A novel class of peptide pheromone precursors in ascomycetous fungi

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Summary
Recently, sexual development in the heterothallic ascomycete Trichoderma reesei (anamorph of Hypocrea jecorina) has been achieved and thus initiated attempts to elucidate regulation and determinants of this process. While the α-type pheromone of this fungus fits the consensus known from other fungi, the assumed α-type peptide pheromone precursor shows remarkably unusual characteristics: it comprises three copies of the motif (LI)GC(TS)VM thus constituting a CAAX domain at the C-terminus and two Kex2-protease sites. This structure shares characteristics of both α- and α-type peptide pheromone precursors. Presence of hybrid-type peptide pheromone precursor 1 (hpp1) is essential for male fertility, thus indicating its functionality as a peptide pheromone precursor, while its phosphorylation site is not relevant for this process. However, sexual development in a female fertile background is not perturbed in the absence of hpp1, which rules out a higher order function in this process. Open reading frames encoding proteins with similar characteristics to HPP1 were also found in Fusarium spp., of which Fusarium solani still retains a putative α-factor-like protein, but so far in no other fungal genome available. We therefore propose the novel class of h-type (hybrid) peptide pheromone precursors with H. jecorina HPP1 as the first member of this class.

Introduction
The mating process in fungi has been subject to elaborate studies for decades (Debuchy and Turgeon, 2006; Aanen and Hoekstra, 2007; Debuchy et al., 2010). Communication between haploid cells of opposite mating type is thereby crucial for the initiation of the mating process in heterothallic fungi. This interaction is predominantly achieved by the secretion of the small, diffusible peptide pheromones, which can act at a distance and relate their signal via specific receptors (Bolker and Kahmann, 1993; Kothe, 1999). Presently, two types of pheromone precursor genes have been identified in fungi, which are related to the MFA and MFα genes of Saccharomyces cerevisiae representing the best-studied examples (Kurjan, 1993; Wang and Dohlman, 2004).

Pheromone signalling causes directed growth of the trichogyne, the female organ, towards the male cells opposite mating-type in heterothallic fungi (Bistis, 1981; 1983). The respective pheromone precursor genes were isolated from several heterothallic filamentous ascomycetes including Cryphonectria parasitica, Magnaporthe grisea, Neurospora crassa and Podospora anserina (Zhang et al., 1998; Shen et al., 1999; Bobrowicz et al., 2002; Coppin et al., 2005). In those 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 the precursor for a short polypeptide with a C-terminal CaaX motif expected to produce a mature pheromone with a C-terminal carboxy methyl isoprenylated cysteine. Structurally similar pheromones were not only found in S. cerevisiae α-strains, S. pombe M-strains and numerous ascomycetes, but also in basidiomycetes (Michaelis and Herskowitz, 1988; Davey, 1992; Spellig et al., 1994; Olesnicky et al., 1999). In addition, the pheromone precursor genes are expressed in a mating-type-specific manner in N. crassa, P. parasitica, P. anserina and M. grisea (Zhang et al., 1998; Shen et al., 1999; Bobrowicz et al., 2002; Coppin et al., 2005). Pheromones have been shown to be essential for male fertility (Coppin et al., 2005). In C. parasitica, which contains two α-type peptide pheromone precursor genes (Zhang et al., 1993; 1998), deletion of one of them does not prevent fertilization, but resulted in barren perithecia and defects in asexual sporulation. However, the defects in asexual...
sporulation may also be due to additional small open reading frames (ORFs; only one of which represents the a-type peptide pheromone precursor gene), in the described deletion strain (Turina et al., 2003). In contrast, it was reported that peptide pheromones are dispensable for female functions during sexual reproduction (Coppen et al., 2005; Kim and Borkovich, 2006). In N. crassa, the appropriate pheromone receptor (Kim and Borkovich, 2004), the heterotrimeric G-α-subunit GNA1 (Ivey et al., 1996) and both the G-protein β- and γ-subunits (Krystofova and Borkovich, 2005; 2006) are necessary for chemotropic behaviour and perithecial development in females.

Although it seemed clear that the major role of pheromones in filamentous heterothallic ascomycetes is the control of recognition between male and female organs of opposite mating types, both types of pheromone precursor genes have also been identified in the homothallic ascomycetes Sordaria macrospora (Poggeler, 2000) and Gibberella zeae (Kim et al., 2008; Lee et al., 2008). Hence, the peptide pheromones and/or their precursor proteins may also have functions beyond attracting a mate. In some fungi, pheromones play a role in post-fusion events as well as cell-cell recognition and fusion. Specifically, pheromones have been implicated in the induction of meiosis in S. pombe (Willer et al., 1995; Chikashige et al., 1997) and stimulation of filamentous growth in Ustilago maydis (Spellig et al., 1994).

For more than 50 years, Trichoderma reesei was known only from one single isolate (QM6a), which was collected as an agent deteriorating cotton material of the US Army in the South Pacific during the World War II. Due to its ability to produce high amounts of cellulases, it served as an ideal progenitor of many mutants that have been used for cellulase production thereafter (El-Gogary et al., 1989). Using molecular diagnostic methods, however, T. reesei was subsequently recognized to be indistinguishable from the tropical ascomycete Hypocrea jecorina (Kuhls et al., 1996). Nevertheless, attempts to induce mating of the original isolate QM6a or its industrially applied progeny had failed thereafter and T. reesei was consequently suggested to be an asexually propagating clonal line of the genus. We recently achieved induction of sexual development of T. reesei/H. jecorina QM6a, which is a MAT1-2 strain, with the MAT1-1 mating type of wild-type isolate H. jecorina CBS999.97 and thus disproved this assumption (Seidl et al., 2009). Upon mating of these strains fertilized stromata and mature ascospores are formed. However, this study also revealed that QM6a, while being male fertile, is female sterile. The identification of a sexual breeding system for this fungus is of utmost importance for strain improvement and initiated more detailed studies on the mechanism of sexual development in T. reesei/H. jecorina. However, while industrial applications of T. reesei and the enzymes produced by this fungus are well studied (Kubicek et al., 2009; Schuster and Schmoll, 2010), research towards elucidation of biology of sexual development is only in its beginnings and reproductive structures as well as the precise mechanism of fertilization remain to be studied.

Here, we report the finding of a protein resembling an a-type peptide pheromone precursor, which also shows characteristics of α-type pheromone precursors. Our results indicate that this unusual a-type peptide pheromone precursor is indeed essential for male fertility upon mating and thus pheromone response although its genomic locus indicates that after loss of the original a-type pheromone precursor this protein might have assumed the respective function. Based on its characteristics and the finding of similar proteins in heterothallic Fusarium spp., we propose a new class of peptide pheromone precursors denominated h-type (hybrid) pheromone precursors.

