Curr Genet (1994) 25: 519–523

Current Genetics

Molecular cloning, sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*

S. Wnendt[1*], N. Ulbrich[2], U. Stahl[1]

[1] Technische Universität Berlin, Fachgebiet Mikrobiologie und Genetik, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany
[2] Deutsches Rheumaforschungszentrum, Berlin, Germany

Received: 18 November 1993

Abstract. The gene encoding the precursor of a small secretory protein with antifungal activity was isolated from *A. giganteus* and characterized by restriction mapping, hybridization and nucleotide sequencing. The promoter contains a typical TATA-box at a distance of 135 bp upstream of the open reading frame. The open reading frame is interrupted by two small introns with conserved splice sites. The precursor of the antifungal protein (AFP) consists of 94 amino acids and appears to be processed to the mature AFP of 51 amino acids by a two-step process. Transfer of the gene into *A. niger* yielded only transformants with a very low expression level, probably because high-expression transformants were counterselected by the antifungal activity of the recombinant protein.

Key-words: Antifungal-protein – Preproprotein – Fungal transformation – Heterologous expression

Introduction

Several small proteins, which are believed to belong to a defense mechanism against phytopathogenic fungi, have been isolated from plants; they include the barley thionins (Bohlmann et al. 1988), stinging nettle lectin (Broekart et al. 1989), radish seed antifungal proteins (Terras et al. 1992), maize zeamatin (Roberts and Seletrennikoff 1990) and rubber-tree hevein (van Parijs et al. 1991). These proteins inhibit fungal growth by diverse molecular modes, e.g., hevein and stinging nettle lectin bind to chitin (Broekart et al. 1989; van Parijs et al. 1991) while zeamatin permeabilizes fungal membranes or cell walls (Roberts and Seletrennikoff 1990).

However, the production of antifungal proteins is not restricted to plant species: a small antifungal protein has been isolated from the imperfect ascomycete *Aspergillus giganteus* along with another inhibitory protein, α-sarcin, a ribosome-inactivating protein (Olson and Goerner 1965; Nakaya et al. 1990). Both proteins are secreted into the culture media. In agar diffusion assays the so-called antifungal protein (AFP) inhibits the growth of a number of filamentous fungi including *Aspergillus niger* and *A. giganteus* itself. It consists of 51 amino acids with a high proportion of lysine residues, leading to an isoelectric point higher than 10.65, and contains eight cysteine residues which are all involved in disulphide bridges (Nakaya et al. 1990). Although the mode of action of the AFP is still to be elucidated, this protein might be useful for food preservation, or in the development of antifungal drugs and the design of plants resistant against phytopathogenic fungi. As a first step in this direction a partial cDNA for the AFP has been isolated and sequenced (Wnendt et al. 1990). In the present report we describe the structural organisation of the gene encoding AFP and the transfer of the gene into another filamentous fungus, *A. niger*.

Materials and methods

Isolation and characterization of the gene encoding AFP. Plaque-lifts from a genomic λEMBL3 library of *A. giganteus* strain MDH 18894 (Wnendt et al. 1993) were hybridized overnight with the radiolabelled AFP cDNA (Wnendt et al. 1990) at 55°C in 4 × SSC, 5 × Denhardt's solution, 0.1% SDS and 100 µg of sonicated single-stranded herring sperm DNA per ml. Filters (Duralose™, Stratagene, Heidelberg, Germany) were washed twice in 2 × SSC and 0.1% SDS at room temperature for 5 min, once in 2 × SSC and 0.1% SDS at 45°C for 15 min, and finally in 1 × SSC and 0.1% SDS at 45°C for 5 min. Among 7.5×10^4 plaques two phages were purified by repeated plating and hybridizational screening under the above conditions. DNA of both phages was isolated using Qiagen columns (Diagen, Düsseldorf, Germany), digested with *Eco*RI and subjected to Southern analysis (Sambrook et al. 1989). In one phage the region of homology to the AFP cDNA was confined to a 2.7-kb *Eco*RI fragment. This fragment was subcloned into pBluescriptKS (+)

