Novel antimycotics and fungicides, processes for their preparation, and their use

Description

The present invention relates to novel antimycotics and fungicides which can be obtained from yeast, processes for their preparation, and their use

Selective antimycotics are extremely important since fungal and/or yeast infections have increased enormously in recent years in humans, and also continue to result in undesired contamination in foods and animal feeds. Mycoses have particularly grave consequences in immunosuppressed patients whose cellular and humoral defense system must be kept at a level which is not fully functional [Anaissie, 1992; Meunier *et al.*, 1992; Wingard, 1995]. Extremely endangered by mycoses are patients infected with *HIV*-1 (AIDS), who very frequently die during a later stage of the disease from opportunistic infections by fungi and/or yeasts which are pathogenic for man [Levy, 1993]. The antimycotics which are currently employed for the therapy of such infections (such as amphotericin B, fluconazole, itraconazole, ketoconazole) cause considerable side effects since they destroy the structural integrity of the eukaryotic cytoplasmic membrane and thus also damage the infected host organism [Hector, 1993]. Moreover, the application of conventional antimycotics has led within only a short time to a rapid increase in fluconazole resistances which spread rapidly among the microorganisms which are pathogenic for man and constitute an ever increasing problem [Cameron *et al.*, 1993; Chavenet *et al.*, 1994; Maenza *et al.*, 1996; Pfaller *et al.*, 1994; Rex *et al.*, 1995; Troillet *et al.*, 1993]. It is therefore an important desire to develop antimycotics which – like bacterial antibiotics – are distinguished by high selectivity and which attack, if possible, only fungi and yeasts which are pathogenic for man. Since, however, most of all cellular processes in higher organisms are governed by gene products which show a high degree in functional homology in eukaryots, the development of "specifically antifungal antibiotics" has hitherto been unsuccessful [Kurz, 1998; Komiyama et al., 1998].

A target of selective antimycotics are the β-1,3-D-glucans of the yeast cell wall which are indispensable for the mechanic and osmotic stability of the

cell, but do not occur in higher eukaryots and, constituting an "Achilles heel", might thus be exploited in the control of pathogenic yeasts [Roemer *et al.*, 1994]. Even though substances which selectively engage in the cell wall structure of yeasts and fungi are thus of great interest, no antibiotic-like inhibitors have been employed as yet for controlling mycoses. While bacterial antibiotic-producers were discovered as early as the beginning of the present century, similar effects in yeasts were only observed at the beginning of the 60's by identifying so-called killer yeasts [Bevan & Makower, 1963]: toxin-producing killer strains of the brewer's yeast *Saccharomyces cerevisiae* produce and secrete proteins termed "killer toxins" which destroy sensitive yeasts in a receptor-dependent process [Bussey, 1991; Tipper & Schmitt, 1991]. In *S. cerevisiae*, the ability of producing toxins is based on infections with reovirus-like double-stranded RNA viruses which stably and in high copy number persist in yeast cytoplasm without noticeably damaging the eukaryotic host cell [Tipper & Schmitt, 1991]. The three killer toxins of the yeast *S. cerevisiae* which are known to date (K1, K2, K28) are unglycosylated α/β-heterodimers which are translated by the infected cell as highly molecular preprotoxins and which are processed during the intracellular secretion pathway by complex modification to give the bioactive killer proteins [Hanes *et al.*, 1986; Dignard *et al.*, 1991; Schmitt & Tipper, 1995]. The toxic effect of the *S. cerevisiae* toxins is based either on a destruction of the membrane integrity (toxins K1, K2) or (as in the case of killer toxin K28) on arresting the cell cycle with a direct inhibition of DNA synthesis [Bussey, 1991; Schmitt & Compain, 1995; Schmitt *et al.*, 1996]. Even though killer toxins of the classes K1, K2 and K28 differ markedly from each other with regard to their modes of action and their physicochemical properties, they share the characteristics of having narrow spectra of action and of predominantly destroying sensitive yeasts of closely related species. This limited spectrum of action is based on the fact that the brewer's yeast killer toxins which have been characterized so far must interact with different receptor populations at the yeast cell wall and cytoplasmic membrane levels in order to be able to destroy sensitive target cells. The primary toxin receptors of the yeast cell wall are either highly branched β-1,6-D-glucans or the outer mannotriose side chains of a cell wall mannoprotein [Bussey, 1991; Schmitt & Radler 1987, 1988].

Apart from the viral protein toxins of the yeast *S. cerevisiae*, *Hanseniaspora uvarum*, *Zygosaccharomyces bailii* and *Ustilago maydis*, killer strains have

also been described in the genera *Debaryomyces*, *Hansenula*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*, *Pichia*, *Kluyveromyces*, *Torulopsis* and *Williopsis* [McCracken *et al.*, 1994; Park *et al.*, 1996; Schmitt & Neuhausen, 1994; Walker *et al.*, 1995]. In these yeasts, however, the genetic base of the killer phenomenon is not viral genomes, but either linear dsDNA plasmids or chromosomal yeast genes [Schründer *et al.*, 1994].

Intensive studies into the molecular biology of various toxin-producing "killer yeasts" have shown that the secretion of toxic proteins ('killer toxins') is widespread in yeasts and constitutes a potential in the development of selective antimycotics which should not be underestimated [Walker *et al.*, 1995; Hodgson *et al.*, 1995; Polonelli *et al.*, 1986; Schmitt & Neuhausen, 1994; Neuhausen & Schmitt, 1996; Schmitt *et al.*, 1997], but it has hitherto been impossible to provide such protein toxins.

It is therefore an object of the present invention to provide suitable antimycotic or fungicidal protein toxins for controlling yeast and/or fungi which are pathogenic for man and plants.

Surprisingly, the killer toxin WICALTIN (also protein toxin) from the wild-type yeast *Williopsis californica* strain 3/57 (DSM 12865), which is produced and secreted in a highly efficient fashion, and the virus-encoded ZYGOCIN (also protein toxin) from the yeast *Zygosaccharomyces bailii* (DSM 12864) prove to be particularly suitable for controlling yeast and/or fungi which are pathogenic for man and plants. Moreover, fungi and harmful yeasts which are a hazard in the food and animal feed sector can also be destroyed. Both protein toxins therefore have the potential of being employed as antimycotics and/or fungicides for controlling yeast and/or fungal infections, in particular mycoses. These indications are verified in the present invention by studies into the mode of action. The toxin genes are cloned and sequenced in a suitable manner for the purposes of the present invention, thus establishing a process for the recombinant production and overexpression of WICALTIN and ZYGOCIN in culture.

A subject matter of the invention therefore relates to protein toxins which can be obtained from *Williopsis californica*, especially preferably strain DSM 12865, and *Zygosaccharomyces bailii*, especially preferably strain DSM 12864. Both strains were deposited on 9th June 1999 at the DSMZ-

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, in 38124 Braunschweig, Mascheroder Weg 1b in compliance with the provisions of the Budapest Treaty (www.dsmz.de).

For the purposes of the present invention, it is in particular DSM 12864 and DSM 12865 which secrete biologically potent protein toxins which, owing to their broad spectrum of action (see Example 4 and 7), also destroy a large number of yeasts and fungi which are pathogenic for man and plants. The invention thus also relates to selective antimycotics or fungicides in the sense of the protein toxins – and the polypeptides hereinbelow according to the invention and their encoding nucleic acids according to the invention, in particular in the functional unit of a toxin gene – being potential *biopharmaceuticals* which, owing to their specific, receptor-medium production, exclusively destroy yeasts and/or fungi and which are thus entirely harmless to higher eukaryots - and thus also to humans and mammalian cells – and to plants, preferably crop plants [cf. Pfeiffer *et al.*, 1988].

The following yeasts and/or fungi which are apathogenic or pathogenic for man and plants can be destroyed selectively:
Zygocin-sensitive yeast species: *Saccharomyces cerevisiae, Candida albicans, Candida krusei, Candida glabrata, Candida vinii, Hanseniaspora uvarum, Kluyveromyces marxianus, Methschnikowia pulcherrima, Ustilago maydis, Debaryomyces hansenii, Pichia anomala, Pichia jadinii, Pichia membranefaciens, Yarrowia lipolytica* and *Zygosaccharomyces rouxii.*
Wicaltin-sensitive yeast species: *Candida albicans, Candida glabrata, Candida tropicalis, Debaryomyces hansenii, Kluyveromyces lactis, Metschnikowia pulcherrima, Pichia anomala, Pichia jadinii, Saccharomyces cerevisiae, Sporthrix spec., Torulaspora delbrueckii, Torulaspora pretoriensis, Yarrowia lipolytica* and *Zygosaccharomyces bailii.*

The particularly high activity of the wicaltin-producing yeast strain DSM 12865 is probably based on its pronounced secretory efficiency, which is markedly more pronounced in comparison with other strains of the same yeast species. The 'killer' property of the zygocin-producing yeast strain DSM 12864 is based on infection with toxin-encoding double-strand RNA viruses (M_{Zb}-dsRNA) which stably persist in the cytoplasm in high copy number and which enable the yeast in question (strain DSM 12864) to produce and secrete zygocin [cf. Schmitt & Neuhausen, 1994]. Other

strains of the same species show no toxic production since they do not harbor toxin-encoding dsRNA viruses in the cytoplasm and are thus to be classified phenotypically as 'non-killer'.