Results

HPP1 encodes a 5.1 kDa peptide pheromone precursor

The gene encoding HPP1 was originally isolated as an EcoRI–cDNA fragment, cfa11C (GenBank Accession No. CF653649), reported to be associated to cellulase signal transduction in H. jecorina (Schmoll et al., 2004). Transcription start point and 3′ UTR were mapped by 5′ and 3′ RACE resulting in a complete cDNA of the respective gene. An upstream in frame stop codon was detected, indicating that RACE amplified the full-length cDNA (Kozak, 1996). The 591 bp full-length cDNA contains a 5′ UTR of 82 nt, an ORF of 144 nt and a 3′ UTR of 347 nt. This unexpectedly long 3′ UTR is not without precedent as such long 3′ UTRs have also been found in a-type pheromone precursor genes of other organisms, for example, with mfa-1 of N. crassa (Kim et al., 2002), mft-2 of C. parasitica (Zhang et al., 1998), MF1-1 of M. grisea (Kim et al., 1999) or ppq2 of S. macrospora (Poggeler, 2000). A polyadenylation signal is lacking. The first ORF starts with an ideal Kozak consensus and encodes a 48 aa polypeptide (Fig. 1A) with a deduced Mr of 5.1 kDa and a predicted isoelectric point of 9.63. It belongs to the α/β-type of proteins as revealed by a Garnier/Robson blot (Garnier et al., 1978) with three predicted β-sheets and two helices (data not shown). Interestingly, the amino acid sequence of all three β-sheets is almost identical: (L/I)GC(S/T)VM.
likely to be farnesylated (Moores et al., 1991). In order to appreciate the particular structure of this unusual peptide pheromone, the gene was named hybrid peptide pheromone precursor gene 1 (hpp1) (GenBank Accession No. AAQ95218).

Interestingly, a second ORF occurs in the 3′ UTR (nts 385–468), which like the first ORF, starts with a good Kozak consensus (Kozak, 2002). According to the rule proposed by Kozak (Kozak, 1996) ‘not the best but the first start codon’ it is unlikely that this ORF is functionally translated. Alternatively, a hypothesis by Turina and coworkers (Turina et al., 2003) suggests that such additional ORFs may be important for asexual sporulation. However, deletion of the area spanning the full mRNA sequence of hpp1 (see below) does not alter asexual sporulation in H. jecorina and hence renders this hypothesis unlikely for this fungus.

Both the size of the polypeptide as well as the C-terminal prenylation signal are consistent with a function of HPP1 as a peptide pheromone precursor. Within the promoter region of hpp1 two pheromone response elements (5′- CAAAG-3′), were found at positions −639 and −610 on the template strand. These sequences were identified in the basidiomycete U. maydis and are frequently present in the vicinity of pheromone inducible genes. Both are necessary and sufficient for pheromone induction (Hartmann et al., 1996; Urban et al., 1996), but so far have not been shown to be functional in ascomycetes.

This region further comprises GATA-factor binding sites (5′- HGATAR-3′) at −716 and −501, a CCAAT-box at −370

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HPP1 represents a novel type of peptide pheromone precursors

As the particular structure of HPP1 suggested that it could be the first representative of a so far unidentified type of peptide pheromone precursors, we were interested whether this type is also present in genomes of other fungi. Therefore, all fungal genome databases available at the BROAD Institute (http://www.broad.mit.edu/annotation/), the Joint Genome Initiative of the US Department of Energy (http://genome.jgi-psf.org/), the Fusarium sporotrichioides EST-database at the University of Oklahoma (http://www.genome.ou.edu/fspsoro_blast.html) and the Epichloe festucae genome database at the University of Kentucky (http://www.endophyte.uky.edu) were screened for proteins with similar characteristics. Hence, the genomes screened cover a broad range of phyla such as basidiomycota, ascomycota, zygomycota and chytridiomycota. Orthologues of HPP1 have so far only been detected in fungi belonging to the order of Hypocreales (Fig. 1A). Analysis of these polypeptides by MEME [(Bailey and Elkan, 1994); http://meme.sdsc.edu] revealed a 6-amino-acid conserved motif with an E-value of 3E-32 (Fig. 1B). Interestingly, this consensus indicates a preference of the first aliphatic amino acid in the CAAX motif for a polar, uncharged one (threonine, serine or asparagine). Therefore, we propose the prenylation site in the MAT1-2 mating system in Hypocreales to be a CPAX motif (p for polar). In agreement with the proposal by Reiss et al. (1991), this CPAX motif may be processed by a farnesyltransferase with different specificity from those dealing with CAAX domains. Nevertheless, a longer stretch of higher sequence similarity between closely related species can be found in some cases. While Hypocreia virens (anamorph Trichoderma virens) has a close orthologue to HPP1, the putative HPP1 orthologue of Hypocreia atrovirdis (anamorph Trichoderma atrovirdide) found at the syntenic locus only comprises one consensus motif and does not share the characteristics of HPP1. The availability of ESTs from both H. virens and H. jecorina confirms that the respective genes are expressed. Besides those closely related species, HPP1 orthologues are also present in the heterothallic Gibberella moniliformis (Fusarium verticioides), Nectria haematococca MPVI (Fusarium solani) and the reportedly asexual Fusarium oxysporum, but not in the homothallic G. zeae (Fusarium graminearum) or the asexual F. sporotrichioides. The occurrence of genes encoding proteins with similar characteristics as HPP1 supports our proposal of the novel class of h-type peptide pheromone precursors (hybrid-type), which is characterized by the occurrence of three copies of the consensus motif, one of them located at the extreme C-terminus thereby creating a CAAX domain for protein prenylation.

Alignment and phylogenetic analysis of HPP1 with its newly identified orthologues and a-type peptide pheromone precursors from other organisms indicates that HPP1 represents a novel variant in the order Hypocreales. However, the low conservation of these proteins considerably decreases the reliability of this analysis (data not shown). Similarly, we also cannot rule out that small proteins with similar characteristics remain to be detected in other fungi, but escaped our screening. HPP1 shows no significant similarity to genes available in GenBank or present in other than the above-mentioned fungal genome databases.

Hypocreia jecorina has a normal α-factor-like peptide pheromone precursor

The finding of an unusual a-type pheromone precursor prompted us to analyse whether the second type of peptide pheromone precursors would match the common characteristics for α-type peptide pheromone precursors. A 286-amino-acid polypeptide with distant homology to other α-type peptide pheromone precursors was found to be encoded by a gene located on scaffold 3 (tre9948; Protein ID: 104292) in the Trichoderma reesi genome database v2.0. An N-terminal signal sequence is predicted for this protein [SignalP; http://www.cbs.dtu.dk/services/SignalP/; (Emanuelsson et al., 2007)]. It comprises four copies of the 11 aa presumed mature pheromone WCYRIGEPCW (Fig. 2), which fits the consensus of WCXGXGXXCW (Zhang et al., 1998; Shen et al., 1999). As in many α-type pheromone precursors, also Kex2-cleavage sites (Rockwell et al., 2002) are present in the respective ORF. The α-type pheromone precursors of S. cerevisiae are known substrates of the Kex2-protease (Fuller et al., 1988)
which has been shown to be essential for normal mating by α-strains in S. cerevisiae (Leibowitz and Wickner, 1976). The promotor of this gene comprises three pheromone response elements (Hartmann et al., 1996; Urban et al., 1996) at −949, −733 and −459 relative to the ATG. Based on these characteristics, we denominated this putative α-type pheromone precursor gene as ppg1 (peptide pheromone precursor gene 1).