* *Present address*: Gruenenthal GmbH, Centre of Research, Aachen, Germany

Correspondence to: U. Stahl

(Stratagene) and the recombinant plasmid was designated pBS71119. A detailed restriction map of the 2.7-kb fragment was constructed and the position of the AFP-encoding gene was determined by Southern analysis. A genomic Southern analysis was performed with *Hin*dIII-, *Bam*HI- and *Eco*RI-digested DNA from *A. giganteus* MDH 18894 using a ^{32}P-labelled *Sph*I/*Eco*RV fragment of the cloned 2.7kb fragment as a probe. The hybridization was performed at 60° C in 4 × SSC, 1% blocking reagent (Boehringer, Mannheim, Germany), 0.02% SDS and 0.1% sarcosyl. The stringency wash was in 1 × SSC, 0.1% SDS at 45° C for 30 min. The nucleotide sequence from both strands of a 1-kb region carrying the AFP-encoding gene was analysed according to Sanger's method (T7 sequencing kit, Pharmacia, Freiburg, Germany; *Taq*-polymerase kit, Stratagene). The partial sequences were determined by using standard primers (SK and KS primer; Stratagene) and restriction fragments subcloned in pBluescriptKS(+), or by using specific oligonucleotide primers. The sequences of the specific primers were deduced from the AFP cDNA sequence and the partial sequences determined in the sequencing process.

Transformation of A. niger. The hygromycin B resistance vector pAN7-1 (Punt et al. 1987) was digested with *Hin*dIII and protruding ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase. This vector was ligated with a 2.1-kb *Sma*I/*Eco*RV fragment of pBS71119 (see Fig. 1 a) which had been eluted from agarose using the Qiaex method (Diagen). The resultant plasmid was designated pANAFP1. *A. niger* ATCC16404 was transformed separately with pAN7-1 and pANAFP1 by protoplast fusion and selection on minimal medium agar containing 100 µg/ml of hygromycin B (Calibiochem, Frankfurt/M, Germany). The transformation was performed as described for *A. giganteus* (Wnendt et al. 1990) except that protoplasts were prepared in a solution of 270 mM $CaCl_2$ and 530 mM NaCl and stored in a modified STC-buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl and 1.2 M sorbitol). Agar medium was also osmotically stabilized with 1.2 M sorbitol. Hygromycin B-resistant transformants were purified by single-spore colony isolation. The fate of the transforming DNA was determined by Southern analysis of the transformants. Genomic DNA was isolated according to Yelton et al. (1984) with the following modifications: the frozen and powdered mycelium from a 3–4 day-old 200-ml culture (2% malt extract, 2% glucose, 0.1% peptone with or without 50 µg of hygromycin B per ml) was resuspended in 15 ml of extraction buffer supplemented with spermine and spermidine [10 mM Tris/HCl, pH 8, 50 mM EDTA, 0.5% SDS, 4 mM spermine (Sigma, Deisenhofen, Germany), 0.4 mM spermidine (Sigma) and 50 µg/ml proteinase K (Merck, Darmstadt, Germany)]. The suspension was kept for 1 h at 56° C, cooled to room temperature and centrifuged for 30 min at 4° C with 40000 *g*. The supernatant was subjected to potassium-acetate precipitation, followed by isopropanol precipitation of nucleic acids. The pellet from the ethanol precipitation was dissolved in 500 µl of TE pH 8 (10 mM Tris/HCl, 1 mM EDTA, pH 8) and extracted twice with TE-satured phenol and once with chloroform/isomyalcohol (24:1) (Merck). Finally the aquous phase was ethanol precipitated and the nucleic acid pellet was dissolved in 150 µl of destilled water.

