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Two pheromone precursor genes are transcriptionally expressed in the homothallic ascomycete *Sordaria macrospora*

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Abstract In order to analyze the involvement of pheromones in cell recognition and mating in a homothallic fungus, two putative pheromone precursor genes, named *ppg1* and *ppg2*, were isolated from a genomic library of *Sordaria macrospora*. The *ppg1* gene is predicted to encode a precursor pheromone that is processed by a Kex2-like protease to yield a pheromone that is structurally similar to the α-factor of the yeast *Saccharomyces cerevisiae*. The *ppg2* gene encodes a 24-amino-acid polypeptide that contains a putative farnesylated and carboxy methylated C-terminal cysteine residue. The sequences of the predicted pheromones display strong structural similarity to those encoded by putative pheromones of heterothallic filamentous ascomycetes. Both genes are expressed during the life cycle of *S. macrospora*. This is the first description of pheromone precursor genes encoded by a homothallic fungus. Southern-hybridization experiments indicated that *ppg1* and *ppg2* homologues are also present in other homothallic ascomycetes.

Key words Pheromone precursor genes · *Sordaria macrospora* · Homothallism

Introduction

In heterothallic fungi, mating occurs only between mycelial structures that are of opposite mating type. As has been well described for the ascomyceteous yeast *Saccharomyces cerevisiae*, mating underlies a complex signal transduction pathway, the pheromone response system (Kurjan 1993). The initial recognition process is mediated by hydrophobic peptide hormones, produced in a mating-type-specific manner. Cells of one mating-type respond exclusively to the pheromone of the opposite mating partner (reviewed in Bölker and Kahmann 1993; Kronstadt and Staben 1997). The regulation of pheromone expression is different in basidiomycetes and ascomycetes. In *S. cerevisiae* two types of pheromone precursor genes are present in the same nucleus, and the production of either pheromone is directly controlled by transcription factors encoded by mating-type genes (Herskowitz 1989). In basidiomyceteous fungi, only one type of pheromone precursor gene(s) is present in the genomic DNA of one cell and the pheromones are produced by the mating-type loci themselves (Bölker et al. 1992; Wendland et al. 1995). As in the yeast *S. cerevisiae*, the expression of pheromone genes in filamentous ascomycetes is supposed to be directly controlled by transcription factors encoded by the mating-type genes (Debuchy 1999).

The mating-type loci, which control sexual development and mating, have been cloned from a number of filamentous ascomycetes (reviewed in Coppin et al. 1997). In the heterothallic species *Neurospora crassa*, these have been extensively analyzed at the molecular level. DNA sequences conferring mating behavior are completely different in *a* and *A* strains and, thus, have been termed idiomorphs, instead of alleles, to denote that the sequences at the same locus in different strains are dissimilar (Glass et al. 1988; Metzenberg and Glass 1990). The molecular characterization of the *N. crassa* mating-type idiomorphs showed that the *a* idiomorph contains only a single gene, *mat a-1*, which encodes the major regulator of mating in *a* strains (Staben and Yanofsky 1990). The *A* idiomorph, however, contains three genes, *mat A-1*, *mat A-2*, and *mat A-3* (Glass et al. 1990; Ferreira et al. 1996). The major regulator of the sexual development in *A* strains is encoded by the *mat A-1* gene (Ferreira et al. 1998). The Mat A-1-polypeptide contains a DNA-binding motif that shows similar-

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ities to a known DNA-binding sequence in the *S. cerevisiae* matα1 mating-type protein.

It has been known for several years that diffusible pheromones are involved in the mating process of filamentous ascomycetes, such as *N. crassa*. Pheromone signalling was suggested to be the cause for directed growth of the trichogyne towards the micro-and macroconidia of the opposite mating-type (Bistis 1981, 1983).