Another subject matter of the present invention is therefore nucleic acids encoding for a protein toxin – with an amino acid sequence in accordance with **SEQ ID No 1 and No 2** and a glucanase activity – or a functional variant thereof, and sections thereof with at least 8 nucleotides, preferably with at least 15 or 20 nucleotides, in particular with at least 100 nucleotides, especially with at least 300 nucleotides (subsequently termed "nucleic acid(s) according to the invention").

The complete nucleic acids encoding for protein toxins which, after intracellular processing and secretion, have a size of 309 amino acids and a molecular mass of 34 kDa (**SEQ ID No 1**) or of 99 amino acids and a molecular mass of 10 kDa (**SEQ ID No 2**). Expression of the nucleic acid in accordance with **SEQ ID No 1** in the yeast *S. cerevisiae* results in a recombinant WICALTIN, which is secreted into the culture supernatant of the yeast as a glycosylated protein with significant β-1,3-D-glucanase activity [cf. Example 10]. Further experiments in accordance with the present invention confirm that the nucleic acids according to the invention are nucleic acids which in the case of **SEQ ID No 1**, encode a protein toxin with glucanase activity and, in the case of **SEQ ID No 2**, a protein toxin which is probably O-glycosylated *in vivo* and is termed ZYGOCIN. The nucleic acids according to the invention can be obtained from DSM 12865 (**SEQ ID No 1**) and DSM 12864 (**SEQ ID No 2**).

In a preferred embodiment, the nucleic acids according to the invention are DNA or RNA, preferably a double-stranded DNA, and in particular a DNA with a nucleic acid sequence in accordance with **SEQ ID No 1** from position 1 to position 947 and in accordance with **SEQ ID No 2** from position 1 to position 713. In accordance with the present invention, the two positions determine the start and the end of the encoding region, i.e. in each case the first and last amino acid of the reading frame in question.

The term "functional variant" is to be understood as meaning in accordance with the present invention a nucleic acid which are functionally related to the nucleic acids according to the invention. Examples of related nucleic acids are nucleic acids from different yeast cells or strains and cultures or

allelic variants. The present invention also encompasses variants of nucleic acids which can be derived from a variety of yeasts/yeast strains or other pathogens such as dermatophytes and molds (in accordance with the DHS system).

The term "variants" in accordance with the present invention is furthermore to be understood as meaning nucleic acids which exhibit a homology, in particular a sequence identity, of approx. 60%, preferably of approx. 75%, in particular of approx. 90% and especially of approx. 95%.

The sections of the nucleic acid according to the invention can be used, for example, for generating individual epitopes, as probes for identifying further functional variants, or as antisense nucleic acids. For example, a nucleic acid of at least approx. 8 nucleotides is suitable as antisense nucleic acid, a nucleic acid of at least approx. 15 nucleotides as primer in the PCR method, a nucleic acid of at least approx. 20 nucleotides for the identification of further variants, and a nucleic acid of at least approx. 100 nucleotides as probe.

In a further preferred embodiment, the nucleic acid according to the invention contains one or more noncoding sequences and/or a poly(A)-sequence, one or more Kex2p endopeptidase recognition sequences (required for intracellular proprotein processing), and one or more potential N-glycosylation sites. The noncoding sequences are regulatory sequences such as promoter or enhancer sequences for the controlled expression of the coding toxin gene containing the nucleic acids according to the invention.

In a further embodiment, the nucleic acid according to the invention is therefore contained in a vector, preferably in an expression vector or in a vector which is effective in gene therapy.

Examples of expression vectors can be, in the case of the nucleic acid in accordance with **SEQ ID No 2**, prokaryotic and/or eukaryotic expression vectors, and, in the case of the nucleic acid in accordance with **SEQ ID No 1**, exclusively eukaryotic expression vectors. Expression of the toxin-encoding nucleic acid in accordance with **SEQ ID No 1** in *Escherichia coli* is not possible since the respective, heterologously expressed protein toxin is toxic to the bacterial cell. Cloning of the WICALTIN-encoding nucleic acid in accordance with **SEQ ID No 1** in *E. coli* is only possible with plasmids

which do not carry a promoter (for example with the aid of derivatives of plasmid pBR322). An example of a prokaryotic vector which allows heterologous expression of the ZYGOCIN-encoding nucleic acid in accordance with **SEQ ID No 2** is the commercially available vector pGEX-4T-1, which allows a glutathione S transferase/ZYGOCIN fusion protein to be expressed in *E. coli*. A further vector for the expression of ZYGOCIN in *E. coli* is, for example, the T7 expression vector pGM10 (Martin, 1996), which encodes an N-terminal Met-Ala-His6 tag which allows an advantageous purification of the expressed protein through an Ni^{2+}-NTA column. Examples of suitable eukaryotic expression vectors for the expression in *Saccharomyces cerevisiae* are the vectors p426Met25 or p426GAL1 (Mumberg et al. (1994) Nucl. Acids Res., 22, 5767), for the expression in insect cells baculovirus vectors such as those disclosed in EP-B1-0127839 or EP-B1-0549721, and for expression in mammalian cells SV40 vectors, which are freely available.

In general, the expression vectors also contain regulatory sequences which are suitable for the host cell, such as, for example, the trp promoter for expression in E. coli (see, for example, EP-B1-0154133), the ADH-2 promoter for expression in yeasts (Russel et al. (1983), J. Biol. Chem. 258, 2674), the baculovirus polyhedrin promoter for expression in insect cells (see, for example, EP-B1-0127839), or the early SV40 promoter, or LTR promoters, for example those of MMTV (Mouse Mammary Tumor Virus; Lee et al. (1981) Nature, 214, 228).

Examples of vectors which are effective in gene therapy are viral vectors, preferably adenoviral vectors, in particular replication-deficient adenoviral vectors, or adeno-associated viral vectors, for example an adeno-associated viral vector which consists exclusively of two inserted terminal repetitive sequences (ITRs).

Suitable adenoviral vectors are described, for example, by McGrory, W.J. et al. (1988) Virol. 163, 614; Gluzman, Y. et al. (1982) in "Eukaryotic Viral Vectors" (Gluzman, Y. ed.) 187, Cold Spring Harbor Press, Cold Spring Harbor, New York; Chroboczek, J. et al. (1992) Virol. 186, 280; Karlsson, S. et al. (1986) EMBO J.. 5, 2377 or WO95/00655.

Examples of suitable adeno-associated viral vectors are described by Muzyczka, N. (1992) Curr. Top. Microbiol. Immunol. 158, 97; WO95/23867;

Samulski, R.J. (1989) J. Virol, 63, 3822; WO95/23867; Chiorini, J.A. et al. (1995) Human Gene Therapy 6, 1531 or Kotin, R.M. (1994) Human Gene Therapy 5, 793.

Vectors which are effective in gene therapy can also be obtained by complexing the nucleic acid according to the invention with liposomes. Suitable lipid mixtures for this purpose are those described by Felgner, P.L. et al. (1987) Proc. Natl. Acad. Sci, USA 84, 7413; Behr, J.P. et al. (1989) Proc. Natl. Acad. Sci. USA 86, 6982; Felgner, J.H. et al. (1994) J. Biol. Chem. 269, 2550 or Gao, X. & Huang, L. (1991) Biochim. Biophys. Acta 1189, 195. When producing the liposomes, the DNA is bound ionically on the liposomal surface in such a ratio that a positive nett charge remains and the DNA is complexed completely by the liposomes.

In a further embodiment, the nucleic acids according to the invention are therefore contained in a vector, preferably in an expression vector for the generation of transgenic plants. Since the above-described killer toxins WICALTIN and ZYGOCIN have a broad spectrum of action and also destroy yeasts and fungi which are pathogenic for plants, it is possible to provide transgenic plants which behave in a resistant fashion for example to an infection with the pathogen *Ustilago maydis*, which is pathogenic for maize. Similar experiments have already been carried out on tobacco plants which, owing to heterologous expression of the *U. maydis* killer toxin KP4, which in nature is encoded by a virus, were capable of secreting the protein toxin in question and thus built up a specific protection from infection with certain phytopathogenic *U. maydis* strains (Park et al., 1996; Kinal et al., 1995; Bevan, 1984). Starting from commercially available transformation systems which are based on modified derivatives of the natural *Agrobacterium tumefaciens* Ti plasmid, the nucleic acids according to the invention, which are also represented in the toxin genes *WCT* and *ZBT*, can be cloned into so-called bidirectional pBI vectors (CLONTECH) and employed for the generation of transgenic plants. To this end, the respective toxin genes *WCT* and *ZBT* are placed under the transcriptional control of the strong cauliflower mosaic virus promoter (CaMV-P). The more detailed construction of the vectors to be constructed is shown schematically in Example 9.