Purification and analysis of the secreted recombinant protein. *A. niger* transformants were each grown for 5 days at 28° C in 100 ml of complete medium (1% beef extract, 2% peptone, 2% starch, 0.5% NaCl; Olson and Goerner 1965) containing 50 µg of hygromycin B per ml. Basic proteins were isolated from culture supernatants by a batch procedure using a cation exchange resin: 7 ml of culture supernatant adjusted to pH 7 with dilute NaOH were mixed with 0.5-ml suspension of carboxymethylcellulose CM-52 (Serva, Heidelberg, Germany) (0.5 g dry weight per ml of TE, pH 7) for 30 min. The resin was washed once with 2 ml of TE, pH 7, and basic proteins were eluted with 250 µl of high salt buffer (10 mM Tris/HCl, pH 7, 1 mM EDTA and 1.5 M NaCl). Eluted proteins were analysed by SDS-PAGE. The polyacrylamide concentration in the separating gel was 15% (w/v). Bands were detected by staining with Coomassie brilliant blue R-250 (Serva).

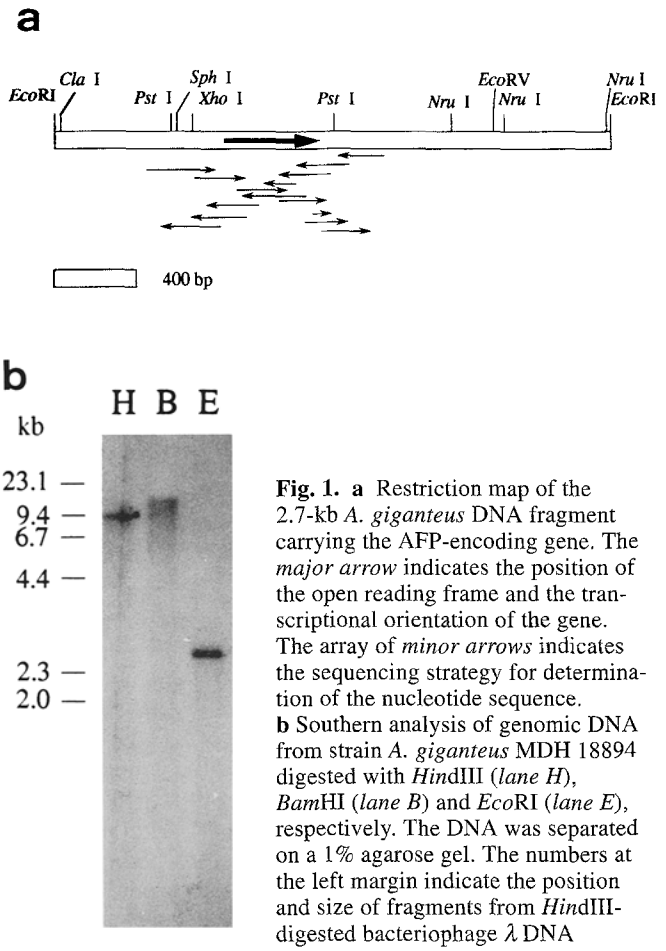

Fig. 1. a Restriction map of the 2.7-kb *A. giganteus* DNA fragment carrying the AFP-encoding gene. The *major arrow* indicates the position of the open reading frame and the transcriptional orientation of the gene. The array of *minor arrows* indicates the sequencing strategy for determination of the nucleotide sequence. **b** Southern analysis of genomic DNA from strain *A. giganteus* MDH 18894 digested with *Hin*dIII (*lane H*), *Bam*HI (*lane B*) and *Eco*RI (*lane E*), respectively. The DNA was separated on a 1% agarose gel. The numbers at the left margin indicate the position and size of fragments from *Hin*dIII-digested bacteriophage λ DNA