Recently, pheromone precursor genes were isolated from the heterothallic filamentous ascomycetes *Cryphonectria parasitica* and *Magnaporthe grisea*, which are closely related to *N. crassa* (Zhang et al. 1998; Shen et al. 1999). In both ascomycetes, one of the precursor genes encodes a polypeptide containing multiple repeats of a putative pheromone sequence bordered by protease-processing sites. These resemble the α-factor precursor gene of *S. cerevisiae* and the P-factor precursor gene of *Schizosaccharomyces pombe*, respectively (Singh et al. 1983; Imai and Yamamoto 1994). The other gene encodes a short polypeptide with a C-terminal CaaX (C = cysteine, a = aliphatic and X = any amino-acid residue) motif expected to produce a mature pheromone with a C-terminal carboxy methyl isoprenylated cysteine. Structurally similar pheromones were not only produced by *S. cerevisiae* a-strains and *S. pombe* M-strains, but also by several basidiomycetes (Michaelis and Herskowitz 1988; Davey 1992; Spellig et al. 1994; Olesnicky et al. 1999). In addition, it was shown for *C. parasitica* and *M. grisea* that the pheromone precursor genes were expressed in a mating-type-specific manner (Zhang et al. 1998; Shen et al. 1999). Significant similarities between the pheromone precursor genes of filamentous ascomycetes and yeasts suggest that features of the mating-type system of *S. cerevisiae* and *S. pombe* have been conserved not only in heterothallic but also in homothallic filamentous ascomycetes. In homothallic fungi, such as the ascomycete *Sordaria macrospora*, a mycelium derived from a uninucleate ascospore is self-fertile and able to complete the sexual cycle without out-crossing. Recently, we showed the presence of a contiguous mating-type locus in *S. macrospora* (Pöggeler et al. 1997a). Interestingly, this locus contains sequences homologous to both the *mat a* and *mat A* idiomorphs of *N. crassa*. In addition, the mating-type locus of *S. macrospora* was expressed in haploid strains of both mating-types of the closely related pseudohomothallic *Podospora anserina*. The pseudohomothallic *P. anserina* develops four-spored asci in which ascospores contain two nuclei, one of each mating-type. A few asci contain five ascospores, three binucleate and two small uninucleate ascopores which produce homokaryons. When the mating-type locus of *S. macrospora* was introduced in these homokaryons the induction of fruiting-body formation has been observed in *P. anserina* homokaryons of both mating-types (Pöggeler et al. 1997a). This indicates that *S. macrospora* mating-type genes are not pseudogenes and are likely to be involved in the mating process in a homothallic fungus. Homothallic species of the genus *Cochliobolus* show the same organization of the mating-type locus. They carry both mating-type genes in a single nucleus, usually closely linked or fused (Turgeon 1998). Recently Yun et al. (1999) have shown that the mechanism of conversion from heterothallism to homothallism is a recombination event between islands of identity in otherwise dissimilar mating-type sequences.

In an attempt to further understand the molecular basis of homothallism, the possibility of pheromone involvement in sexual reproduction of the homothallic *S. macrospora* was considered. The results presented here show that *S. macrospora* contains two pheromone precursor genes encoding pheromone precursors with structural characteristics and processing signals similar to those reported from *C. parasitica* and *M. grisea*. In contrast to these heterothallic ascomycetes, both pheromone precursor genes are expressed in the homothallic mycelium. The expression is sustained throughout the entire sexual reproductive phase of *S. macrospora*. In addition, it was demonstrated that homologues of both *S. macrospora* pheromone precursor genes are present in the genomic DNA of other homothallic ascomycetes. The putative functions of pheromones in homothallic fungi are discussed.

Materials and methods

Fungal strains and media

The *S. macrospora* strain K (isolate L3346) from our laboratory collection displays a wild-type phenotype. For DNA isolation the following fungal strains were used: *Sordaria fimicola* (FGSC #2918), *N. crassa* (FGSC #4317), *N. crassa* (FGSC #4347) *Neurospora africana* (FGSC #1740), *Neurospora pannonica* (FGSC #7221), *Neurospora terricola* (FGSC #1889). All strains were cultivated on corn-meal medium (Esser 1982).

PCR amplification

PCR amplification of genomic DNA and cosmid pools was utilized to generate fragments for sequencing and cloning. Double-stranded amplification was performed in a GeneAmp PCR System 9600 thermocycler (Perkin Elmer). The reaction profile included one initial denaturation at 94 °C for 2 min, followed by 40 cycles with annealing at 50 °C for 1 min, extension at 72 °C for 1 min and denaturation at 94 °C for 1 min; a final elongation at 75 °C for 15 min closed the amplification. The following oligonucleotides have been used as primer pairs for the generation of *ppg1* gene fragments: pheba-5 [5′ATGAAGTTCACTCTCCCTCTT-3′] and pheba-3 [5′-TCCTCCCTCCTCCTCGGTGGAGGG-3′]; for the generation of *ppg2* gene fragments: phesa-5 [5′-CCCACAAACC-ATCAACATGCC-3′] and phesa-3 [5′-GCCCTGTGAGGTTAT-TTACAT-3′]. PCR primers were synthesized by MWG-Biotech (Ebersberg, Germany).

Preparation of nucleic acids and hybridization

Preparation of DNA was done according to Pöggeler et al. (1997b). Total RNA was isolated from *S. macrospora* using the method of Hoge et al. (1982). Southern and Northern blotting were performed according to Sambrook et al. (1989). DNA gels were soaked in 0.1 M HCl prior to denaturation.

