEPITENFE*      77%  -  (4) Saccharomyces cerevisiae
401............................SAE..G.FEG..EEAB*                   78%  -  (5) Chlamydomonas reinhardtii
402............................TA...D.YD...EQVYES*                 77%  -  (6) Arabidopsis thaliana
401............................TA..QG.FE..GEED.A*                  82%  -  (7) Gallus gallus
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Fig. 5. Comparison of the β-tubulin sequences from different eukaryotic sources. Similarity values are given between the *A. chrysogenum* tubulin and other eukaryotes. The numbers indicate references as follows: (*1*) this paper; (*2*) Orbach et al. (1986); (*3*) May et al. (1987); (*4*) Neff et al. (1983); (*5*) Youngblom et al. (1984); (*6*) Silflow et al. (1987); (*7*) Valenzuela et al. (1981)

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                         MET Ala Thr Phe Ser Val

   Wild type: 5'- G ATG GCC ACC TTC TCC GTC G -3'


Oligon. no. 375: 5'  G ATG GCC ACC TAC TCC GTC G  3'

                                    Tyr
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Fig. 6. Nucleotide sequence of codons 164 to 169 of the β-tubulin wild-type sequence and of oligonucleotide no. 375 which was used for in-vitro mutagenesis to change the TTC-Phe codon no. 167 into a TAC-Tyr codon

DNA, while no resistant colonies were found on control plates with DNA-untreated protoplasts. The selection of transformants was done on plates with a final concentration of 10 μg of benomyl/ml of medium and these colonies became visible after 5–8 days of cultivation. Protoplasts were treated in parallel with plasmid pCN1 which contains the wild-type β-tubulin gene. In this case no benomyl-resistant colonies appeared on selective media, indicating that under our selection conditions the mutation rate was low enough to distinguish between spontaneous benomyl-resistant colonies and DNA-mediated transformants. The mutated β-tubulin gene can therefore be used as a dominant benomyl resistance gene to transform any strain of *A. chrysogenum*, irrespective of whether it is derived from type-culture collections or from industrial screening programs (Walz and Kück, unpublished). An homologous transformation system based on the β-tubulin gene was used for high-frequency transformation of *N. crassa* (Orbach et al. 1986; Vollmer and Yanofsky 1986). In comparison to these data, the frequencies obtained here seem to be rather low. However, similar frequencies have been obtained when *N. crassa* tubulin was used in heterologous hosts (for review see Finkelstein 1992). It seems to be a general problem to obtain higher transformation rates in *A. chrysogenum*, irrespective of which dominant marker gene was used to select for DNA transformants, (e.g., Isogai et al. 1987; Skatrud et al. 1987; Whitehead et al. 1990; Gutiérrez et al. 1991; Walz and Kück 1993).

The β-tubulin gene has been used in different fungal transformation systems to transfer non-selectable genes into the recipient strain by co-transformation procedures (e.g., Austin and Tyler 1990). We have tested whether vector pCN3 is suitable for efficient co-transformation of another recombinant plasmid pMW1 (Kück et al. 1989). This plasmid contains the bacterial hygromcyin B phosphotransferase gene (*hph*) under the control of the *A. chrysogenum pcbC* promoter and has recently been used successfully in transforming *A. chrysogenum* to hygromycin B resistance (Walz 1992; Walz and Kück 1993). The presence of plasmid pMW1 in benomyl-resistant co-transformants can be tested easily when clones are transferred to hygromycin B-containing selective media. Colonies which showed moderate or strong growth on both selective media were counted as co-transformants. Testing all benomyl-resistant transformants, we have achieved a frequency of up to 38% co-transformants using the procedure described in the Materials and methods section. This result can be enhanced when the amounts of