For example, the nucleic acids according to the invention can be synthesized chemically, for example following the phosphotriester method,

with reference to the sequences disclosed in **SEQ ID No 1 and No 2** or with reference to the peptide sequences disclosed in **SEQ ID No 1 and No 2**, taking into consideration the genetic code (see, for example, Uhlman, E. & Peyman, A. (1990) Chemical Reviews, 90, 543, No. 4). Another possibility of obtaining the nucleic acid according to the invention is the isolation of a suitable gene bank with the aid of a suitable probe (see, for example, Sambrook, J. et al. (1989) Molecular Cloning. A laboratory manual. 2nd Edition, Cold Spring Harbor, New York). Suitable probes are, for example, single-stranded DNA fragments with a length of approx. 100 to 1000 nucleotides, preferably with a length of approx. 200 to 500 nucleotides, in particular with a length of approx. 300 to 400 nucleotides, whose sequence can be deduced from the nucleic acid sequence in accordance with **SEQ ID No 1 and No 2.**

Another subject matter of the present invention are the polypeptides as such with an amino acid sequence in accordance with **SEQ ID No 1 and No 2** or a functional variant thereof, and portions thereof with at least six amino acids, preferably with at least 12 amino acids, in particular with at least 65 amino acids, and especially with 309 amino acids **(SEQ ID No 1)** and with 99 amino acids **(SEQ ID No 2)** (hereinbelow "polypeptide(s) according to the invention"). For example, a polypeptide which is approximately 6-12, preferably approx. 8 amino acids in length, may contain an epitope which, after coupling to a support, serves for the production of specific polyclonal or monoclonal antibodies (see, in this context for example US 5,656,435). Polypeptides with a length of at least approx. 65 amino acids can also serve directly for the preparation of polyclonal or monoclonal antibodies, without support.

The term "functional variants" for the purposes of the present invention is to be understood as meaning polypeptides which are functionally related to the peptide according to the invention, i.e. which exhibit glucanase activity. Variants are also understood as meaning allelic variants or polypeptides which may be derived from various yeasts/yeast strains or other infective agents such as dermatophytes, molds (in accordance with the DHS system).

In the wider sense, they are also to be understood as meaning polypeptides which have a sequence homology, in particular a sequence identity of approx. 70%, preferably of approx. 80%, in particular of approx.

90%, especially of approx. 95%, with the polypeptide with the amino acid sequence as shown in Figure 2. This term also includes deletion of the polypeptide in the region of approx. 1 - 60, preferably of approx. 1 - 30, in particular of approx. 1 - 15, especially of approx. 1 – 5, amino acids. For example, the first amino acid methionine may be absent without this considerably altering the function of the polypeptide. Besides, it also includes fusion proteins which contain the above-described polypeptides according to the invention, it being possible for the fusion proteins themselves to have a glucanase function or only to acquire the specific function after the fusion portion has been split off. Especially, these include fusion proteins which contain in particular non-human sequences of approx. 1 - 200, preferably of approx. 1 - 150, in particular of approx. 1 - 100, especially of approx. 1 – 50, amino acids. Examples of non-human peptide sequences are prokaryotic peptide sequences, for example from the E. coli galactosidase, or a so-called histidine tag, for example a Met-Ala-His_6 tag. A fusion protein with a so-called histidine tag is particularly advantageously suited for the purification of the expressed protein through metal ion-containing columns, for example through an Ni^{2+}-NTA column. "NTA" indicates the chelator nitrilotriacetic acid (Qiagen GmbH, Hilden). In this respect, the invention also encompasses those polypeptides according to the invention which are masked in the sense of a proprotein or, in the wider sense, as pre-drug.

The portions of the polypeptides according to the invention represent, for example, epitopes which can be recognized specifically by antibodies.

The polypeptides according to the invention are prepared by methods generally known to the skilled worker, for example by expression of the nucleic acid according to the invention in a suitable expression system such as already described above. Host cells which are suitable for the preparation of correctly processed, and thus bioactive, protein toxins are exclusively eukaryotic organisms, preferably the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*.

In particular the abovementioned portions of the polypeptide can also be synthesized with the aid of traditional peptide synthesis (Merrifield technique). They are particularly suitable for obtaining antisera, with the aid of which suitable gene expression libraries can be screened in order to

arrive at further functional variants of the polypeptide according to the invention.

A further subject matter of the present invention thus relates to a process for the preparation of a polypeptide according to the invention, wherein a nucleic acid according to the invention is expressed in a suitable host cell and, if appropriate, isolated.

Very especially preferred is the fission yeast *Schizosaccharomyces pombe*, since this yeast is WICALTIN- and ZYGOCIN-resistant by nature and has already been repeatedly employed successfully for the heterologous expression of foreign proteins [Giga-Hama & Kumagai (1997), in "Foreign Gene Expression in Fission Yeast: *Schizosaccharomyces pombe*", Springer Verlag]. As exemplified in Example 11, the toxin-encoding nucleic acids in accordance with **SEQ ID No 1** and **SEQ ID No 2** can be cloned, for example, into the *S. pombe* vector pREP1 [Maundrell (1990), J. Biol. Chem. 265:10857-10864], in which they are under the transcriptional control of the thiamine-regulated *nmt1* promoter of the fission yeast [*nmt* = '*n*o *m*essage with *thiamine*']. Yeasts which are transformed with such a vector express the foreign gene in question as a function of the respective thiamine concentration in the culture medium of the yeast. If desired, this allows the yeast growth phase to be separated in time from the phase during which the foreign protein is produced, so that in principle it is also possible to express proteins which are toxic to the yeast. To allow simultaneous secretion and thus a considerably easier purification of the toxins WICALTIN and ZYGOCIN, which are expressed heterologously in *S. pombe*, we already have constructed an expression/secretion vector [Vector pTZα/γ; see Example 11] which contains secretion and processing signals of the viral K28 preprotoxin gene [Schmitt & Tipper, 1995] and thus allows effective secretion of the respective foreign protein which is arranged downstream in-frame.

Another subject matter of the present invention also relates to antibodies which specifically react with the polypeptide according to the invention, it being possible for the abovementioned portions of the polypeptide either to be immunogenic themselves or to be made immunogenic, or improved in their immunogenicity by coupling to suitable carriers such as, for example, bovine serum albumin.

The antibodies are either polyclonal or monoclonal. The preparation, which also constitutes a subject matter of the present invention, is carried out for example by generally customary methods by immunizing a mammal, for example a rabbit, with the polypeptide according to the invention or the abovementioned portions thereof, if appropriate in the presence of, for example, Freund's adjuvant and/or aluminum hydroxide gels (see, for example, Diamond, B.A. et al. (1981) The New England Journal of Medicine, 1344). The polyclonal antibodies formed in the animal owing to an immunological reaction can subsequently readily be isolated from the blood by generally customary methods and purified, for example by column chromatography. It is preferred to subject the antibodies to an affinity purification, where for example the antigen in question (ZYGOCIN or WICALTIN) is coupled covalently with a CnBr-activated Sepharose matrix which is freely available and employed for purifying the antibodies, which are in each case toxin-specific.

Monoclonal antibodies can be prepared for example by the known methods of Winter & Milstein (Winter, G. & Milstein, C. (1991) Nature, 349, 293).

Another subject matter of the present invention is a drug product which comprises the nucleic acids according to the invention or the polypeptides according to the invention (individually or in combination) and, if appropriate, suitable additives or adjuvants, and a process for the preparation of a drug product for treating mycoses such as superficial, cutaneous and subcutaneously dermatomycoses, mycoses of the mucous membranes and systemic mycoses, especially preferably Candida mycoses, wherein a nucleic acid according to the invention or a polypeptide according to the invention is formulated together with pharmaceutically acceptable additives and/or adjuvants.

Example 12 exemplifies that the toxin WICALTIN, which is produced by the strain DSM 12865 and purified, even has a markedly more potent toxicity to yeasts than the topical antimycotics clotrimazole and miconazole which were tested for comparison reasons and are frequently employed in the therapy of mycoses.

The invention thus also relates to a drug product in the above sense, comprising an antimycotic or a protein toxin obtainable from DSM 12864

and/or DSM 12865 and/or antimycotically active polypeptides according to the invention.