Plasmid construction and DNA sequencing

PCR products were cloned into pMON38201 (a plasmid for *Xcm*I-based cloning of PCR products, as described by Borovkov and Rivkin 1997). Cosmid clone H4 isolated from pool VI 1733–1828 of the indexed cosmid library (Pöggeler et al. 1997b) carries the *ppg1* gene, and cosmid clone F8 from the same pool carries the *ppg2* gene. Subcloning of gene fragments containing either the *ppg1* or the *ppg2* gene using the vector pBCKS(+) (Stratagene) was done by standard techniques (Sambrook et al. 1989). The subcloned fragments were sequenced by MWG-Biotech Customer Service (Ebersberg, Germany). Nucleotide sequences were deposited in the EMBL database under the following accession numbers: *ppg1* (AJ249863), *ppg2* (AJ259862).

Results

Cloning of the pheromone precursor genes

In order to isolate fragments of putative pheromone precursor genes from *S. macrospora*, a PCR-based assay was performed. The heterothallic ascomycete *N. crassa* is a close relative of the homothallic *S. macrospora*. Recent evolutionary analysis revealed that the conserved *gpd* gene encoding glyceraldehyde 3′ phosphate dehydrogenase, show a nucleotide identity of over 90% between these two species (Pöggeler 1999). Therefore, primers for the amplification of pheromone precursor genes were designed after extracting nucleotide sequences encoding putative pheromone precursor genes from the Expressed Sequences Tag (EST) library of the *N. crassa* genome (Nelson et al. 1997). For this purpose, the conserved sequences of known pheromone genes from *C. parasitica* and *M. grisea* were used. The **a**-factor-like pheromone precursors of the heterothallic ascomycetes *C. parasitica* and *M. grisea* contain a conserved C-terminal XYCV/IVM region, whereas the α-factor-like pheromone precursors carry a repeated WCXXGXXCW motif (Zhang et al. 1998; Shen et al. 1999). These conserved peptide motifs of *C. parasitica* and *M. grisea* were used to carry out a tblastn search in the *N. crassa* EST library at http://molbiol.aph.pccunm.edu. Applying this strategy, several *N. crassa* cDNA clones were identified, which showed either structural similarities to α-factor-like or **a**-factor-like pheromone precursor genes. The DNA sequences of the EST clone NM6E5-T7 (Nelson et al. 1997), containing conserved peptide sequences of the α-factor-like precursor, were used to design the primer pair pheba-5/pheba-3, which was used to amplify an α-like pheromone gene from *S. macrospora* genomic DNA. The PCR experiment carried out with these primers yielded one amplicon of approximately 330 bp (data not shown). Sequence analysis of the amplified fragment revealed that the amplified DNA encodes a polypeptide with significant amino-acid homology to the pheromone precursors of *C. parasitica* and *M. grisea* and, in addition, a sequence identity of 91% to the *N. crassa* EST clone NM6E5-T7.

Sequences for primers phesa-5 and phesa-3 for the **a**-factor-like pheromone precursor gene were designed according to the sequences of the *N. crassa* EST clone NP4F7-T3 (Nelson et al. 1997), which harbors the conserved CaaX motif of the **a**-factor-like percursors. One PCR product of approximately 100 bp was obtained when the genomic DNA of *S. macrospora* was used as a template (data not shown). Sequencing of this PCR amplicon revealed that the amplified DNA encodes a polypeptide, which shows structural similarities to the **a**-factor-like pheromone precursors of *C. parasitica* and *M. grisea* and, additionally, a nucleotide identity of 98% when compared to the EST sequences of putative pheromone precursor genes of *N. crassa*.

Since both primer pairs were suitable for the amplification of pheromone precursor genes of *S. macrospora,* these primer pairs were subsequently used to perform a high-throughput PCR screening (Bottoli et al. 1999) of pooled cosmid DNA derived from an indexed *S. macrospora* genomic cosmid library (Pöggeler et al. 1997b). Using this fast PCR approach, at least one single cosmid clone was identified with each primer pair and was further used for subcloning of the putative pheromone precursor genes. The α-like and **a**-like pheromone precursor genes were named pheromone precursor gene 1 (*ppg1*) and pheromone precursor gene 2 (*ppg2*), respectively. Sequence analysis of *ppg1* indicated that this gene encodes a putative pheromone precursor, which beares structural features similar to the *Mf1/1* gene from *C. parasitica* and the *MF2-1* gene from *M. grisea*. Within the 277-amino-acid polypeptide deduced from the nucleotide sequence (Fig. 1) five repeats of the undecapeptide sequence QWCRIHGQSCW were identified. All five copies of the undecapeptide are surrounded by maturation signals similar to those of the α-factor pheromone precursors of *S. cerevisiae* (Julius et al. 1983, 1984). The five repeats are preceded by two to eight repeats of a dipeptide XA (or XP). All of these dipeptide stretches, except the first, were again preceded by the basic dipeptide KR. With the signal-peptide-prediction program SignalP V1.1 (Nielsen et al. 1997), a putative cleavage site was identified between amino-acid position 16 and 17 (Fig. 1). The motif that follows the undecapeptide is different for all of the five repeats, but always contains the dipeptide KR (Fig. 1).