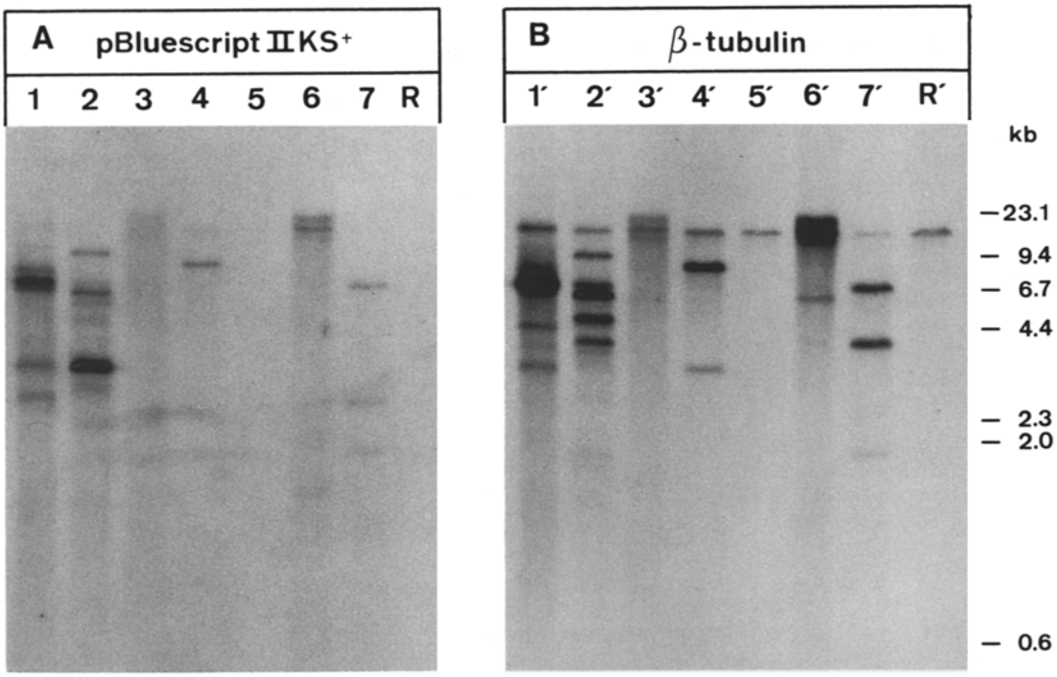

Fig. 7 A, B. Southern hybridization analysis of *A. chrysogenum* transformants. *Eco*RI-restricted DNA from the recipient strain (*R*), as well as from seven randomly-selected transformed (*T1–T7*), was subjected to gel electrophoretic separation and blotted on nitrocellulose filters. *T1/T2* (*lanes 1, 1′, 2, 2′*) resulted from co-transformation with both pCN3 and pMW1; strains *T3–T5* (*lanes 3, 3′, 4, 4′, 5, 5′*) were transformed with undigested plasmid pCN3, and *T6/T7* (*lane 6, 6′, 7, 7′*) with *Eco*RI/XbaI-restricted plasmid DNA. Identical filters were probed with radiolabelled-plasmid pBlueskriptIIKS+ DNA (**A**) and with the β-tubulin probe (**B**)

transforming DNA are decreased in comparison to the amount of co-transforming DNA. Frequencies of 75–80% were obtained, for example, when plasmid pMW1 was used together with non-selectable genes in co-transformation experiments (Menne and Kück, unpublished). Our data are similar to those from co-transformations of other filamentous fungi (*N. crassa*, Vollmer and Yanofsky 1986; *A. nidulans*, Timberlake et al. 1985; Wernars et al. 1987; *Podospora anserina*, Osiewacz et al. 1991) or even with animals cells (Wigler et al. 1979). The co-transformation approach that is presented here, allows the integration of any foreign gene into the *A. chrysogenum* chromosomal DNA. These findings are relevant for industrial strains of *A. chrysogenum* for which mutant recipient strains are not usually available to select for transformants showing a wild-type phenotype.