Results and discussion

Restriction map of the gene encoding AFP and genomic Southern analysis

A 2.7-kb *Eco*RI DNA fragment of *A. giganteus* DNA hybridising to the partial AFP cDNA was isolated as described under Materials and methods. A preliminary restriction map of the genomic *Eco*RI fragment carrying the gene encoding AFP has been already been published (Wnendt et al. 1990). The restriction map presented in Fig. 1 a differs in one point from the map previously presented: the positions of the *Pst*I and *Sph*I restriction sites in the proximity of the unique *Xho*I site are interchanged. A Southern analysis was performed with genomic DNA from *A. giganteus* strain MDH 18894. The internal *Sph*I/*Eco*RV fragment of the cloned 2.7-kb fragment was used as a probe. This probe hybridized to a 10-kb *Hin*dIII fragment, a 13-kb *Bam*HI fragment and, as was expected, to an *Eco*RI fragment of 2.7-kb (Fig. 1 b). The same result was obtained when the partial cDNA was used as probe (Wnendt et al. 1990). The fact that each restriction enzyme gave only one band indicates that *A. giganteus* contains only one copy of the AFP-encoding gene per genome. Fine mapping of the cloned genomic *Eco*RI fragment excluded the possibility of a tandem array of the gene (Fig. 1 a).

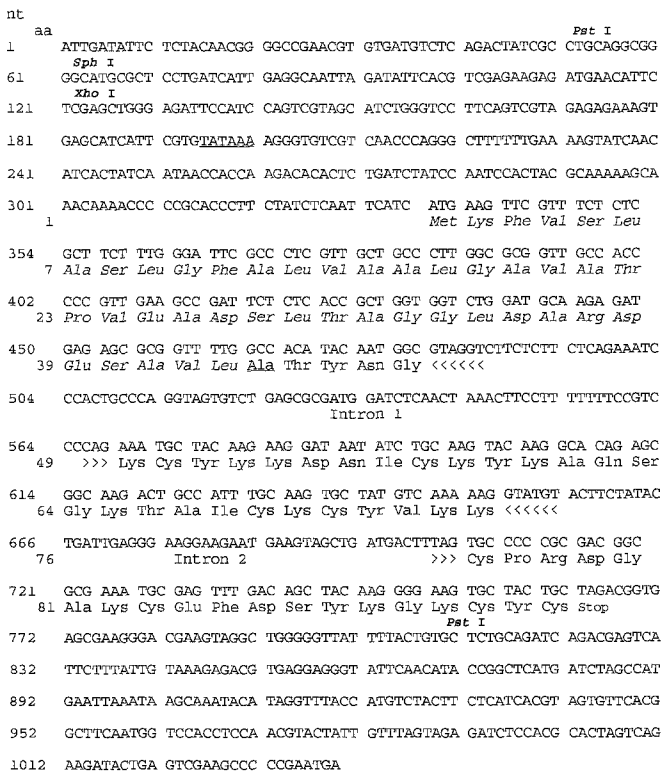

Fig. 2. Nucleotide sequence of the gene encoding AFP. The deduced aa sequence of the gene is listed below the corresponding codons. The putative presequence is in *italics*. The amino-terminal aa of the mature AFP is *underlined* (*Ala*). <<< and >>> represent 5′ and 3′ splice sites, respectively. The TATA-box (nt 195 – 200) is *underlined*. Relevant restriction enzyme recognition sites are indicated above the nt sequence. The sequence of the AFP gene is deposited under accession number X60771 with EMBL Data Library, Heidelberg, Germany

Nucleotide sequence of the gene encoding AFP and putative functional elements

The nucleotide sequence of the AFP gene (Fig. 2) was determined from overlapping partial sequences (for sequencing strategy see Fig. 1 a). The gene contains an open reading frame of 282 nt encoding the amino-acid sequence of an AFP precursor. The amino-acid sequence encoded by nt 465–762 is identical with the sequence of the mature AFP (Nakaya et al. 1990). The context of the putative translational start codon (5′-CATC*ATG*, nt 332 to 338) corresponds to the eukaryotic consensus structure: cytosine in position -1 and -4, and adenosine in position -3 relative to the putative start codon (Kozak 1984). Within the sequenced region there are three ATGs upstream of the assumed translational start codon (nt 34–36, nt 64–66 and nt 111–113) but their contexts are different from the consensus sequence of eukaryotic start codons. Additionally, two ATGs (nt 439–441 and nt 448–450), located between the assumed translational start codon and the first amino acid of the mature AFP (Nakaya et al. 1990), are out of frame. Taken together it appears most likely that the ATG in position 336 to 338 is the translational start codon.