The *ppg2* gene encodes a short polypeptide of 24 amino acids that ends with a CaaX motif (Fig. 2). This motif is also found in the precursors of several fungal pheromones that have been shown to be farnesylated and carboxy methyl-esterified (Andregg et al. 1988; Davey 1992; Spellig et al. 1994; Olesnicky et al. 1999). In contrast to the PPG1 precursor, hydrophobic signal peptide sequences could not be detected with the program SignalP V1.1 (Nielsen et al. 1997) in the N-terminus of PPG2. The flanking sequences of the proposed ATG of *ppg1*, as well as of *ppg2*, showed a high level of similarity to the translation initiation sites from other *S. macrospora* genes (Pöggeler 1997).

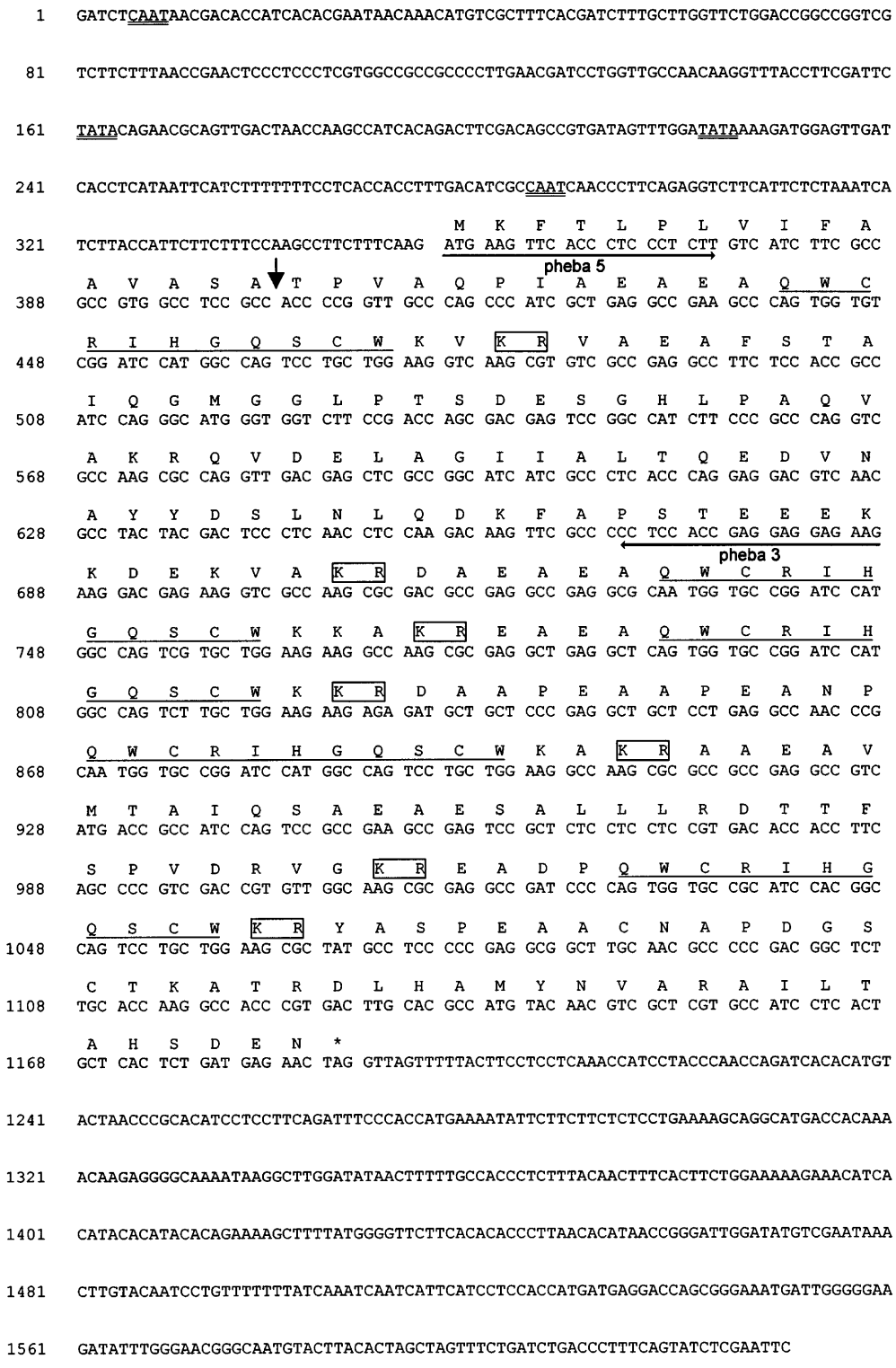

Fig. 1 Nucleotide sequence of the *S. macrospora* pheromone-precursor gene *ppg 1*. Putative TATA box and CAAT box sequences are *double underlined*. The derived amino-acid sequence is indicated above the nucleotide sequence; (*), denotes the stop codon. The five repeats of the undecapeptides are *underlined*, KR dipeptides are *framed*. The putative cleavage site of the signal sequence is marked by a *vertical arrow*. Locations of the primers pheba-5 and pheba-3 used to amplify a part of the gene are indicated by *horizontal arrows*

Transcriptional expression of pheromone precursor genes

In heterothallic ascomycetes, the pheromone precursor genes are expressed in a mating-type-specific manner (Zhang et al. 1998; Shen et al. 1999). In order to examine the expression pattern of the pheromone precursor genes *ppg1* and *ppg2* in the homothallic *S. macrospora*, Northern-blot analysis of total RNA isolated during different stages of sexual development was performed. As shown in Fig. 3, both pheromone precursor genes are expressed in *S. macrospora*. A transcript of 1.55 kb was detected with a *ppg1*-specific probe, whilst the *ppg2*-specific probe labelled a transcript of

Fig. 2 Nucleotide sequence of the *S. macrospora* pheromone precursor gene *ppg 2*. Putative TATA box sequences are *double underlined*. The derived amino-acid sequence is indicated above the nucleotide sequence; (*) denotes the stop codon. The prenylation signal sequence (CaaX) is *underlined*. Locations of the PCR primers phesa-5 and phesa-3 are indicated by *horizontal arrows*