No further gene corresponding to the characteristics of α-type pheromone precursors was found in the genome sequence of H. jecorina. We conclude that it is the only α-type pheromone precursor gene of H. jecorina.

The locus of hpp1 differs from those found in related species

As the gene encoding the respective a-factor pheromone precursor gene was found in the vicinity of a cyanate lyase gene in N. crassa, M. grisea and G. zeae (F. graminearum) (http://www.broad.mit.edu/annotation/fgi/), we expected the locus of hpp1 to be similar to those fungi. We performed a detailed analysis and comparison of the respective area with the loci in N. haematococca, G. moniliformis (F. verticillioides), F. oxysporum, G. zeae (F. graminearum), E. festucae and N. crassa including four flanking genes: one encoding a MYND-zinc finger protein (contains domain IPR002893), one homologue of the rRNA processing protein 2 (EBP2) and two hypothetical proteins designated A and B. This analysis revealed that neither a cyanate lyase homologue nor hpp1 nor another CAAX-domain protein was present within this locus in H. jecorina, while the flanking genes (hypothetical proteins A and B) are syntenic with N. haematococca, G. zeae and E. festucae (Fig. 3; for genomic coordinates of the respective genes see Table S1). A similar genomic structure as in the Fusarium spp. was detected in M. grisea and Chaetomium globosum. Also in these genomes potential a-type pheromone precursors comprising CAAX domains were found downstream of the respective cyanate lyases (C. globosum: MPSTTTQTKVPQSTNFNGY; M. grisea: MSPSTKNIAPVAGRAPHYCVM; see also Table S1). In P. anserina, the arrangement is slightly altered, but the locus comprises the same genes as mentioned above with the peptide pheromone precursor downstream of cyanate lyase and the EBP2 and MYND-zinc finger protein homologues next to each other (Table S1). The arrangement of the genes in this area in H. jecorina suggests a genomic rearrangement event, which led to the loss of both the cyanate lyase homologue and the original a-factor peptide pheromone precursor gene in H. jecorina (Fig. 3).

Because of the assumed genome rearrangement or recombination event it cannot be excluded that an ORF encoding a small CAAX-domain protein might have been retained within the area upstream of the promotor of the EBP2 homologue. We therefore searched all six reading frames within 1.5 kb of this area, but did not find ORFs
meeting the requirements for a putative a-factor pheromone precursor. The same analysis was done for H. atroviridis, H. virens and all other fungi included in Fig. 3. If a cyanate lyase was found in the respective organism also the genomic area downstream of this gene was screened. The area between the cyanate lyase orthologue and the EBP2 orthologue in N. haematococca comprises an ORF coding for a protein with intriguingly similar characteristics to HPP1, i.e. the repeated motif and the CAAX domain. Nevertheless, within the respective region also one additional small CAAX domain (Fig. 1A, in brackets) protein is encoded, which could also function as a peptide pheromone precursor, although the respective HPP1 orthologue better fits the a-type consensus. Besides this gene, we did not detect a further pheromone precursor candidate in this analysis in any fungus analysed.

hpp1 is located at an unusual locus in the genomes of Hypocrea (Trichoderma) spp.

Interestingly, the loci containing the Hypocrea h-type peptide pheromone precursors are not syntenic with Neuropora, Epichloe or Fusarium spp. Within the H. jecorina genome, hpp1 is located on scaffold 18 between tre22465, a putative homologue of S. cerevisiae sec23 possibly involved in ER-Golgi trafficking and a yet uncharacterized 14-3-3 domain protein probably involved in intracellular signalling (Fig. 4). Homologues of the genes flanking hpp1 have been found in H. virens and also a small ORF encoding a CAAX-domain protein with certain similarity to HPP1 (44% identity) was detected. The same analysis was done with the genome of the more distantly related H. atroviridis, but despite an expected nt-sequence similarity of approximately 80% to H. jecorina no ORF with significant similarity to hpp1 was found in this fungus. Nevertheless the area between the respective sec23 and the 14-3-3 orthologue revealed a CAAX-domain protein of comparable size with HPP1 and also at approximately the same distance to its flanking genes, but as expected without significant similarity to HPP1 (Figs 1A and 4). This protein contains only one h-type consensus motif and a recognition sequence for the Kex2-protease. Moreover, ESTs available on the H. atroviride genome homepage do not unequivocally support the functional transcription of this gene, because they would suggest transcription of another gene on the opposite strand. Therefore, devoid of further experimental support, assignment of this protein to h-type peptide pheromone precursors must be considered preliminary. Further analyses are necessary in order to elucidate whether these CAAX-domain proteins also assumed the function of the a-type peptide pheromone precursor in H. virens and H. atroviridis. In order to clarify, whether in N. crassa, E. festucae and Fusarium spp. the area around...
the genes homologous to those flanking hpp1 could also be a potential site of (presumably) dormant CAAX-domain protein encoding genes, we searched the respective regions and found appropriate ORFs in N. crassa (sequence: MFIIRIRRAYVASSRLERRIVGCVLT; ORF found in reverse orientation within the second intron of the ORF of NCU01317, a gene encoding a 60S ribosomal protein L12) downstream of the sec23 homologue, as well as two such ORFs in the vicinity of the sec23 homologue (sequence: MPPLLMPLKLCWID; upstream) and the 14-3-3 homologue (sequence: MVASRAQSCALK; downstream) in G. zeae (F. graminearum). Those genes however, did not comprise the triple motif of HPP1. Moreover the ORFs found in G. zeae are upstream (sec23) or downstream (14-3-3) of the respective gene, which – considering the locus in Hypocrea spp. – does not fit the order of genes in the hpp1 locus. No ORFs for CAAX-domain proteins were found in these regions in E. festucae and Aspergillus nidulans.

Hence, the different loci yet similar characteristics of the h-type peptide pheromone precursors in Hypocrea spp. and Fusarium spp. rule out that the presence and functionality (see below) of HPP1 in H. jecorina represents an evolutionary artefact due to the deletion in the original a-type locus in Hypocrea spp.

HPP1 is essential for male fertility

Although female sterility of the T. reesei/H. jecorina nature isolate QM6a causes certain inconveniences with the application of sexual development for strain improvement, the female sterile background of QM6a and all its progeny (including the frequently used QM9414) allows for investigation of the influence of putative regulators or conditions on male fertility. In case of a negative effect of a deletion on male fertility we would observe complete sterility of the respective strain. Thus, by comparison with the effect in the female fertile CBS999.97, it is possible to unequivocally distinguish between influences on male and/or female fertility.

For a functional characterization of hpp1, we deleted the genomic region spanning the whole cDNA of hpp1 in the female sterile strain QM9414 (MAT1-2) (Fig. 5) and retransformed the respective deletion mutant with a wild-type copy. No significant effects on growth or conidiation could be detected in any of these strains.