The sequence determined for the open reading frame of the structural gene differs in nt 458–459 from the previously published cDNA sequence (Wnendt et al. 1990). Due to a mistake there is an extra guanosine at this position in the published cDNA sequence which led to the assumption of a too short of secretion signal sequence. However, repeated sequence determination of the cloned genomic fragment verified the nucleotide sequence presented in Fig. 2. The identified open reading frame is interrupted by two introns (intron 1, nt 480–568; intron 2, nt 650–705) which are absent in the cloned cDNA (Wnendt et al. 1990). The small size of both of these (89 and 56 nt, respectively) compared to mammalian introns is a typical feature of fungal genes (Gurr et al. 1987). The 5′- and 3′-ends of both introns are very similar (see Fig. 2) and in complete agreement with the consensus splice sequences for fungal introns (5′-consensus: 5′-GTANGT, 3′-consensus: 5′-YAG; Gurr et al. 1987).

Upstream of the translational start codon is a TATA-box (nt 195–200) which complies in its sequence (5′-TATAAA, and the following three nucleotides: AGG) with the TATA-box found in the promoter region of the gene encoding α-sarcin (Wnendt et al. 1993). Upstream of the putative TATA-box there are two sets of relatively-long direct repeats: 5′-GATATTC, nt 3–10 and nt 91–97, and 5′-CAGTCGTAG, nt 141–149 and nt 163–171. However, it remains to be determined by promoter deletion studies whether the TATA-box and the repeats, respectively, are functional in the transcriptional process.

Amino-acid sequence of the AFP precursor

The open reading frame encodes a 94-aa-long precursor of the AFP. The first part of the presequence (aa 1–26) has several features of a secretion signal sequence (von Heijne 1985): a hydrophilic, cationic N-terminus with a lysine in position 2, a hydrophilic core region from aa 3 to 21, and a polar C-terminal region, extending from aa 22 to 26. The function of the remaining presequence (aa 27–43) is not obvious. With respect to the antifungal activity of the mature protein it is tempting to speculate that this part of the presequence maintains the precursor in an inactive form until it is further processed to the mature and active AFP. There are other inhibitory proteins which are produced as preproproteins, e.g., the killer toxins from *Saccharomyces cerevisiae* or *Ustilago maydis* (Bostian et al. 1984; Tao et al. 1990). However, to support this hypothesis further data are necessary which confirm the presence of an intermediate of the AFP precursor and describe its activity.

Expression of the AFP-encoding gene in A. niger

The cloned gene encoding AFP was transferred to another *Aspergillus* species in order to demonstrate its function. *A. niger*, like *A. giganteus*, is a sexually-imperfect asomycete, which can easily be transformed with plasmid DNA (Punt et al. 1987) and belongs to the group of safe recipient strains for gene-transfer experiments. *A. niger* does not secrete a basic protein with the molecular mass of the AFP, nor does it contain genomic DNA which hybridizes to the ^{32}P-labelled AFP cDNA in Southern analysis (data not

shown). Therefore, the gene encoding AFP was inserted into the hygromycin B resistance vector pAN7-1 (Punt et al. 1987) and the resultant plasmid, pANAPF1, was used to transform *A. niger* to hygromycin B resistance; pAN7-1 was used as a control plasmid in *A. niger* transformation. Seven pANAFP1 and three pAN7-1 transformants were randomly selected and spore suspensions were replated in dilutions on selective minimal medium in order to isolate homokaryotic single-spore colonies. Two transformants from the pANAFP1 group did not regenerate. Southern analysis of the remaining five pANAFP1 transformants showed that the plasmid DNA is either integrated as a single copy or as one or two tandemly-arranged copies into the host genome (data not shown). Integration and tandem arrangement are common features of ascomycete transformation and have been described before (Ballance 1991).