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1    GACTCACTATAGGGATCGATAATAGCAAGGCAAGATTGGAGGAGATAGCTTCAGACTGACAGTTCGTTAGGAGATTCAGC

81   GCAGTGGAAAATCTATAAGAACGTCAGTCTCCTCCTGCCCAACTCTGGATTTCCTTTTCCTCCTCATCCCACAACAGCCT

                                                    M   P   S   T   A   A   S   T   K   V
161  TTGAATCCATTTATCAAACAACCCTAAAACCATCAAC    ATG CCT TCC ACC GCT GCT TCC ACC AAG GTC
                                  phesa 5
      P   Q   T   T   M   N   F   N   G   Y   C   V   V   M   *
228  CCC CAG ACC ACC ATG AAC TTC AAC GGC TAC TGC GTT GTT ATG TAA ATAACCTCACAGTTGTCTTG
                                                          phesa 3

293  AAGGGCAACGAGAGCATTGAGAGGTGGATTCCAACCAGCTTTCCTCCAGCAATCTTCCGACAAACCACCAATATCACTCA

373  CCGAGATCCACGGGAGAATCGGTATCTCGCCTCTCAACGTCAGACCAGCAACCTTCTTTTCCCCTCTCTCTCGGGAAAGC

453  CCAACTCTTGTTTCGCCACAACAGCAACCTCGCCTAGGAAGTTTCTTGTTCTGCTTTCATGGTGGCGCTCTGCACAATGG

533  GAGCACAACCACTCGACAACACCAGAGCGGTGAGGATCTTTCCTTGATGGGAATGGAAAAGAAACTTTCGTCAACCGGGG

613  ATATACATCCGACACATGTCACACACCATCCTTCATACACGATCTTCGCTTCTTTTTGTTGGGGATAACTTCACACGGGA

693  CCATTTTCGTCCACACGGAGCCTTCACCTTCTTCACCTTTTGACACTCGGGCAACAACTTCATCACCCTTTTCTTTCTTT

773  TCGTCGTTGACAATCATCAAACACTCTTCGCAAACTGAGGAAATCGCCGGCGGGAAAACGATACAGCAACTTCTTTTCGG

853  TACTCAACTTTCGGCAAACTACACTCCTGTGAGTCGGCGGACTCGGACCTCCAGTCTCGACTTGTTTTCATCAGCTGGAA

933  GGGAGCTATTTCGCGACATGCTTCTCTCTTTTCTCGGTGGAGCTGGAAGGGGTTGGTTTCGGC
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1.0 kb. Under our experimental conditions, sexual development started 2–4 days after inoculation ended, with the discharge of mature ascospores from perithecia 7 days after inoculation (Nowrousian et al. 1999). In order to determine levels of *ppg1* and *ppg2* mRNA during the sexual phase of the *S. macrospora* life cycle, the amount of both transcripts was monitored by means of a Northern-blot analysis of total RNA isolated at different times during sexual development. Figure 3 clearly shows that transcripts of the *ppg1* and *ppg2* genes can be detected during the entire sexual phase. During this time course, maximum expression of both pheromone precursor genes occurred 4 days and 7 days after inoculation.