We screened for differences in the behaviour of H. jecorina CBS999.97 MAT1-1 upon confrontation with the female sterile strain QM9414 (MAT1-2) containing wild-type hpp1 or the mutant strain deleted for the putative a-type peptide pheromone gene hpp1 respectively, as
described by Seidl et al. (Seidl et al., 2009). Additionally, we used a strain in which the cAMP-dependent phosphorylation site of HPP1 was rendered unfunctional (hpp1T40A), in order to test whether phosphorylation of HPP1 is relevant for its function in sexual development. Replacement of the threonine within the putative cAMP-dependent phosphorylation site of HPP1 by alanine prevents phosphorylation and hence renders this phosphorylation site unfunctional.

As analysis of transcription of hpp1 in the strain env1PAS- (Schmoll et al., 2005), which lacks the PAS domain of the light regulatory protein ENV1, revealed a strong upregulation of hpp1-transcription upon cultivation in light (Fig. 6) we also added this strain to our analysis. In this test we observed that – in accordance with our earlier study (Seidl et al., 2009) – fruiting body formation on the border of confrontation with H. jecorina CBS999.97 started after day 5 for QM9414. Figure 7 shows the beginning of fruiting body formation on day 6 and mature fruiting bodies on day 14 after inoculation. The formation of stromata did not occur with the hpp1 deletion strain Δhpp1, whereas it was enhanced and started earlier (as can be observed on day 6) as compared with the wild-type strain QM9414, with the strain env1PAS- that overexpresses hpp1 (see also below). However, we presently cannot be sure whether this is a direct or indirect effect of env1, because this gene has numerous regulatory targets (Schuster et al., 2007). Fruiting body development and ascospore discharge were indistinguishable from wild type in hpp1T40A and we therefore conclude that the cAMP-dependent phosphorylation site of HPP1 is not relevant for sexual development.

In order to obtain information on the potential processing of HPP1, we tested whether the two KEX2-protease processing sites might be essential for the function of HPP1. As loss of the positive charge at amino acid position P1 results in a drastic drop in catalytic efficiency at this site (Rockwell et al., 1997; 2002), we exchanged the P1 residue in the respective site for asparagine (uncharged) in each site individually, resulting in strains hpp1R22N and hpp1K38N and in both sites, resulting in strain hpp1DX. We used two to six independent transformants for every mutation to check for fertility by crossing with CBS999.97 MAT1-1. Crosses were repeated twice.

Fig. 6. Transcription of hpp1 in wild type and an ENV1 mutant strain env1PAS-. Strains were grown for 24 h in constant darkness and mycelia were harvested after illumination for the time given in the figure. For Northern blots, 20 μg of RNA were loaded per lane, and hybridizations were performed with PCR fragments spanning the ORF of hpp1. Hybridization with an 18S rRNA probe is shown as a control.

Fig. 7. Fruiting body formation in mutants of hpp1.
A. Schematic representation of the experimental setup for induction of sexual development between CBS999.97 (MAT1-1) and MAT1-2 strains derived from QM9414 to be tested for fruiting body formation.
B. Perithecia formation on the border of confrontation between H. jecorina CBS999.97 (MAT1-1), the wild-type strain H. jecorina QM9414 (MAT1-2), Δhpp1 (MAT1-2), hpp1T40A (MAT1-2) and the mutant deleted for the PAS domain of the light regulatory protein ENV1, env1PAS- (MAT1-2). Strains were grown on malt extract agar for the time indicated. The plate assays were done at least in triplicates; one representative plate is shown in the figure.

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Despite lack of one or both KEX2-processing sites, none of the mutations abolished fruiting body formation or ascospore discharge (Fig. S1). Hence, we conclude that either the KEX2-processing sites in HPP1 are not essential for processing or the unprocessed pheromone precursor is still recognized by the mating partner.

Wild-type behaviour was completely restored in an hpp1 retransformant. Thus, we conclude that HPP1 represents the only functional a-type peptide pheromone precursor of *H. jecorina* that obviously has assumed a-type function and is essential for male fertility of QM9414. As neither fruiting body formation nor ascospore discharge was altered in a strain deleted for ppg1 (Fig. S2), this gene – as expected – does not play a role in male fertility of QM9414 (MAT1-2).

**HPP1 does not generally interfere with sexual development**

As deletion of hpp1 abolished mating of QM9414 (MAT1-2) with *H. jecorina* CBS999.97 MAT1-1, the question arose, whether it could have a more general function in sexual development and would possibly be essential for this process. Because of the female sterility of QM9414 [which is a derivative of the female sterile QM6a described in (Seidl et al., 2009)] we could not distinguish, if hpp1 would only be essential for male fertility (as would be expected for an a-type peptide pheromone) or if it would have a more general function in sexual development. Therefore, we constructed deletion strains of hpp1 in a female fertile background (*H. jecorina* CBS999.97 MAT1-1 and MAT1-2) and analysed the influence of lack of hpp1 on sexual development in these strains. In both cases, we found that sexual development (fruiting body formation and ascospore discharge) is not perturbed (Fig. 8) and hence we conclude that hpp1 is essential for male fertility, but not sexual development in general. This result further confirms that despite its unusual structure, hpp1 has assumed the function of an a-type peptide pheromone in *H. jecorina*.

**hpp1 and ppg1 are transcribed during sexual development**

For the heterothallic ascomycetes *N. crassa* (Bobrowicz et al., 2002), *M. grisea* (Shen et al., 1999) and *C. parasitica* (Zhang et al., 1998), it was shown that the peptide pheromone precursors are expressed in a mating-type-specific manner. In order to test whether this is also the case for the heterothallic *H. jecorina*, we analysed transcription of both peptide pheromone precursors during development in QM9414 (MAT1-2), QM9414 Δhpp1 (MAT1-2), CBS999.97 MAT1-1 and deletion mutants of hpp1 in CBS999.97 MAT1-1 and MAT1-2 in daylight before contact (day 3), at contact (day 4), after contact (day 5/6) and after fruiting body formation (day 7/8) (Fig. 9).

Interestingly, we found that both hpp1 and ppg1 are transcribed during development of *H. jecorina* and that this transcription is not dependent on the mating type, neither for hpp1 nor for ppg1. Nevertheless, transcription of hpp1 appears to be slightly enhanced in MAT1-2 strains. In support of the enhanced fruiting body formation upon confrontation of *env1<sup>PAS</sup>* (MAT1-2) with CBS999.97 MAT1-1 as described above, transcription of hpp1 is enhanced in *env1<sup>PAS</sup>* as compared with CBS999.97 MAT1-1 under these conditions. Presence or absence of hpp1 in the genome of the mating partner only had a slight, if any influence on transcription of hpp1 or ppg1 in the other partner.

Interestingly, we found pheromone response elements both in the promoters of hpp1 and ppg1 (see above), thereby offering a possible explanation for the transcription of ppg1 in this strain, which would not be expected in
Nevertheless, it should be kept in mind that the function of pheromone response elements has only been shown in the basidiomycete U. maydis (Hartmann et al., 1996; Urban et al., 1996) and is not necessarily comparable in ascomycetes.