Suitable for use in human gene therapy is especially a drug product which comprises the nucleic acid according to the invention in naked form or in the form of one of the above-described vectors which are effective in gene therapy or in the form of complexes with liposomes.

Examples of suitable additives and/or adjuvants are a physiological saline, stabilizers, proteinase inhibitors, nuclease inhibitors and the like.

Another subject matter of the present invention is also a diagnostic comprising a nucleic acid according to the invention, a polypeptide according to the invention or an antibody according to the invention and, if appropriate, suitable additives and/or adjuvants, and a process for the preparation of a diagnostic for diagnosing mycoses such as superficial, cutaneous and subcutaneous dermatomycoses, mycoses of the mucous membranes and systemic mycoses, especially preferably Candida mycoses, wherein a nucleic acid according to the invention, a polypeptide according to the invention or antibodies according to the invention are combined with suitable additives and/or adjuvants.

For example, a diagnostic based on the polymerase chain reaction (PCR diagnostic for example in accordance with EP-0200362) or on a Northern and/or Southern Blot, as described in greater detail in Example 13, can be prepared in accordance with the present invention with the aid of the nucleic acid according to the invention. These tests are based on the specific hybridization of the nucleic acid according to the invention with the complementary strand, conventionally the corresponding mRNA. The nucleic acid according to the invention can also be modified, as described, for example, in EP0063879. Preferably, a DNA fragment according to the invention is labeled by generally known methods by means of suitable reagents, for example radiolabeled with α-P^{32}-dATP or provided with a non-radioactive biotin label, and incubated with isolated RNA which has preferably been bound to suitable membranes, for example of cellulose or nylon. In addition, it is advantageous to separate the isolated RNA prior to hybridization and binding to a membrane according to size, for example by means of agarose gel electrophoresis. If the amount of test RNA from each

tissue sample is identical, the amount of mRNA which has been labeled specifically by the probe can thus be determined.

Another diagnostic comprises the polypeptide according to the invention or the immunogenic portions thereof which have been described above in greater detail. The polypeptide or portions thereof, which are preferably bound to a solid phase, for example of nitrocellulose or nylon, can be contacted in vitro for example with the body fluid to be examined, such as blood, in order to be able to react with, for example, antibodies. The antibody/peptide complex can subsequently be detected, for example with the aid of labeled anti-human-IgG or anti-human-IgM antibodies. The label is, for example, an enzyme such as peroxidase which catalyzes a color reaction. Presence and quantity of autoimmune antibodies can thus be detected easily and rapidly via the color reaction.

Another diagnostic comprises the antibodies according to the invention themselves. These antibodies allow for example a human tissue sample to be examined easily and rapidly for the presence of the polypeptide in question. In this case, the antibodies according to the invention are labeled, for example with an enzyme as already described above. The specific antibody/peptide complex can thus be detected easily and equally rapidly via an enzymatic color reaction.

Another subject matter of the invention relates to a fungicide which comprises the nucleic acids according to the invention and/or the polypeptides according to the invention, singly or in combination, and, if appropriate, suitable additives or adjuvants, and to a process for the preparation of a fungicide for controlling harmful yeasts and harmful fungi, wherein a nucleic acid according to the invention or a polypeptide according to the invention is formulated together with agriculturally acceptable additives and/or adjuvants.

As already described, a transgenic plant is generated in a preferred embodiment which expresses the protein toxin according to the invention. The invention therefore also relates to plant cells and inherently to the transgenic plant as such comprising the polypeptides and/or protein toxins according to the invention.

Another subject of the present invention also relates to an assay for identifying functional interactors such as, for example, inhibitors or stimulators comprising a nucleic acid according to the invention, a polypeptide according to the invention or the antibodies according to the invention and, if appropriate, suitable additives and/or adjuvants.

A suitable assay for identifying functional interactors, in particular those which interact in the sensitive yeast cell with the protein toxin ZYGOCIN in accordance with **SEQ ID No 2** is, for example, the two-hybrid system (Fields, S. & Sternglanz, R. (1994) Trends in Genetics, 10, 286). In this assay, a cell, for example a yeast cell, is transformed or transfected with one or more expression vectors which express a fusion protein comprising the polypeptide according to the invention and a DNA binding domain of a known protein, for example Gal4 or LexA from E. coli, and/or express a fusion protein comprising an unknown polypeptide and a transcription activation domain, for example of Gal4, Herpes virus VP16 or B42. In addition, the cell comprises a reporter gene, for example the E. coli LacZ gene, green fluorescence protein or the yeast amino acid biosynthesis genes His3 or Leu2, which reporter gene is controlled by regulatory sequences such as, for example, the lexA promoter/operator or by a yeast upstream activation sequence (UAS). The unknown polypeptide is encoded for example by a DNA fragment which originates from a gene library, for example a human gene library. Usually, a cDNA gene library is first produced in yeast with the aid of the expression vectors described, so that the assay can be carried out immediately thereafter.

In a yeast expression vector, for example, the nucleic acid according to the invention is cloned in functional unit on the nucleic acid encoding the LexA DNA binding domain so that a fusion protein of the polypeptide according to the invention and the LexA DNA binding domain is expressed in the transformed yeast. In another yeast expression vector, cDNA fragments of a cDNA gene library are cloned in functional unit on the nucleic acid encoding the Gal4 transcription activation domain, so that a fusion protein of an unknown polypeptide and the Gal4 transcription activation domain is expressed in the transformed yeast. The yeast, for example $Leu2^-$, which is transformed with both expression vectors additionally comprises a nucleic acid which encodes Leu2 and which is controlled by the LexA promoter/operator. In the event of functional interaction between the polypeptide according to the invention and the unknown polypeptide, the

Gal4 transcription activation domain binds to the LexA promoter/operator via the LexA DNA binding domain, thus activating the LexA promoter/operator and expressing the Leu2 gene. As a consequence, the $Leu2^-$ yeast is capable of growth on minimal medium which does not contain leucin.

When using the LacZ or green fluorescence protein reporter gene instead of an amino acid biosynthesis gene, transcriptional activation can be detected by the formation of colonies which fluoresce blue or green. However, the blue or green fluorescent stain can also be quantified easily in a spectrophotometer, for example at 585 nm in the case of blue staining.

In this manner, expression gene libraries can be screened easily and rapidly for polypeptides with interact with the polypeptide according to the invention. The novel polypeptides which have been found can subsequently be isolated, and characterized further.

Another possible use of the two-hybrid system consists in influencing the interaction between the polypeptide according to the invention and a known or unknown polypeptide by other substances, such as, for example, chemicals. This also allows novel valuable active ingredients to be found which can be synthesized chemically and employed as therapeutics. The present invention is therefore not only intended for a method of finding polypeptide-like interactors, but also extends to a method of finding substances which are capable of interacting with the above-described protein/protein complex. Such peptide-like, and chemical, interactors are therefore termed functional interactors for the purposes of the present invention which can have an inhibitory or stimulatory action.

Another subject matter of the invention relates to a process for the preparation of protein toxins by culturing and secreting the protein toxins into a medium which constitutes a synthetic culture medium (BAVC medium), which considerably facilitates chromatographic purification of the secreted toxins, for example by means of ultrafiltration and cation exchange chromatography and/or affinity chromatography on laminerin-Sepharose and/or mannoprotein-Sepharose [cf. Example 1 and Appendix to the Examples]. In the case of WICALTIN, which is produced and secreted by strain DSM 12865, the toxin production can be increased further by supplementing the medium with an addition of the plant-derived (and readily available) β-1,3-D-glucan laminarin in a final concentration of 1%.

As exemplified in Example 14, the addition of laminarin to the culture medium leads to induction of the WICALTIN production, and Northern analyses allowed this to be attributed to transcriptional induction.

Synthetic B medium can be employed to produce the toxin ZYGOCIN, which is secreted by DSM 12864 [cf. Radler et al., 1993].

The examples which follow are intended to illustrate the invention without restricting the invention to these examples.