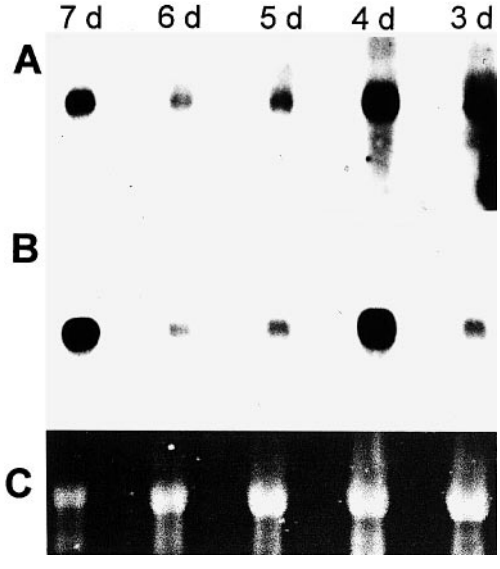

Fig. 3A, B Expression of pheromone precursor genes. **A** a Northern blot containing total RNA isolated at different times during the sexual development of *S. macrospora* was probed with a *ppg1* gene-specific probe and then stripped and re-probed with a *ppg2* gene-specific probe (**B**). **C** gel picture of the 25S rRNA as a loading control

Presence of *ppg1* and *ppg2* gene homologues in other homothallic ascomycetes

In order to determine the copy number of both genes in the genome of *S. macrospora* genomic DNA, and to identify *ppg1* and *ppg2* homologues in other homothallic ascomycetes, genomic DNA isolated from *S. macrospora* and various other fungi was used to perform Southern hybridizations. Two probes containing the entire *ppg1* coding region or the entire *ppg2* coding region were used for analysis. With each probe, only one hybridization signal was obtained from the genomic DNA of *S. macrospora*. Thus, the genome of *S. macrospora* carries only one copy of *ppg1* and *ppg2*, respectively (Fig. 4). Genomic DNA from homothallic *Sordaria* and *Neurospora* species also gave strong signals with both probes, indicating that precursor genes for both pheromones are present in these fungi. As a control, genomic DNA of a *N. crassa A* and *a* strain was hybridized with the *ppg1* and *ppg2* probes. As expected from the EST sequences extracted from the *N. crassa* EST library (see

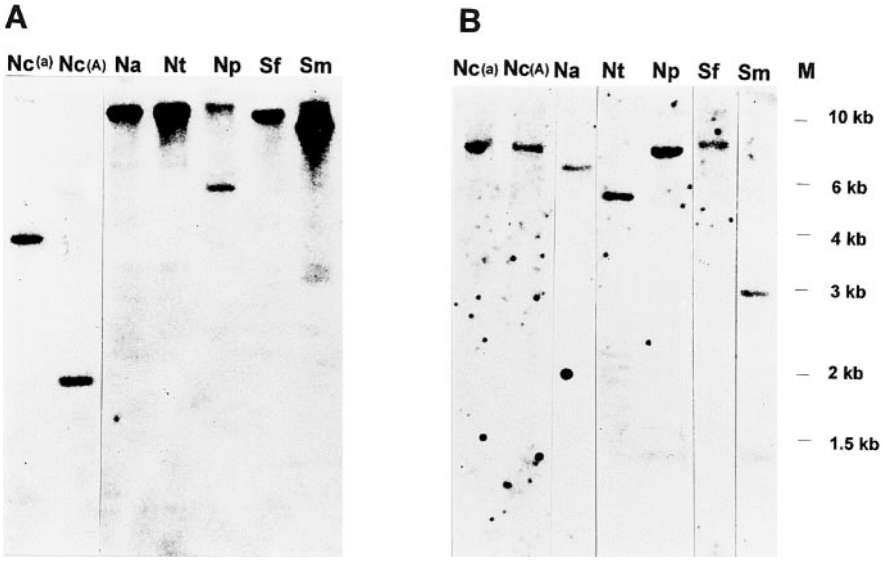

Fig. 4 Hybridization of fungal genomic DNAs with *S. macrospora ppg1* (**A**) and *ppg2* (**B**) gene-specific probes. The *Eco*RI-digested DNA was isolated from strains as indicated. Nc (A), *Neurospora crassa mat A*; Nc (a), *Neurospora crassa mat a*; Na, *Neurospora africana*; Np, *Neurospora pannonica*; Nt, *Neurospora terricola*; Sf, *Sordaria fimicola*; Sm, *Sordaria macrospora*. Sizes of marker fragments are given on the right