In order to test whether the AFP-encoding gene is expressed in *A. niger* the transformants were grown in a complete medium for 5 days, basis proteins were isolated from culture supernatants and then separated on SDS-PAGE (Fig. 3). There is a protein of about 7.5 kDA secreted in very low amounts by the pAN7-1 control transformant (control) and by pANAFP1 transformants T1 (T1, lanes a and b). This protein was also found in samples from untransformed *A. niger* (Wnendt et al. 1993). A basic protein with the molecular mass of AFP was isolated from four out of five pANAPF1 transformants (T2–T5 but not transformant T1). Western analysis showed that this protein reacted with serum from rabbits immunized with a mixture of AFP and α-sarcin from *A. giganteus* (data not shown). The molecular mass of the recombinant protein from the pANAFP1 transformants (T2–T5, Fig. 3) and its immunoreactivity suggest that this protein is identical with AFP. The concentration of recombinant AFP found in parallel cultures of the respective transformants was very variable (consider for example transformants T3 and T4) and there was no correlation between the expression level and the copy number of vector DNA as determined by Southern analysis (data not shown). The maximal concentration of recombinant protein was about 350 µg/l (transformants T4 and T5) which is much less than that observed with the natural producer which secretes between 30 and 40 mg/l. A very-low heterologous expression in *A. niger* was also observed with the α-sarcin gene in *A. giganteus*. In that case only one out of four *A. niger* transformants carrying the α-sarcin gene produced significant amounts of recombinant protein (Wnendt et al. 1993). With respect to the inhibitory properties of α-sarcin and the AFP it is conceivable that during regeneration of transformed *A. niger* protoplasts only those clones were viable which produced very-low amounts of α-sarcin or AFP. The observation that two of seven primary pANAFP1 transformants did not regernate after replating (see above) seems to support this hypothesis. However, it is also possible that specific factors for transcriptional activation of the gene, or proteins for efficient processing of the AFP-precursor, are missing in *A. niger*. The gene encoding AFP was also expressed in an *A. giganteus* isolate which naturally secretes relatively-low amounts of AFP. The homologous expression led to a drastic increase from about 5 mg/l to about 30 mg/l AFP (data not shown). This observation might argue for the role of species-specific gene expression.

In order to use the gene as a new resistance trait future work will have to analyse the molecular mechanism of the antifungal activity of AFP and show whether the AFP gene can be expressed in plants.

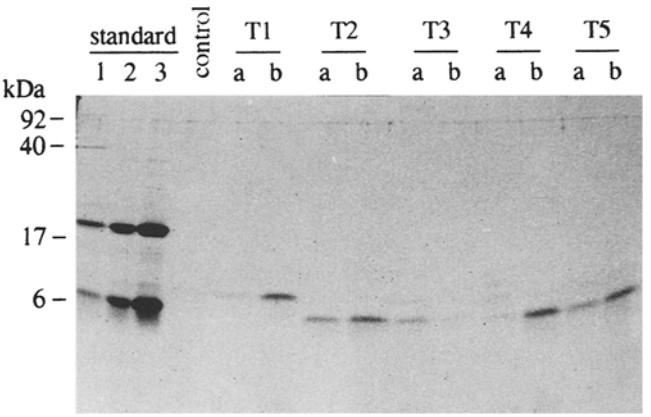

Fig. 3. Expression of the AFP-encoding gene in *A. niger*. SDS-PAGE (15% polyacrylamide, Coomassie stain) of basic proteins secreted by *A. niger* transformants T1 to T5 and a pAN7-1 transformant (control). The strains T1 to T5 were grown and analysed in parallel duplicates (*a and b*). Each sample corresponds to 2.8 ml of culture supernatant. Standards: 0.5 µg (*1*), 1.5 µg (*2*) and 4.5 µg (*3*) each of α-sarcin (Olson and Goerner 1965) and AFP