**Discussion**

Sexual development represents one of the most important accomplishments in evolution. It is predominantly meant to improve the chances of survival of a species in a MAT1-2 strain. Nevertheless, it should be kept in mind that the function of pheromone response elements has only been shown in the basidiomycete U. maydis (Hartmann et al., 1996; Urban et al., 1996) and is not necessarily comparable in ascomycetes.
changing environment, because recombination through meiosis enables enlarging the gene pool and hence acquiring features crucial for adaptation to the altered conditions. Thus, sexual development is a significant evolutionary benefit of a given organism and specifically in fungi. In contrast, numerous examples of obviously only asexually propagating fungi are known. Although this might be due to inappropriate experimental conditions in screening assays, it is equally possible that sexual development indeed does not occur or is only a very rare event with these organisms and thus of low relevance for survival. Also, the existence of truly asexual organisms is being challenged because of the lack of conclusive survival. Also, the existence of truly asexual organisms is with these organisms and thus of low relevance for development indeed does not occur or is only a very rare event with these organisms and thus of low relevance for survival. Also, the existence of truly asexual organisms is being challenged because of the lack of conclusive methods to prove asexuality (Taylor et al., 1999). T. reesei was initially only known from a single isolate (QM6a) and all strains currently in use in research and industry are derived from this single isolate. Despite the elucidation of the teleomorph of T. reesei more than a decade ago (Kuhls et al., 1996), sexual development of the fungus has only recently received closer attention. In this respect the case of H. jecorina (T. reesei) QM6a is intriguing, as this wild-type isolate is female sterile (Seidl et al., 2009) and appears to have lost its original a-type peptide pheromone precursor, the latter phenomenon being a common characteristic in the genus Hypocrea.

HPP1 was initially identified in a study towards elucidation of the signalling process related to cellulase induction (Schmoll et al., 2004). It was found to be differentially regulated between a cellulase producing and a non-producing strain. This raises the question as to whether hpp1 may – besides acting as a pheromone – also be involved in the regulation of expression of cellulase genes. Although some physiological functions beyond mating have been suggested for peptide pheromone precursors (Spellig et al., 1994; Willer et al., 1995; Chikashige et al., 1997; Debuchy, 1999), an involvement in regulation of biosynthesis of extracellular enzymes has not been reported so far. Hence, it cannot be excluded that initiation of fertilization and availability of nutrients might be connected by some regulatory pathways.

Divergence between related species has been suggested to proceed by inversions of small genomic regions, which is supported by studies on yeasts (Seoighe et al., 2000; Huynen et al., 2001) as well as by comparison of the genomes of P. anserina and N. crassa (Silar et al., 2003). These genomic rearrangements, which affect the architecture of a genome and represent important evolutionary events are suggested to primarily occur at the replication forks and cause inversions, translocations and deletions (Andersson, 2000; Tillier and Collins, 2000). The fact that an essentially similar locus and also a similar rearrangement of genes in the expected a-type pheromone locus in H. jecorina was found both in H. virens and H. atroviridis, eliminates the possibility that the loss of an original a-type peptide pheromone precursor gene occurred during strain improvement of the parent strain H. jecorina QM6a to QM9414, the strain used in this study. This locus, as well as the potential translocation of hpp1, seems to be unique to Hypocrea; hence, these characteristics rather represent an early evolutionary development in this genus. As such translocation sites are often flanked by inversions or losses and gains of single genes (Tillier and Collins, 2000), this would also be a consistent explanation for the obvious deletion of the cyanate lyase and potentially the CAAX-domain protein in its vicinity observed in the locus syntenic to the a-type peptide pheromone precursor gene locus in other fungi. However, given the altered structure of HPP1 we cannot be sure whether the original a-type peptide pheromone precursor of H. jecorina was lost during this rearrangement and HPP1 has assumed its function or if a translocation event only led to the locus different from its orthologues (with similar characteristics) in other fungi. Interestingly, despite the synteny of the flanking genes (the sec23 orthologue and the 14-3-3 protein encoding gene) in Hypocreales, no indications for the involvement of a transposon have been detected. The genome of H. jecorina indeed contains traces of different types of transposons, but no active transposon was found (Martinez et al., 2008) and none of the candidates is located in proximity to the hpp1 locus.

Pheromones are conserved proteins because of their similarity in other homothallic and heterothallic fungi (Poggeler, 2000). A comparison of the a-type peptide pheromone precursors from several organisms with the here proposed h-type peptide pheromones shows that the consensus for the a- and h-type pheromone precursors (so far only detected in closely related species) is rather loose, which contrasts with the high conservation of the α-type pheromone. It therefore seems possible that further h-type peptide pheromone precursors comprising three similar motifs, one of them constituting a CAAX domain at the C-terminus exist in other fungi, but have escaped detection so far because their repeated motif does not exactly match the consensus sequence developed in this study. At the present stage, we cannot rule out that the h-type peptide pheromone precursors described here represent a special variety of a-type peptide pheromone precursors, as they seem to have replaced the typical a-type gene in most cases. Moreover, investigation of maturation of these pheromone precursors will reveal
whether their processing rather resembles α- or α-type pheromone precursors. Nevertheless, their distinctive structure stands out and necessitates unequivocal differentiation from known peptide pheromone precursors, even if they would turn out to be closely related to the known proteins.

It has been suggested for α-factor export that recognition by the responsible yeast transporter (Ste6p) may be solely dependent on the prenyl- and methyl-CAAX modifications, which would thus tolerate considerable variability in the structure of the α-type pheromones, although also a certain influence of the sequence has been reported (Huyer et al., 2006). This hypothesis was also supported by the finding that S. cerevisiae could process and functionally express the Coprinopsis cinerea α-factor pheromone precursor (Olesnicky et al., 1999). Therefore, processing of the HPP1-precursor protein resulting in a prenylated CAAX domain is likely to result in the functional pheromone.

While efforts to elucidate the nature of the natural mature and functional HPP1 are currently in progress, determination of the mechanism of HPP1 processing and export will still require future studies, because several possibilities for this process could be deduced from literature: It could be assumed that due to the detected KEX2-peptidase sites, intrinsically characteristic for α-type pheromone precursors, the maturation process of HPP1 resembles that of α-type peptide pheromones. If so, the mature pheromone would not comprise the C-terminus including the CAAX domain, which is essential for α-type peptide pheromone precursors and thus for the observed function of HPP1. Moreover, this processing would not result in similar peptides characteristic for α-type pheromones. Consequently our results, that the KEX2-peptidase sites are not essential for the function of HPP1 are in agreement with the hypothesis of an alternative maturation pathway which, however, does not rule out that these peptidase sites have functions other than in sexual development.