Examples

Example 1:

Isolation, concentration and purification of the anti-*Candida* toxin WICALTIN from culture supernatants of the killer yeast *W. californica* strain 3/57 (DSM 12865)

In the agar diffusion test on Methylene Blue agar against sensitive yeasts, the killer toxin WICALTIN secreted by the killer yeast *W. californica* 3/57 shows an optimal inhibitory action at pH 4.7 and 20°C. In synthetic liquid medium, the killer yeast *W. californica* strain 3/57 shows maximum toxin production when grown in BAVC medium (pH 4.7). For the purposes of toxin concentration, the killer yeast was first incubated for 24 hours in 5 ml of YEPD medium at 30°C with shaking, then all of it was transferred into 200 ml of BAVC medium and again cultured for 48 hours at 20°C on the shaker (140 rpm). Four main cultures of 2.5 l BAVC medium each (pH 4.7 in 5-l Erlenmeyer flasks) were inoculated with the second preculture (1% inoculum) and incubated for five days at 20°C with gentle shaking (60 rpm). To concentrate the secreted killer toxin, the cell-free culture supernatant was concentrated 200-fold to a volume of 50 ml by means of ultrafiltration on polysulfonic acid membranes ('EasyFlow' [Fa. Sartorius]; exclusion limit 10 kDa) at +4°C and a pressure of 1 bar. To remove low-molecular-weight compounds and to desalinify the concentrate thus obtained, the toxin was dialyzed overnight at +4°C in a dialysis tube (exclusion limit 10-20 kDa) against 5 mM citrate/phosphate buffer (pH 4.7). To store the toxin concentrate, the dialyzed product was filter-sterilized through a 0.2-µm membrane and frozen at -20°C in 1-ml aliquots.

The toxin activity was detected and standardized in an agar diffusion test on Methylene Blue agar (MBA; pH 4.7) against the sensitive indicator yeast *Saccharomyces cerevisiae* 192.2d. To this end, logarithmic dilution steps of the toxin concentrate were prepared in 0.1 M citrate/phosphate buffer (pH 4.7), and 100-µl aliquots were pipetted into wells (well diameter 9 mm)

which had previously been punched into an MBA plate inoculated with the sensitive indicator yeast (2×10^5 cells/ml). After the plates had been incubated for three days at 20°C, the inhibition zones, which were clearly visible, were measured. It emerged that a linear relationship exists between the inhibition zone diameter and the logarithm of the toxin concentration. An arbitrary toxin activity of $1x10^4$ units/ml was assigned to an inhibition zone diameter of 20 mm (corrected by the well diameter).

The concentrated WICALTIN was purified either by cation exchange chromatography on Bioscale-S (FPLC) or by affinity chromatography on an epoxy-activated Sepharose-6B matrix (Pharmacia) to which the plant-derived β-1,6-D-glucan pustulan had previously been coupled. The toxin preparation (**Table 1**), which had thus been enriched 625-fold in its specific activity, was gel-electrophoretically pure and, after SDS-PAGE (in a 10-22% gradient gel), only showed a single band at approximately 37 kDa, which was detectable both with Coomassie Blue (protein stain) and periodic acid - Schiff stain (PAS; carbohydrate stain). The positive PAS stain suggests a potential N-glycosylation of the anti-*Candida* toxin WICALTIN. Treatment of the purified toxin with endoglycosidase-H confirmed that WICALTIN has an N-glycosidically linked carbohydrate moiety of approximately 3 kDa, whose size, in yeast, also suggests a single N-glycosylation site in the protein toxin. Since the deglycosylated WICALTIN shows markedly restricted toxicity, it can be deduced that the carbohydrate moiety of WICALTIN is probably necessary for binding to the sensitive target cell and thus indirectly affects the bioactivity of the toxin.

Table 1: Concentration of WICALTIN from the culture supernatant of the killer yeast *Williopsis californica* [UF, Ultrafiltration]

Preparation	Volume [ml]	Total protein [mg]	Total toxin activity [E]	Specific toxin activity [E/mg]	Activity yield [%]	Purification factor
Culture supernatant	10,000	24,600	7.9×10^5	3.2×10^1	100	1
UF retentate	50	162	6.3×10^5	3.9×10^3	80	122
Lyophilized dialysate	25	45.8	3.1×10^5	6.8×10^3	39	213
Bio-Scale S (cation exchange)	64	1.28	2.5×10^4	2.0×10^4	3.2	625

Example 2:

Determination of the NH_2-terminal amino acid sequence of WICALTIN, and detection of an enzymatic β-1,3-glucanase activity

The first ten amino acids were determined by sequencing the N-terminal amino acids of the purified killer toxin. As can be seen from **Figure 1**, the N-terminus of WICALTIN shows significant homology to the amino terminus of the endo-β-1,3-glucanase encoded by the *BGL2* gene of the yeast *Saccharomyces cerevisiae*.

Since the homology of WICALTIN and Bgl2 had been determined, the possibility of detecting a glucanase activity in the unpurified toxin concentrate and in the purified toxin preparation was investigated. In the WICALTIN preparations, a pronounced β-1,3-D-glucanase activity was detected both in the enzyme assay with the β-1,3-D-glucan laminarin as substrate and in the fluorescence assay with 4-methyl-umbelliferyl-β-D-glucoside (MUC) as substrate; the β-1,6-D-glucan pustulan, which was also tested, was not hydrolyzed by WICALTIN.

Example 3:

Survival rates of WICALTIN-treated yeast cells in the presence and absence of cell wall glucans: competition analyses

Sensitive yeast cells of strain *S. cerevisiae* 192.2d which are grown in YEPD liquid medium (pH 4.7) at 20°C in the presence of $1x10^5$ U/ml purified WICALTIN showed the kill kinetics shown in **Figure 2**. Addition of the plant-derived β-1,6-D-glucan pustulan allowed the survival rate of toxin-treated yeast cells to be increased significantly and fully reversed WICALTIN toxicity when added at concentrations of 10 mg/ml. As opposed to pustulan, the β-1,3-D-glucan laminarin was not capable of increasing the survival rate of the toxin-treated yeasts (**Figure 2**).

The findings shown therefore allow the conclusion that the action of WICALTIN requires a binding to β-1,6-D-glucans which act as primary docking sites (toxin receptors) of the yeast cell wall. In agreement with this finding, it was shown that yeasts with a deletion in the chromosomal *KRE1* gene locus show toxin resistance, but regain toxin sensitivity when retransformed with an episomal vector which carries *KRE1* (**Figure 3**). The toxin resistance in *kre1* mutants is based on a markedly reduced β-1,6-D-glucan content and thus a reduced toxin binding to the yeast cell surface, which is required for the lethal action.

Example 4:

Spectra of action and kill spectra of WICALTIN

In the agar diffusion test, the purified *W. californica* toxin WICALTIN exhibited a pronounced toxicity against the yeasts shown in **Table 2**. With the exception of three strains of the yeast *Candida krusei,* all the 22 clinical patient isolates which were tested and all the other control strains of *Candida* species which are pathogenic for man were destroyed by WICALTIN in a highly efficient manner. With 14 toxin-sensitive yeast species from a total of 10 different genera, WICALTIN shows a spectrum of action which is unusually broad for killer toxins.

Table 2: Spectrum of action of WICALTIN on pathogenic and apathogenic yeasts of different genera. All strains were tested in the agar diffusion test (MBA; pH 4.7) against purified WICALTIN. The toxin activity applied was $1x10^6$ U/ml. The strain *C. tropicalis* (patient number 541965) was obtained from the Department of Medical Microbiology and Hygiene of the University Hospital Mainz.

Yeast strain	Pheno-type	Inhibitory zone diameter [mm]
Candida albicans ATCC 10231	S	11
C. glabrata NCYC 388	S	12
C. krusei 185	R	0
C. tropicalis patient number 541965	S	11
Debaryomyces hansenii 223	S	16
Hanseniaspora uvarum ATCC 64295	R	0
Hasegawaea japonica var. Versatilis 191	R	0
Kluyveromyces lactis CBS 2359/152	S	22
K. marxianus C 8,1	R	0
Metschnikowia pulcherrima K/3I B6	S	8
Pichia anomala 245	S	17
P. farinosa 258	R	0
P. jadinii 251	S	6
P. kluyveri ATCC 64301	R	0
P. membranaefaciens NCYC 333	R	0
Saccharomyces cerevisiae		
192.2d	S	30
381	S	23
ATCC 42017 (K1 superkiller)	S	19
NCYC 738 (K2 killer)	S	14
452 (= NCYC 1006)	S	16
Saccharomycodes ludwigii 240	R	0
Schizosaccharomyces pombe CBS1042	R	0
Sporothrix **spec.** 1129	S	11
Torulospora delbrueckii 208	S	18
T. pretoriensis 186	S	10
Yarrowia lipolytica 271	S	8
Zygosaccharomyces bailii 412	S	23

Example 5:

Cloning, sequencing and molecular characterization of the WICALTIN-encoding *WCT* gene of the yeast *W. californica* strain 3/57 (DSM 12865)

Starting with the N-terminal amino acid sequence of WICALTIN, specific DNA oligonucleotides were generated which led to the identification and cloning, and to the characterization of the molecular biology of the toxin

gene *WCT*, which is located chromosomally. The DNA sequence of *WCT* (**SEQ ID No. 1**) shows a single open reading frame which encodes a potentially N-glycosylated protein of 309 amino acids and a calculated molecular weight of 34,017 Da. Studies into the action of the *WCT* encoded killer toxin showed that WICALTIN is a glycoprotein which is extremely toxic to yeasts and whose primary targets are the cell wall β-1,3-D-glucans found in yeasts. Its selective toxicity to yeasts and fungi is based on WICALTIN destroying the cell wall structure and/or integrity in the sensitive target cell, and thus attacking yeasts where they are most sensitive, finally killing them.