above), both pheromone precursor genes were identified in the genomic DNA of *N. crassa A* and *a* strains. Remarkably, the hybridization signals obtained with the *ppg1* probe labelled fragments of different sizes in *A* and in *a* strains, respectively (Fig. 4A). This finding corresponds with reports on a putative pheromone gene being physically linked to the *A* mating-type locus of *N. crassa* (Bölker and Kahmann 1993, Beatty et al. 1994). However, with the *ppg2* probe no mating-type specific hybridization signals were observed.

Discussion

The structure of the *S. macrospora* pheromone precursor genes shows similarities to pheromone genes from heterothallic fungi

Two putative pheromone precursor genes are present in the genomic DNA of the homothallic ascomycete *S. macrospora*. A peptide pheromone structurally similar to the *S. cerevisiae* α-factor is encoded by the *ppg1* gene, which encodes a 277-amino-acid precursor. This precursor contains five identical repeats of the putative pheromone sequence QWCRIHGQSCW. Homologues of the *ppg1* gene in the closely related heterothallic filamentous ascomycetes *C. parasitica* (MF1/1) and *M. grisea* (MF2–1) contain seven copies of identical decapeptides and four copies of undecapeptides, respectively (Zhang et al. 1998; Shen et al. 1999). In the *S. macrospora* PPG1 precursor, the repeats are separated by downstream-located KR dipeptides, which are potential Kex2 protease-processing sites (Julius et al. 1984). These dipeptide motifs also occur in the pheromone precursors of *C. parasitica* and *M. grisea*. In common with the *Mf1/1* gene product of *C. pararsitica*, the five undecapeptide repeats within the *S. macrospora* PPG1 precursor are preceded by two to eight copies of the dipeptide XA or XP. Such dipeptide repeats are cleavage signals for the Ste13p dipeptidyl aminopeptidase, which in the case of the yeast α-factor precursor removes EA repeats from the N-terminus of the pheromone (Julius et al. 1983). In yeast, the Ste13p processing results in an α-factor peptide with a KR C-terminal extension, which is subsequently removed by the activity of the carboxypeptidase Kex1p (Cooper and Bussey 1989). Only the fifth repeat of the *S. macrospora* precursor has a C-terminal KR extension, whereas the other four repeats end either with KVKR, KKAKR, KKR or KAKR. It is possible that these peptide extensions are also processed by a Kex1p homologue or, alternatively, all five repeats produce pheromones with different C-termini. The putative pheromone is most-likely secreted out of the cell via the classical secretory pathway. A putative N-terminal signal peptide leader enabling transit into the endoplasmatic reticulum is common to most secretory proteins, e.g. the yeast α-factor precursor (Waters et al. 1988). Its existence was predicted for PPG1 by the program SignalP V1.1 (Nielsen et al. 1997). The putative signal peptide was predicted to be cleaved between amino-acid positions 16 and 17 (Fig. 1).

The second pheromone precursor gene of *S. macrospora*, *ppg2*, encodes a polypeptide of 24 amino acids. The most significant feature of the *ppg2* gene product is the presence of a C-terminal CaaX box representing a signal for modification of polypeptides with an isoprenoid group (Schafer and Rine 1992). Despite inherent differences within the amino-acid sequences, the characteristic CaaX box is common to all known fungal lipopeptide pheromone precursors. After complex N- and C-terminal post-translational processing, mature fungal lipopeptide pheromones, which have been biochemically characterized, contain a variable amino-acid sequence of 9 to 15 residues and a hydrophobic carboxyl terminus including a methylester and a farnesyl group (Caldwell et al. 1995). Since N-terminal cleavage cannot be inferred by comparison with those of other known fungal pheromone sequences, the sequence of the mature

S. macrospora lipopeptide pheromone cannot be predicted. However, it seems very likely that the C-terminus of the mature pheromone encoded by *ppg2* is a farnesylated and carboxy methyl-esterified cysteine. In *S. macrospora*, the final amino acid of the CaaX box is a methionine. It has been shown that this final amino acid in the CaaX sequence is a major determinant of the type of prenyl modification attached to the cysteine. When precursors contain alanine, serine, glutamine, cysteine or methionine as the final residue X of the CaaX box, the peptide is usually farnesylated (Moores et al. 1991). The lack of a hydrophobic signal sequence at the N-terminus of the translated product of *ppg2* suggests that in *S. macrospora*, the mature lipopeptide pheromone might be exported through a mechanism similar to that described for the yeast **a**-factor pheromone, which is secreted by the specialized Ste6p transporter (Caldwell et al. 1995).