For Coprinus cinereus the presence of an acidic-basic pair was shown to be essential for pheromone function of the peptide, while C-terminal prenylation only increased the efficiency of the pheromone (Olesnicky et al., 1999). As neither the acid-basic pair nor the KEX2-protease sites have been detected in all proteins fulfilling the h-type characteristics, we consider it possible, that – alternative to the suggested processing sites – a maturation process comparable with that of rhodotorucine A in the basidiomycetous yeast Rhodosporidium toruloides (Akada et al., 1989) and to the peptide pheromone precursors in Tremella spp. (Sakagami et al., 1981) may take place. The precursor protein of rhodotorucine A is a mixture of tandem pheromone sequences separated by CAAX-like spacers and represents an apparent union of the two separate forms of pheromone gene structure adopted by S. cerevisiae for the α- and α-factor genes. Processing of these precursors results in multiple mature peptide pheromones. Considering the characteristics of HPP1, a similar mechanism could also lead to the production of up to three prenylated peptides from the h-type pheromone precursor proteins. Analysis of the structures of the h-type peptide pheromones identified in this study (Fig. S3) showed that comparison with the structure and maturation of rhodotorucine A allows for a hypothesis on mature peptide pheromones in Fusarium spp. However, for Hypocrea spp. we can only speculate that the peptidase responsible for processing should act upstream of the consensus motif in a way that the mature peptide would comprise several amino acids additional to the consensus motif after prenylation. Still, in both cases, experimental information on mature peptide pheromones will be necessary to understand the respective maturation process. Unfortunately the enzymes responsible for the maturation of rhodotorucine A are not known and thus the presence and function of such a mechanism in species containing h-type pheromone precursor genes can at this time only be subject to speculation. Nevertheless, the structural similarities in peptide pheromone precursors between these basidiomycetes and species belonging to Hypocreales raise the question whether lateral transfer from basidiomycetes to an early ancestor of Hypocreales might be the reason for the presence of h-type peptide pheromone precursors within ascomycetes.

Interestingly, we found that both types of peptide pheromone precursors are transcribed during development in H. jecorina. In case of hpp1, this could be attributed to the unusual locus of the gene, which might have resulted in an altered expression pattern. However, although hpp1/HPP1 shows some unusual characteristics, our data provide substantial evidence that HPP1 has assumed the function of an α-type peptide pheromone precursor. Hence, its atypical expression pattern is unlikely to be due to HPP1 not being an α-type peptide pheromone precursor. Also the more or less constitutive transcription of ppg1 in both QM9414 and CBS999.97 was rather unexpected. Nevertheless, ppg1 seems not to interfere with the initiation of a mating response, which is in accordance with a study on post-transcriptional regulation of peptide pheromone expression in P. anserina (Coppin et al., 2005). Concordant with these findings, the result, that deletion of hpp1 in a female sterile background abolishes perithecia formation upon contact with fertile strains as well as the phenotype of the ppg1 deletion strain, rule out that PPG1 plays a role in the interaction of the MAT1-2 strain QM9414 with CBS999.97 (MAT1-1). As both peptide pheromone precursors are obviously not transcribed in a strictly mating-type-dependent manner, this can be considered rather a characteristic of H. jecorina.
than a hint as to a function other than pheromone precursors. It will be interesting to learn, how the presence of the mature HPP1 peptide as a mating signal is perceived, because it cannot unequivocally be assigned to either a- or α-type peptide pheromones and thus it can hardly be predicted which receptor is responsible for reception of this signal.

The lack of a common a-type peptide pheromone precursor gene in the Hypocrea spp. analysed in this study, initially suggested a fortunate recovery of the sex pheromone, which had been lost in a previous genomic rearrangement or recombination event. However, as proteins with similar characteristics as HPP1, also sharing the consensus sequence have been found in other fungi, which retained the genomic region presumably lost in Hypocrea, this hypothesis is highly unlikely. Most importantly, these genes are located within the syntenic genomic area, which comprises the a-type peptide pheromone precursor in closely related fungi, such as N. crassa and M. grisea. With the exception of N. haematococca, this locus did not contain an additional, typical a-type peptide pheromone precursor, if an h-type was detected. Notably, the CAAX-domain protein of N. haematococca located downstream of the h-type protein, does not correspond well to the consensus sequence published for a-type peptide pheromone precursors (Zhang et al., 1998; Shen et al., 1999) and may thus be non-functional.

Interestingly, all fungi containing an HPP1 orthologue are heterothallic, except F. oxysporum. However, this reportedly asexual fungus contains a set of MAT genes highly similar to those of the heterothallic Gibberella fujikuroi and unexpectedly these MAT genes are also expressed in F. oxysporum (Yun et al., 2000). Therefore, F. oxysporum may share characteristics of heterothallic fungi, although sexual development has not been reported so far. G. zeae, however, has a peptide pheromone precursor strikingly similar to the other Fusarium spp., but this protein comprises only one copy of the h-type consensus sequence. Therefore, it would not be considered an h-type peptide pheromone precursor, which would require three copies. Hence, we hypothesize that HPP1 could be a heterothallism-specific peptide pheromone precursor. Nevertheless, no protein with h-type characteristics has been detected in the heterothallic E. festucae, but again we found only one h-type consensus motif. Therefore, the occurrence of the h-type pheromones seems to be not a strict rule for heterothallic Hypocreales, but rather a peculiarity within this group. Consequently, only a detailed analysis of the genomes of several further homothallic and heterothallic species will reveal whether this hypothesis is admissible.

While the functionality of H. jecorina HPP1 as a peptide pheromone has been shown in this study, a similar function remains to be established for the other members of the novel group of h-type peptide pheromones. It will also be interesting to learn, whether proteins sharing these characteristics are present in other fungi and if their specific structure indicates functions beyond attracting a mating partner. Their size, and – besides the triple motif – low conservation as well as the lack of characterized domains in these small proteins may have prevented their discovery, as potential members of this class are unlikely to be found using standard tblastn searches. With the availability of further genomes of homothallic, heterothallic and asexual fungi it will also be possible to evaluate the specificity of h-type pheromones for heterothallic fungi. Further studies on these proteins and their functions will presumably reveal intriguing new insights into the mechanisms and determinants of mating in fungi.

Experimental procedures

Microbial strains and culture conditions

Hypocrea jecorina (T. reesei) wild-type strain QM9414 (ATCC 26921) as well as a strain lacking the PAS domain of ENV1 [env1PAS-; (Schmoll et al., 2005)] were used throughout this study. For the analysis of sexual development and mating response strain H. jecorina CBS999.97 (CBS, Utrecht, the Netherlands) was used. This strain (originally named G. J. S. 97-38) is phylogenetically close to H. jecorina QM6a, the predecessor of QM9414 and produces perithecia in axenic culture (Lieckfeldt et al., 2000). As the initial isolate deposited at CBS is a mixture of both mating types, purified and confirmed MAT1-1 or MAT1-2 strains derived from this isolate (Seidl et al., 2009) were used for this study. Strains were propagated on 3% (w/v) malt extract agar plates at 22°C in daylight to promote fruiting body formation. For transcript analysis, plates were covered with cellophane to facilitate harvesting of mycelia.