Example 6:

Concentration and purification of the viral toxin ZYGOCIN from culture supernatants of the killer yeast *Z. bailii* strain 412 (DSM 12864)

The virus-encoded killer toxin ZYGOCIN of the yeast *Z. bailii* strain 412 was isolated from the culture supernatant of the killer yeast by the method described by Radler *et al.* (1993), concentrated by ultrafiltration and finally purified by affinity chromatography. The one-step purification of ZYGOCIN, which was developed in the present study, exploits the natural affinity of the toxin to cell wall mannoproteins of sensitive yeasts. The mannoprotein, which was isolated and partially purified from *S. cerevisiae* strain 192.2d by a method described by Schmitt & Radler (1997), was coupled covalently to an epoxy-activated Sepharose-6B matrix (Pharmacia) and employed by means of FPLC for purifying the toxin by column chromatography. Following SDS-PAGE, the highly bioactive ZYGOCIN which had been purified in this manner showed a single protein band with an apparent molecular weight of approximately 10 kDa (**Figure 4**).

Example 7:

Spectrum of action and kill spectrum of ZYGOCIN

The spectrum of action of viral ZYGOCIN of the yeast *Z. bailii* 412 (DSM 12864) which was determined in the agar diffusion test comprises pathogenic and apathogenic yeast genera, amongst which *Candida albicans* and *Sporothrix schenkii* are important pathogens in humans and animals, and *Ustilago maydis* and *Debaryomyces hansenii* are important harmful yeasts in agriculture and in the food sector (**Tab. 3**).

Table 3: Spectrum of action of ZYGOCIN to pathogenic and apathogenic yeasts of different genera. All strains were tested in the agar diffusion test

(MBA; pH 4.5) against the ZYGOCIN preparation with an activity of $1x10^4$ U/ml.

ZYGOCIN-sensitive yeasts	Relative degree of sensitivity
Saccharomyces cerevisiae	++
Candida albicans	+
Candida krusei	++
Candida glabrata	++
Candida vinii	+
Hanseniaspora uvarum	++
Kluyveromyces marxianus	+
Metschnikowia pulcherrima	+
Ustilago maydis	++
Debaryomyces hansenii	++
Pichia anomala	++
Pichia jadinii	+
Pichia membranefaciens	+
Yarrowia lipolytica	+
Zygosaccharomyces rouxii	++

Example 8:

Cloning and sequencing of the ZYGOCIN-encoding *ZBT* gene (ZBT) of the yeast *Z. bailii* strain 412 (DSM 12864)

The cDNA of the toxin-encoding double-stranded RNA genome of the killer yeast *Z. bailii* 412 was synthetized out by a method similar to that described by Schmitt (1995) using purified M-dsRNA which had been denatured with methylmercury hydroxide as template and various hexanucleotides as primers. After ligation into the EcoRI-restricted vector pUC18, transformation in *E. coli* and isolation of the recombinant plasmids identified, several cDNA clones were isolated and sequenced. The cDNA sequence of the ZYGOCIN-encoding reading frame (**SEQ ID No 2**) contains the genetic information for a precursor protein (pro-toxin) of 238 amino acids, which carries a potential Kex2-endopeptidase cleavage site in the amino acid position RR^{139}. The bioactive ZYGOCIN, whose molecular weight (10 kDa; 99 amino acids) and N-terminal amino acid sequence exactly agree with the data determined for the purified ZYGOCIN, is formed

by Kex2-mediated pro-ZYGOCIN-processing, which takes place *in vivo* during the late Golgi stage.

Owing to the toxicity of ZYGOCIN, heterologous expression of the *ZBT*-cDNA in the yeast *S. cerevisiae* resulted in the transformed yeasts killing themselves by their own toxin. A future aim will be heterologous ZYGOCIN expression in the toxin-resistant fission yeast *Schizosaccharomyces pombe* since, as has already been demonstrated by way of example of the viral K28 toxin, the fission yeast is particularly suitable for expressing or secreting foreign proteins.

Example 9:

Expression of the toxin genes *WCT* and *ZBT* in transgenenic plants

Since the above-described killer toxins WICALTIN and ZYGOCIN have a broad spectrum of action and also destroy plant-pathogenic yeasts and fungi, it should be possible to construct transgenic plants which show resistance to, for example, an infection with the maize pathogen *Ustilago maydis*. Similar experiments have already been carried out on tobacco plants which, owing to heterologous expression of the *U. maydis* killer toxin KP4, which is encoded virally in nature, were capable of secreting the killer toxin in question and which thus generated a specific protection from infection with certain phytopathogenic *U. maydis* strains (Park et al., 1996; Kinal et al., 1995; Bevan, 1984). Starting with commercially available transformation systems based on modified derivatives of the natural *Agrobacterium tumefaciens* Ti-Plasmid, it is possible to clone the toxin genes *WCT* and *ZBT*, which we have cloned, into so-called bidirectional pBI vectors (CLONTECH) and to use them for the generation of transgenic plants. To this end, the toxin genes in question, *WCT* and *ZBT*, are placed under the transcriptional control of the strong cauliflower mosaic virus promoter (CaMV-P). The construction of the vectors to be constructed is shown schematically in **Figure 5**.

Example 10:

Heterologous expression of the WICALTIN-encoding *WCT* gene of the yeast *W. californica* 3/57 (DSM 12865) in *S. cerevisiae*

To express the *WCT* gene heterologously in the yeast *S. cerevisiae*, the WICALTIN-encoding *WCT* gene was cloned as a 930 bp *Eco*RI/*Sma*I fragment into the 2μ vector pYX242, which is generally available. The resulting vector pSTH2 (**Figure 6**) comprises the toxin gene under the transcriptional control of the yeast's triose phosphate isomerase promoter

(*TPI*) and thus allows the constitutive expression of WICALTIN after transformation into yeast (*S. cerevisiae*). An analysis by gel electrophoresis of the culture supernatant of the yeast transformants obtained in this manner showed that the recombinant WICALTIN is secreted into the external medium and has a β-1,3-D-glucanase activity which corresponds to that of the homologous WICALTIN (from wild-type strain DSM 12865); (**Figure 6**).

Example 11:

Experiments on the heterologous expression of WICALTIN and ZYGOCIN in the fission yeast *Schizosaccharomyces pombe*

Since the fission yeast shows resistance to WICALTIN and ZYGOCIN, both as intact cell and as a cell-wall-free spheroplast, it is suitable as host for the heterologous expression of the toxins in question. To ensure that the recombinant toxins are not only expressed by the fission yeast, but simultaneously also fed into the intracellular secretional pathway and thus secreted into the external medium, a vector was constructed (pTZα/γ; **Figure 7**) which carries a secretion and processing signal (S/P) which is functional in *S. pombe* and which is derived from the cDNA of the viral K28-preprotoxin gene of the yeast *S. cerevisiae* [c.f. Schmitt, 1995; Schmitt & Tipper, 1995]. The secretion and processing signal ensures that the foreign protein, which is arranged downstream in-frame, is imported in the fission yeast into the lumen of the endoplasmatic reticulum and thus fed into the secretional pathway of the yeast. The Kex2p cleavage site which is present on the C-terminus of the S/P-region causes the desired foreign protein to be cleaved off from its intracellular transport vehicle in a late Golgi compartment by the yeast's Kex2p-endopeptidase, and it can finally be secreted into the external medium as bioactive protein (ZYGOCIN and/or WICALTIN).

Example 12:

Comparative bioactivities of purified WICALTIN and the topical antimycotics clotrimazole and miconazole

Since purified WICALTIN has a broad spectrum of action and also efficiently kills yeasts and/or fungi which are pathogenic for man it is important as a candidate antimycotic. Thus, comparative studies were carried out on WICALTIN with the topical antimycotics clotrimazole and miconazole, which are currently widely employed. First, the toxic effect of clotrimazole and miconazole against *Sporothrix* spec. as indicator yeast

was tested in the MBA agar diffusion test. To this end, clotrimazole was dissolved in ethanol (96%) in a concentration of 10 mg/ml; this stock solution was diluted with ddH_20 and employed in the MBA test in concentrations of 0.1 to 10 mg/ml per 100 µl. When an amount of 10-50 µg of clotrimazole was employed, the inhibitory zone diameters were between 12 and 32 mm. Miconazole was used to prepare a stock solution of 100 µg/ml in DMSO (100%), and this was tested in the same manner as clotrimazole in the MBA test for bioactivity against *Sporothrix* spec. In the bioassay, the use of 0.08-0.3 µg of miconazole resulted in inhibitory zones between 22 and 36 mm. The bioactivities of 10 µg of clotrimazole and 0.08 µg of miconazole thus correspond to the toxicity of 2 µg of purified WICALTIN. A comparison based on the molecular weight of the three test compounds shows that even at a concentration of 0.07 pmol WICALTIN shows the same activity as 0.2 pmol miconazole and 29 pmol clotrimazole; WICALTIN is thus an extremely potent antimycotic (**Figure 8**).