Putative functions of pheromones in homothallic ascomycetes

In *S. macrospora*, as well as in the closely related heterothallic *M. grisea*, only one copy of each pheromone precursor gene can be detected by Southern-blot analysis of the genomic DNA (Fig. 4) (Shen et al. 1999). In contrast, two genes that encode the **a**-factor and two genes that encode the α-factor are present in the genomic DNA of *S. cerevisiae*, whilst the genome of *C. parasitica* harbors two copies of the lipopeptide pheromone-encoding gene and one copy of the gene encoding the α-factor-like pheromone (Zhang et al 1998).

Since gene-disruption experiments have not been successful in *S. macrospora*, direct experimental evidence, proving that the pheromone genes identified in *S. macrospora* are functional, is missing. However, Northern-blot analyses revealing that both genes are transcriptionally expressed during the sexual development of *S. macrospora*, suggest that the *S. macrospora* genes are not pseudogenes. The expression of both *S. macrospora* precursor genes seems to be developmentally regulated, being highest at the beginning and at the end of the sexual phase (Fig. 3). In contrast to this finding, transcription of the pheromone precursor genes depends on mating-type in heterothallic ascomycetes, so that only one or the other type of pheromone precursor gene is expressed. The transcription factors encoded by the mating-type genes are supposed to control this mating-type-specific expression (Zhang et al. 1998; Shen et al. 1999). In heterothallic ascomycetes, either *A* or *a* specific mating-type transcription factors are present in *A* or *a* strains, whilst in the homothallic *S. macrospora* both types of mating-type regulators are expressed in the same individual (Pöggeler et al. 1997a). For this reason, not surprisingly, both pheromone precursor genes are transcriptionally expressed in this homothallic fungus. However, the actual function of pheromones in homothallic fungi remains speculative. Homothallic *Neurospora* and *Sordaria* species lack conidiospores, spermatia and trichogynes, which, in heterothallic species, are sent out from protoperithecia and grow towards a conidium of the opposite mating-type. Therefore, homothallic ascomycetes are probably not in need of pheromones for sensing the mating partner and for initial fertilization events. It was demonstrated in the yeast *Saccharomycopsis lipolytica* by protoplast fusion that fusion products of cells carrying the same mating-type allele can undergo the sexual cycle and produce ascospores. Thus mating-types control only the initial steps of the mating sequence, cell recognition and agglutination, but not karyogamy and meiosis (Stahl 1976). However, apart from this initial recognition function, it has been repeatedly proposed that pheromones are also necessary for post-fertilization events (Bölker and Kahmann 1993; Debuchy 1999). In the sexual development of both homothallic and heterothallic fungi, an important post-fertilization event takes place in the development of dikaryotic ascogenous hyphae in order to enable karyogamy and meiosis. In the pseudohomothallic ascomycete *P. anserina*, the mating-type genes are involved in the establishment of nuclear identity, which seems to be a prerequisite for the internuclear recognition of two nuclei within the syncytial cell and for the migration of two different nuclei into the ascogenous hyphae (Zickler et al. 1995; Arnaise et al. 1997). Recently, Debuchy (1999) has proposed that a pheromone/receptor system controlled by the mating-type genes might be involved in the internuclear recognition in ascomycetes. In heterothallic fungi, as well as in homothallic fungi, exactly two nuclei have to migrate into the ascogenous hyphae in order to allow an ordered meiosis, However, in a homothallic mycelium individual nuclei are not distinguished by different mating-type information. Glass et al. (1990) and Coppin et al. (1997) discussed the possibility of nuclei of homothallic fungi being functionally heterothallic in order to circumvent the recognition problem. Taking their considerations into account, it seems likely that homothallic ascomycetes have evolved a mechanism that allows alternate expression of either mating-type to guarantee nucleus recognition before migration of the nuclear pairs into the ascogenous hyphae. As proposed for the heterothallic ascomycete *P. anserina* (Debuchy 1999), a mating-type-controlled pheromone/receptor system may also be of importance for the nuclear recognition process in a homothallic fungus. Future investigations should include functional analysis of the pheromone genes and identification of the pheromone receptors in order to either substantiate their involvement in the sexual development of homothallic ascomycetes or to identify them as remnants of a former heterothallic life style.

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