Molecular analysis of A. chrysogenum transformants and co-transformants

In order to investigate the fate of the transforming DNA, total genomic DNA from different benomyl-resistant strains was subjected to DNA hybridization analysis using the purified β-tubulin gene and vector pBluescriptIIKS+ as probes. Representative data from three different transformation experiments are presented, in which circular plasmid DNA, *Eco*RI/*Xba*I-linearized plasmid DNA, or two different vector molecules (pCN3, pMW1), were used for DNA-mediated transformations. As can be seen in Fig. 7, most strains show a rather complex pattern of hybridizing fragments, indicating major rearrangements in the integrated vector DNA. The comparison of data from the two autoradiograms shown in Fig. 7A and B demonstrates that most transformants contain copies of the mutated β-tubulin gene in addition to the wild-type gene. The recipient strain shows only a single 20-kb *Eco*RI fragment in Fig. 7B and most probably carries only a single β-tubulin gene. Similarly, only a single restriction fragment was detected when *Xba*I/*Eco*RI-restricted DNA was used for hybridization experiments (data not shown). A fragment of identical size can be seen in all lanes when DNA from different transformants was probed with the β-tubulin gene (Fig. 7B). A similar result was obtained when labelled oligonucleotide 340 was used as a hybridization probe (data not shown). Thus, an homologous recombination event, leading to the replacement of the wild-type β-tubulin gene, seems to be rather rare in *A. chrysogenum*, as was also recently found for the *pcbC* gene from *A. chrysogenum* (Walz and Kück 1993). It has been shown for several filamentous fungi, as well as for yeast, that transforming DNA integrates by illegitimate recombination into ectopic sites of the chromosomal DNA (e.g, Wright et al. 1986; Binninger et al. 1987; Asch et al. 1992). Another result which becomes evident from the hybridization experiments is the rather complex pattern which can be seen in the DNA from two co-transformants. Vectors pCN3 and pMW1 show extensive homology with the two hybridization probes and this can be detected in the two autoradiograms of Fig. 7. The use of two transforming plasmids seems to increase the recombination process during chromosomal integration in fungal transformants, which might be due to the presence of *A. chrysogenum* sequences in both plasmids. In order to

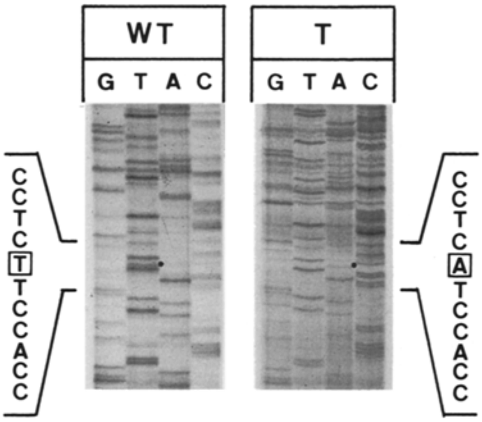

Fig. 8. Sequence analysis of PCR-amplified DNA fragments from a randomly-selected benomyl-resistant *A. chrysogenum* transformant (*T*) in comparison to the recipient strain (*WT*). Both sequences can be distinguished by a T to A substitution in codon 167 of the *β*-tubulin gene

verify the introduction of the mutated *β*-tubulin in benomyl-resistant transformants, the chromosomal DNA was subjected to DNA sequence analysis. A fragment of the *β*-tubulin gene containing the codon for amino-acid residue 167, was amplified using oligonucleotides 341 and 376 as primers. Direct sequencing of the PCR amplification products revealed the sequences shown in the autoradiogram of Fig. 8. The recipient strain shows only the wild-type codon (TTC-Phe) while the mutated codon (TAC-Tyr) is found when DNA was used from benomyl-resistant strains. From the hybridization data both gene copies may be expected in the fungal transformants. However, high-copy integration of the mutated *β*-tubulin gene in the transformants (Fig. 7) most probably results in preferential amplification of the mutated *β*-tubulin sequence. Therefore, transformed and non-transformed strains can be distinguished by a T to A base-pair substitution in the *β*-tubulin gene.

In conclusion, we have developed an homologous transformation system which is adaptable to any strain of *A. chrysogenum* and which can be considered as safe in terms of the regulations for in-vitro recombinant experiments. We are currently using this approach to transfer non-selectable genes into the *A. chrysogenum* genome for investigations on the mechanisms controlling gene expression in this filamentous fungus.

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