Example 13:

Detection of the WICALTIN-encoding *WCT* gene of the yeast *W. californica* 3/57 (DSM 12865) by Southern hybridization with a gene-specific DNA probe.

To prove that the nucleic acid in accordance with **SEQ ID No. 1** can be employed to generate a WICALTIN-specific DNA probe for a subsequent Southern hybridization, a DIG-labeled 930 bp DNA probe was employed for detecting the *WCT* gene which had been cloned into the vector pSTH1. The constructed vector pSTH1 represents a derivative of the procaryotic cloning vector pBR322, which is generally available.

The agarose gel electrophoresis shown in **Figure 9** and the corresponding Southern blot show beyond doubt that the nucleic acid probe can be used to detect the WICALTIN-encoding *WCT* gene.

Example 14:

Northern blot analysis for detecting a transcriptional induction of the WICALTIN-encoding *WCT* gene of the yeast *Williopsis californica* 3/57 (DSM 12865) by β-1,3-D-glucans

To detect a β-1,3-D-glucan-induced *WCT* transcription, the yeast strain DSM 12865 was grown in 300 ml of BAVC medium or in BAVC medium supplemented with 0.03% of the plant-derived β-1,3-D-glucan laminarin for 48 hours at 20°C and gentle shaking (60 rpm) and, after different intervals, used for preparing total RNA. Before the RNA isolation, all samples (10 ml)

were brought to an identical cell density of 1.8 x 10^8 cells/ml and separated by electrophoresis in denaturing agarose formaldehyde gels. As can be seen from **Figure 10**, a size of 1100 bases was detected for the *WCT* transcript both under noninducing conditions (BAVC medium without supplementation) and in the laminarin supplemented BAVC medium. Without addition of glucan, maximum *WCT* expression was achieved toward the end of the exponential growth phase (after 19 hours); the hybridization signals, which turn markedly weaker in the stationary growth phase, suggest a reduced transcription. Under inducing culture conditions (in the presence of laminarin), the *WCT* transcript shows a much higher intensity after 10 hours than in the noninduced culture, allowing the conclusion that transcription of the WICALTIN-encoding *WCT* gene can be induced by addition of β-1,3-D-glucans.

Appendix to the examples:

Media and solutions used in the examples:

a.) BAVC medium

glucose	50 g/l
D,L-malate	20 g/l
trisodium citrate	0.5 g/l
$(NH_4)_2SO_4$	1.5 g/l
$MgSO_4$	1.0 g/l
$CaCl_2$	0.5 g/l
myo-inositol	0.04 g/l
amino acid stock solution (10 x)	200 ml/l
trace element stock solution (100 x)	10 ml/l
vitamin stock solution (100 x)	20 ml/l

with:

b.) Amino acid stock solution (10 x)

alanine	0.75 g/l
arginine monohydrochloride	3.5 g/l
aspartic acid	0.5 g/l
glutamic acid	3 g/l
histidinium monochloride	0.2 g/l

methionine	0.4 g/l
serine	0.5 g/l
threonine	2 g/l
tryptophan	0.4 g/l

c.) Trace element stock solution (100 x)

boric acid	200 mg/l
$FeCl_3 \times 6\ H_2O$	200 mg/l
$ZnSO_4 \times 7\ H_2O$	200 mg/l
$AlCl_3$	200 mg/l
$CuSO_4 \times 5\ H_2O$	100 mg/l
$Na_2MoO_4 \times 2\ H_2O$	100 mg/l
$Li_2SO_4 \times H_2O$	100 mg/l
KI	100 mg/l
potassium hydrogen tartrate	2 g/l

d.) Vitamin stock solution (100 x)

4-aminobenzoic acid	20 mg/l
biotin	2 mg/l
folic acid	2 mg/l
nicotinic acid	100 mg/l
pyridoxin hydrochloride	100 mg/l
riboflavin	50 mg/l
thiamineium dichloride	50 mg/l
calcium D-pantothenate	100 mg/l

Biotin: dissolve in 5 g KH_2PO_4/50 ml distilled water.
Folic acid: dissolve in 50 ml of distilled water with addition of a few drops of dilute NaOH.
Riboflavin: dissolve in 500 ml of distilled water and a few drops of HCl with heating.
The remaining vitamins can be dissolved in a little distilled water.

The pH of the BAVC medium was brought to pH 4.7 by addition of KOH. The glucose and stock solutions were sterilized separately. Amino acid, vitamin and trace element stock solutions were sterilized for 20 minutes at

100°C with the valve open and were then added to the autoclaved BAVC medium.

Figures and the most important sequences

SEQ ID No. 1: DNA sequence and deduced amino acid sequence of the *WCT*-encoded protein toxin WICALTIN of the yeast *Williopsis californica* strain 3/57.

SEQ ID No. 2: cDNA sequence and deduced amino acid sequence of the *ZBT*-encoded protein toxin ZYGOCIN of the yeast *Z. bailii*

Figure 1: N-terminal amino acid sequences of the *W. californica* toxin WICALTIN and of the endo-β-1,3-glucanase Bgl2 of the yeast *S. cerevisiae*. The only deviation of the subsequences, which are otherwise identical, is shown in bold (Bgl2p sequence after Klebl & Tanner, 1989)

Figure 2: Kill kinetics of WICALTIN-treated cells of the sensitive yeast *S. cerevisiae* 192.2d in the presence (2a) and absence (2b) of the β-D-glucans laminarin (L) and pustulan (P). The toxin employed had a total activity of 4.0×10^5 U/ml at a specific activity of 4.2×10^5 U/mg protein.

Figure 3 (a,b,c,d): Agar diffusion test for detecting a WICALTIN sensitivity/resistance in $Kre1^+$ and $Kre1^-$ strains of the yeast *S. cerevisiae*. Transformation of the WICALTIN-resistant *kre1* zero-mutant *S. cerevisiae* SEY6210[Δ*kre1*] with the *KRE1*-carrying vector pPGK[*KRE1*] fully restores the WICALTIN sensitivity.

Figure 4: (A) Analysis by gel electrophoresis (SDS-PAGE) of the ZYGOCIN produced and secreted by the yeast *Z. bailii* strain 412 (DSM 12864) after affinity chromatography on mannoprotein-Sepharose. (B) Agar diffusion test for detecting the bioactivity of the purified killer toxin ZYGOCIN.

Figure 5: Schematic construction of a *ZBT*- or *WCT*-carrying expression vector for the generation of transgenic plants.
[Key: RB, LB: right and left border sequences of the natural Ti-plasmid of *Agrobacterium tumefaciens*; CaMV-P: cauliflower mosaic virus 35S promoter; NOS-P, NOS-T: nopalin synthase transcription promoter and

terminator; kan^R: *Streptococcus* kanamycin resistance gene for selection in *E. coli*; NPT-II: neomycin phosphotransferase gene from transposon Tn5 for selection in the plant].

Figure 6: (A) Partial restriction map of the episomal vector pSTH2 for the heterologous expression of the WICALTIN-encoding toxin gene *WCT* in the yeast *Saccharomyces cerevisiae*. Vector pSTH2 is a constructed plasmid based on the commercially available 2μ multi-copy vector pYX242 into which the *WCT* gene from strain DSM 12865 was cloned as a 930 bp *Eco*RI/*Sma*I fragment. The toxin gene in question is under the transcriptional control of the yeast's *TPI* promoter and thus allows the strong and constitutive expression of WICALTIN after transformation into *S. cerevisiae*.

(B) Analysis by gel electrophoresis (SDS-PAGE; 10-22.5% gradient gel) of concentrated culture supernatants of *S. cerevisiae* after transformation with the constructed WICALTIN expression vector pSTH2 (lane 1) and the basic vector pYX242 (lane 2). The WICALTIN which has been expressed heterologously in *S. cerevisiae* is marked by an arrow.

(C) Detection of extracellular β-1,3-D-glucanase activity of the yeast *S. cerevisiae* after transformation with the WICALTIN-expressing yeast vector pSTH2. To determine the exo-β-1,3-D-glucanase activity, the yeast colonies which have been grown on leucin-free SC agar were sprayed with 0.04% 4-methylumbelliferyl-β-D-glucoside (MUG) in 50 mM sodium acetate buffer (pH 5.2). After incubation at 37°C for 30 minutes, the agar plates were irradiated with UV light (wavelength 254 nm). Glucanase activity was detected by the fluorescence owing to MUG hydrolysis.