Acknowledgements. We thank the Deutsche Gesellschaft für Chemisches Apparatewesen, Chemische Technik und Biotechnologie e. V. (DECHEMA), Frankfurt/M, Germany, for supporting this work with a PhD student grant to S. W. The support of N. U. from Hoechst AG, Germany, and the Fonds der Chemischen Industrie is also gratefully acknowledged.

References

Ballance DJ (1991) Transformation systems for filamentous fungi and an overview of fungal gene structure. In: Leong SA, Berka RM (eds) Molecular industrial mycology. Marcel Dekker, New York, pp 1–29

Bohlmann H, Clausen S, Behnke S, Giese H, Hiller C, Reimann-Philipp U, Schrader G, Barkholt V, Apel K (1988) Leaf-specific thionins of barley – a novel class of cell-wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. EMBO J 7:1559–1565

Bostian KA, Eliott Q, Bussey H, Burn V, Smith A, Tipper DJ (1984) Sequence of the preprotoxin dsRNA gene of type-I killer yeast: multiple processing events produce a two-component toxin. Cell 36:741–751

Broekart WF, Van Parijs J, Leyns F, Joos H, Peumans WF (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science 245:1100–1102

Gurr SJ, Unkles SE, Kinghorn JR (1987) The structure and organization of nuclear genes of filamentous fungi. In: Kinghorn JR (ed) Gene structure in eukaryotic microbes. Special publications of the Society for General Microbiology, vol 22. IRL Press, Oxford, pp 93–139

Heijne G von (1985) The limits of variation. J Mol Biol 184:99–105

Kozak M (1984) Compilation and anlysis of sequences upstream form the translational start site in eukaryotic mRNAs. Nucleic Acids Res 12:857–872

Nakaya N, Omata K, Okahashi I, Nakamura Y, Kolkenbrock HJ, Ulbrich N (1990) Amino-acid sequence and disulphide bridges of an antifungal-protein isolated from *Aspergillus giganteus*. Eur J Biochem 193:31–38

Olson BH, Goerner GL (1965) Alpha sarcin, a new antitumor agent. I. Isolation, purification, chemical composition, and the identify of a new amino acid. Appl Microbiol 13:314–321

Parijs J van, Broekart WF, Goldstein IJ, Peumans WJ (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta 183:258–264

Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJ J (1987) Transformation of *Aspergillus* based on a hygromycin B resistance marker from *E. coli*. Gene 56:117–124

Roberts WK, Seletrennikoff CP (1990) Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J Gen Microbiol 136:1771–1778

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Tao J, Ginsberg I, Banerjee N, Held W, Koltin Y, Bruenn J (1990) *Ustilago maydis* KP6 killer toxin: structure, expression in *Saccharomyces cerevisiae*, and relationship to other cellular protein. Mol Cell Biol 10:1373–1381

Terras FRG, Schoofs HME, De Bolle MFC, Van Leuven F, Rees SB, Vanderleyden J, Cammue BPA, Broekart WF (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J Biol Chem 267:15301–15309

Wnendt S, Ulbrich N, Stahl U (1990) Cloning and nucleotide sequence of a cDNA encoding the antifungal-protein of *Aspergillus giganteus* and preliminary characterization of the native gene. Nucleic Acids Res 18:1653

Wnendt S, Felske-Zech H, Henze P-PC, Ulbrich N, Stahl U (1993) Characterization of the gene encoding α-sarcin, a ribosome-inactivating protein secreted by *Aspergillus giganteus*. Gene 124:239–244

Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a trpC plasmid. Proc Natl Acad Sci USA 81:1470–1474

Communicated by K. Wolf