Escherichia coli JM109 (Yanisch-Perron et al., 1985) and E. coli ER1647 (Novagen, Madison, WI, USA) were used for DNA manipulations.

Analysis of fruiting body formation

The strains were grown on malt extract agar. The test for initiation of fruiting body formation was done as described in (Seidl et al., 2009). Strains were inoculated on opposite sites of a malt extract agar plate and growth in daylight at 22°C was monitored for 14 days. All experiments were done at least in triplicates.

Screening of a chromosomal library of Hypocrea jecorina QM9414

Isolation of chromosomal clones was performed according to the manufacturer’s protocol (ΔBlueSTAR™ Vector System, Novagen). The Trichoderma reesei genome database (http://genome.jgi-psf.org/Trire2/Trire2.home.html) was used to evaluate the sequence.

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Nucleic acid isolation and blotting

Stains grown on malt extract agar plates in daylight at 22°C were harvested every 24 h as indicated in the legend to the figure. For DNA isolation, strains were grown in liquid malt extract medium, harvested by filtration and washed with tap water. Mycelia were snap frozen and ground in liquid nitrogen. DNA and RNA were isolated as described previously (Schmoll et al., 2004). Standard methods (Sambrook et al., 1989) were used for electrophoresis, blotting and hybridization of nucleic acids.

Reverse transcriptase polymerase chain reaction

Five micrograms of total RNA was treated with DNase I (Fermentas, Vilnius, Lithuania), then the reaction was terminated by adding EDTA to a final concentration of 2.5 mM and incubation at 65°C for 10 min. The RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) was used for first strand synthesis according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplification was done with GoTaq Polymerase (Promega, Madison, WI, USA) and primers hppF and hppR spanning the reading frame of hpp1 for 31 cycles, primers ppg1F and ppg1R for amplification of ppg1 for 33 cycles and primers tefF and tefR for amplification of tef1 for 25 cycles. For all primer sequences see Table 1. Total RNA (containing residual DNA) was used as positive control and DNase I treated RNA as negative control for PCR. Thirty-three PCR cycles were used for these controls. Individual samples were used for the positive control and yielded a fragment in the expected size. For the negative control pooled samples were used, along with a no template control, none of which showed an amplified fragment.

Preparation of hpp1 deletion strains and retransformation

To obtain a deletion mutant of hpp1, QM9414 was transformed with plasmid pDELhpp1 in which the sequence spanning the complete mRNA of hpp1 as determined by RACE is replaced by the E. coli hph gene under H. jecorina expression signals.

Table 1. Primers used throughout the study.

| Name         | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| hppF         | 5′-AATTCACAATGGCTCAAC-3′                                                   |
| hppR         | 5′-GCTTGAAGCTGTGTGTTACGTG-3′                                              |
| ppg1F        | 5′-TGGAGAGCAGAAGGGAAGACTG-3′                                              |
| ppg1R        | 5′-GGCAGTGTGGTGTAGGGA-3′                                                  |
| tefF         | 5′-AGAAGGTCGCTCTTACCCCCC-3′                                               |
| tefR         | 5′-GGTATCGGGAAGGACTCCTTC-3′                                               |
| DELHPP5F     | 5′-ATTGTCACCGCTTGTTATGG-3′                                                |
| DELHPP5R     | 5′-AGAAGTTCGTGGCCCTCCTG-3′                                                |
| DELHPP3F     | 5′-GTCAGGTGGCAAGAGAGCAG-3′                                               |
| DELHPP3R     | 5′-CAGGAGGCCTGCAAGGACTTTC-3′                                             |
| matA2fw      | 5′-GCCACACGCTATTCTAGTGG-3′                                                |
| matA2rv      | 5′-AGCTTGAAGGACTCCTTC-3′                                                  |
| hppT40A3F    | 5′-AGAAGTTCGTGGCCCTCCTG-3′                                                |
| hppX5F       | 5′-GTCAGGTGGCAAGAGAGCAG-3′                                               |
| hppT40A5R    | 5′-CAGGAGGCCTGCAAGGACTTTC-3′                                             |
|hppORF1F      | 5′-ATTTCACAATGGCTCAAC-3′                                                   |
| hppORF1R     | 5′-AGGCTTGAAGGACTCCTTC-3′                                                 |
| HPaFull3F    | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPAx3R       | 5′-CAACCTACCGACTGAAAGACG-3′                                               |
| HPAx5F       | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPAFull5R    | 5′-GTCAGGTGGCAAGAGAGCAG-3′                                               |
| HPA1NF       | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPA1NR       | 5′-CAACCTACCGACTGAAAGACG-3′                                               |
| HPakr3F      | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPakr5R      | 5′-CAACCTACCGACTGAAAGACG-3′                                               |
| HPAkks5F     | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPAkk3F      | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPP1RACE-A   | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPP1RACE-N   | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| RASH18PB     | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| RACE-N       | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |
| HPP1RACE3    | 5′-TCGAGAGGAGAACCTTATGGCTGCAGCGTCATGAC                                   |

Restriction site introduced to facilitate vector construction are underlined; mutations are given bold and underlined.
(Mach et al., 1994). This vector was constructed as follows: a 769 bp fragment of the 5′ flanking region of hpp1 was amplified by PCR using primers DELHPPS5F and DELHPPS5R, the amplicons cleaved with Acc65I and Xhol (all restriction enzymes by Fermentas) and cloned into the Acc65I–Xhol sites of pBSXH (Schmoll et al., 2005), which contains the pkI promoter cassette from pRLMex30 resulting in pDELhpp1H5. Thereafter, a 1275 bp fragment of the 3′ flanking region of hpp1 was amplified by PCR using primers DELHPP3F and DELHPP3R and cloned into the HindIII–PstI sites of pBluescript SK+®. The resulting plasmid cleaved with HindIII and SpeI, and the excised fragment thus obtained finally cloned into the respective sites of pDELhpp1H5 resulting in pDELhpp1. The plasmid was linearized by restriction digestion with SpeI and 10 μg were used for transformation (Gruber et al., 1990). Transformants were selected on plates containing 50 μg ml−1 hygromycin B (Calbiochem, Merck KGaA, Darmstadt, Germany). Fungal DNA was isolated from transformants using standard protocols and subjected to Southern analysis (using AccI) to verify replacement of the gene and the presence of no additional copies of the deletion cassette (Fig. 5). Two deletion strains (∆hpp1-26 and ∆hpp1-32) were used and yielded similar results.

Complementation of ∆hpp1 with the hpp1 wild-type gene was achieved by cotransformation of a PCR fragment amplified with primers DELHPPS5F and DELHPP3R comprising the entire gene and the amdS marker cassette (Penttilä et al., 1987; Seiboth et al., 1997). Successful complementation was confirmed by PCR using primers hppF and hppR (see above). Wild-type behaviour was restored in the transformants.