[Key: 1 and 4, *S. cerevisiae* transformed with a vector (pEP-WCT) which expresses the WICALTIN-encoding *WCT* gene under its own promoter; 2, wild-type yeast *W. californica* 3/57 (DSM 12865); 3, wild-type yeast *W. californica* 3/111; 5, *S. cerevisiae* after transformation with the WICALTIN-expressing vector pYX-WCT; 6, *S. cerevisiae* transformed with the basic vector pYX242 (without toxin gene)]

Figure 7: Scheme of the structure of vector pTZα/γ for the heterologous expression and secretion of foreign proteins (in particular WICALTIN and ZYGOCIN) in the fission yeast *Schizosaccharomyces pombe*.

[Key: P_{nmt1}, T_{nmt1}, transcription promoter and transcription terminator of the thiamine-regulated *nmt1* gene of the fission yeast *S. pombe*; S/P, secretion and processing sequence of the viral K28 preprotoxin of the

budding yeast *S. cerevisiae*; *ars1*, autonomously replicating sequence from chromosome 1 of the fission yeast; *leu2*, leucine-2 marker gene for the selection of leucine-prototrophic *S. pombe* transformer]

Figure 8: Comparison of the bioactivities of purified WICALTIN, clotrimazole and miconazole; in the bioassay (agar diffusion test) against the sensitive indicator yeast *Sporothrix* spec., the molar quantities indicated produce an inhibitory zone diameter of 12 mm.

Figure 9: Detection of the WICALTIN-encoding *WCT* gene of the yeast *W. californica* 3/57 (DSM 12865), cloned into pSTH1 (pBR322 derivative), by agarose gel electrophoresis (A) and Southern hybridization with a DIG-labeled *WCT* probe (B).
[Key: M, DIG-labeled DNA size standard II; lane 1, pSTH1 restricted with *Eco*RI and *Sal*I; lane 2, "smart ladder" DNA marker]

Figure 10: Northern analysis of the transcriptional induction of the WICALTIN-encoding *WCT* gene of the yeast *W. californica* 3/57 (DSM 12865) under noninducing culture conditions in BAVC medium (A) and under inducing conditions in BAVC medium supplemented with 0.03% laminarin (B). The total RNA isolated from strain DSM 12865 was separated by electrophoresis in a denaturing agarose/formaldehyde gel at constant voltage (7 V/cm). The RNA was hybridized on a nylon membrane against a WICALTIN-specific, DIG-labeled DNA probe (630 bp) and detected by chemiluminescence.
[Key: M, DIG-labeled RNA size standard I; lanes 1-8 correspond to the sampling times to isolate total RNA: lane 1, 10 hours; lane 2, 15 hours; lane 3, 19 hours; lane 4, 24 hours; lane 5, 33 hours; lane 6, 38 hours; lane 7, 43 hours; lane 8, 48 hours]

Abbreviations used in the text:

WCT	Williopsis Californica Toxin
ZBT	Zygosaccharomyces Bailii Toxin
ZYGOCIN	Proper name; secreted toxin from DSM 12864
WICATIN	Proper name; secreted toxin from DSM 12865

Deposits

The following microorganisms used for the purposes of the present invention were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), - Maschenroder Weg 1b, 38124 Braunschweig, Federal Republic of Germany – which is recognized as international depository in compliance with the provisions of the Budapest Treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure (deposit number; deposit date):

Williopsis californica strain 3/57	(DSM 12865)	(09.06.1999)
Zygosaccharomyces bailii strain 412	(DSM 12864)	(09.06.1999)

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We claim:

1. A protein toxin which can be obtained from *Williopsis californica* and/or *Zygosaccharomyces bailii*.

2. A protein toxin as claimed in claim 1, which can be obtained from DSM 12864 and/or DSM 12865 .

3. A protein toxin as claimed in claims 1 and 2, which has an antimycotic and/or fungicidal action.

4. A protein toxin as claimed in any of claims 1 to 3 with glucanase activity.

5. A protein toxin as claimed in claim 4, which binds to β-1,6-D-glucans and has β-1,3-D-glucanase and/or β-1,3-glucanosyl transferase activity.

6. Nucleic acid encoding a glucanase and/or a protein toxin as claimed in any of claims 1-5 with an amino acid sequence in accordance with **SEQ ID No 1** or **SEQ ID No 2** or a functional variant thereof, and portions thereof with at least 8 nucleotides, where **SEQ ID No 1** or **SEQ ID No 2** is part of the claim.

7. A nucleic acid as claimed in claim 6, wherein the nucleic acid is a DNA or RNA, preferably a double-stranded DNA.

8. A nucleic acid as claimed in claim 6 or 7, which is a DNA with a nucleic acid sequence in accordance with **SEQ ID No 1** of base position 1 to 951 or **SEQ ID No 2** of base position 1 to 717, where **SEQ ID No 1** or **SEQ ID No 2** is part of the claim.

9. A nucleic acid as claimed in claim 8, which contains one or more regulatory regions (promoter, enhancer, terminator) and/or a 3'-terminal poly-A sequence and/or a Kex2p endopeptidase cleavage site which is necessary for the intracellular protoxin processing and/or one or more potential N-glycosylation sites.

10. A nucleic acid as claimed in any of claims 8-9 which can be obtained from DSM 12864 and/or DSM 12865.

11. A nucleic acid as claimed in any of claims 6-10, which is contained in a vector, preferably in an expression vector or in a vector which is effective in gene therapy.

12. A process for the preparation of a nucleic acid as claimed in any of claims 6-10, wherein the nucleic acid is synthesized chemically or isolated from a gene library with the aid of a probe.

13. A polypeptide with an amino acid sequence in accordance with **SEQ ID No 1** or **SEQ ID No 2** or a functional variant thereof, and portions thereof with at least 6 amino acids.

14. A process for the preparation of a polypeptide as claimed in claims 1-5 and 13, wherein a nucleic acid as claimed in any of claims 6-11 is expressed in a suitable host cell.

15. An antibody against a polypeptide as claimed in any of claims 1-5 and 13.

16. A process for the preparation of an antibody as claimed in claim 15, wherein a mammal is immunized with a polypeptide as claimed in claim 7 and, if appropriate, the antibodies formed are isolated.

17. A drug product comprising a nucleic acid as claimed in any of claims 6-10 or a polypeptide as claimed in any of claims 1-5 and 13 or antibodies as claimed in claim 15 and, if appropriate, pharmaceutically acceptable additives and/or adjuvants.

18. A process for the preparation of a drug product for the treatment of mycoses such as superficial, cutaneous and subcutaneous dermatomycoses, mycoses of the mucous membranes and systemic mycoses, especially preferably *Candida* mycoses, wherein a nucleic acid as claimed in any of claims 6-10 or a polypeptide as claimed in any of claims 1-5 and 13 or antibodies as claimed in claim 15 is/are

formulated together with a pharmaceutically acceptable additive and/or adjuvant.

19. A diagnostic comprising a nucleic acid as claimed in any of claims 6-10 or a polypeptide as claimed in any of claims 1-5 and 13 or antibodies as claimed in claim 15 and, if appropriate, suitable additives and/or adjuvants.

20. A process for the preparation of a diagnostic for diagnosing mycoses such as superficial, cutaneous and subcutaneous dermatomycoses, mycoses of the mucous membranes and systemic mycoses, especially preferably *Candida* mycoses, wherein a nucleic acid as claimed in any of claims 6-10 or a polypeptide as claimed in any of claims 1-5 and 13 or antibodies as claimed in claim 15 is/are combined with a pharmaceutically acceptable carrier.

21. An assay for identifying functional interactors comprising a nucleic acid as claimed in any of claims 6-10 or a polypeptide as claimed in any of claims 1-5 and 13 or antibodies as claimed in claim 15 and, if appropriate, suitable additives and/or adjuvants.

22. The use of a nucleic acid as claimed in any of claims 6-10 or of a polypeptide as claimed in any of claims 1-5 and 13 for identifying functional interactors.

23. The use of a nucleic acid as claimed in any of claims 6-10 for finding variants, which comprises screening a gene library with the abovementioned nucleic acid and isolating the variant which has been found.

24. The use of a polypeptide as claimed in any of claims 1-5 and 13 for controlling harmful yeasts and fungi in foods and animal feeds.

25. A process for growing DSM 12864 and DSM 12865, which comprises growing them in synthetic B and/or BAVC medium.

26. The use of the nucleic acids as claimed in any of claims 6-11 for the generation of transgenic plants and plant cells.