Deletion of hpp1 in H. jecorina CBS999.97 (MAT1-2) was accomplished essentially as described above for H. jecorina QM9414. To obtain a deletion mutant of hpp1 in H. jecorina CBS999.97 (MAT1-1) the single spore isolate of the primary deletion strain H. jecorina CBS999.97 (MAT1-2) ∆hpp1 was crossed with the H. jecorina CBS999.97 (MAT1-1) wild-type strain. Progeny was isolated as described previously (Seidl et al., 2009). Hygromycin resistant single-ascospore clones were analysed by PCR to verify the deletion of hpp1. In order to determine the mating type of ascospore clones, primer pairs binding within the MAT1-1 or MAT1-2 mating-type loci were chosen. For the MAT1-1 mating-type locus the primers matA1F and matA1R were used. For 5′ and 3′ RACE, first strand synthesis was performed using a gene-specific primer (HPP1RACE-A). Single-stranded DNA of QM9414 and 32 as positive and negative controls was used and yielded similar results.

Construction of strains lacking one or both KEX2-processing sites

The vectors for complementation of ∆hpp1 with expression of altered versions of HPP1 lacking the first, the second or both KEX2-processing sites were constructed using a similar approach as described above.

For deletion of both KEX sites the PCR products amplified with primers HPAPsU5F and HPAx5R or HPAx5F and HPAPsU5R respectively, were purified, combined in equimolar amounts and used as template for a nested PCR using primers HPA1NF and HPA1NR. The resulting fragment was digested with BsiWI and Sall and ligated into Acc65I/Xhol digested pBSamdS, which contains an Xbal/Sall amdS fragment integrated into the corresponding sites of pBluescript SK+® (Stratagene).

Strategies for deletion of every individual KEX2 site were similar except that for deletion of the first KEX2 site (KR), primers HPAx5F and HPAkr5R or HPAkr3F and HPAx3R were used for primary PCR reactions. For deletion of the second KEX2 site (KK) primers HPAx5F and HPAkk5F or HPAkk3F and HPAx3R were used for primary PCR reactions. HPA1NF and HPA1NR were used for the secondary PCR in both cases and ligation into pBSamdS was done as described above.

All constructs were sequenced to confirm deletion of the KEX2 sites. Transformation cassettes were linearized using NotI and used for transformation into ∆hpp1-32 as described above.

5′ and 3′ RACE

For 5′ RACE, first strand synthesis was performed from 3 μg total RNA using the Reverse Transcription System (Promega) at 42°C for 1 h according to the manufacturers instructions with a gene-specific primer (HPP1RACE-A). Single-stranded DNA was digested using RNase A (Sigma-Aldrich, St Louis, MO, USA) and afterwards the reaction mixture was purified using the QIAquick PCR Cleanup Kit (QIAGEN). The DNA–RNA hybrid fragments were ligated into EcoRV digested pBLUESCRIPT (Stratagene, La Jolla, CA, USA) and PCR was performed with one nested gene-specific primer (HPPRACE-N) and RASH1PB, which binds to pBLUESCRIPT using Q-Taq and Q-Solution (QIAGEN). As a control chromosomal DNA was used to rule out, that contaminations of chromosomal DNA in the RNA-preparation result in false positives.

For 3′ RACE, first strand synthesis was performed using the Reverse Transcription System (Promega) according to the manufacturers protocol at 42°C for 1 h with primer sequences. The resulting plasmid (pHPPaaT40A) was linearized and used for cotransformation (Gruber et al., 1990) of ∆hpp1-32 with the amdS-marker gene cassette (Penttilä et al., 1987; Seiboth et al., 1997). Integration of the mutated copy of hpp1 into QM9414 ∆hpp1 was analysed by PCR using hppORF1F and hppORF1R, which amplify a 167 bp fragment comprising the coding region of hpp1, along with DNA of QM9414 and ∆hpp1-32 as positive and negative controls respectively.

Construction of strains bearing an amino acid exchange in HPP1 and retransformation

For the mutated copy bearing a non-functional phosphorylation site a T→A amino acid exchange at position 40 was introduced using a PCR-based strategy. Pfu-polymerase (Fermentas) was used for amplification of the overlapping fragments. An 897 bp 3′ fragment was amplified using primers hppT40A3F and hppX3R, and a 1089 bp 5′ fragment using primers hppX5F and hppT40A5R. The fragments were purified using QiAquick PCR purification columns (QIAGEN, Hilden, Germany), combined and used as template for amplification of the complete 1966 bp mutated copy of hpp1 with primers hppX5F and hppX3R. In this step, GoTaq Polymerase (Promega) was used to facilitate ligation.

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RACE-N. The reaction mixture was cleaned up using the QIAquick PCR Cleanup Kit (QIAGEN). PCR and Nested PCR were performed according to standard protocols using a gene-specific primer (HPPRACE3). The resulting PCR fragments were ligated into pGEM-T (Promega) and sequenced.

Bioinformatic analyses

Analysis of the genomic locus of hpp1 and the loci of peptide pheromone precursors in related organisms was done by the aid of the genome databases published by the Joint Genome Institute of the US Department of Energy (http://genome.jgi-psf.org) and the Broad Institute (http://www.broad.mit.edu/annotation/fungi/). All fungal genome databases available on these sites including ascomycetes, basidiomycetes, zygomycetes and chytridiomycetes were analysed. Additionally, the F. sporontrichoides EST-database at the University of Oklahoma (http://www.genome.ou.edu/) and the Epichloë festucae genome database at the University of Kentucky (http://www.endophyte.uky.edu) were used. Blast searches were done with the NCBI Blast server (http://www.ncbi.nlm.nih.gov/BLAST/). In any case, tblastn searches were done with the NCBI Blast server (http://www.ncbi.nlm.nih.gov/BLAST/). In any case, tblastn (E-value threshold set to 10.0; BLOSUM62, PAM70 or PAM30 matrix) was applied in order to prevent a false negative result due to the small size and low conservation of the respective proteins, which may thus have escaped automatic annotation. Bidirectional blast was applied to confirm homology. Search for pseudoreplications.

For the phylogenetic analysis amino acid sequences were aligned with CLUSTAL X 1.81 (Thompson et al., 1997) and then visually adjusted using GeneDoc (Nicholas and Nicholas, 1997). Phylogenetic analyses were performed in MEGA 4 (Tamura et al., 2007) using the minimum evolution (ME) approach. The reliability of nodes was estimated by ME bootstrap percentages (Felsenstein, 1985) obtained after 500 pseudoreplications.

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References

Aanen, D.K., and Hoekstra, R.F. (2007) Why sex is good. In Sex in Fungi – Molecular Determination and Evolutionary Implications. Heitman, J., Kronstad, J.W., Taylor, J.W., and Casselton, L. (eds). Washington, DC: ASM Press, pp. 527–534.

Akada, R., Minomi, K., Kai, J., Yamashita, I., Miyakawa, T., and Fukui, S. (1989) Multiple genes coding for precursors of rhodotoricin A, a farnesyl peptide mating pheromone of the basidiomycetous yeast Rhodosporidium toruloides. Mol Cell Biol 9: 3491–3498.

Andersson, S.G. (2000) The genomes gamble. Nat Genet 26: 134–135.

Bailey, T.L., and Elkan, C. (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology. Altman, R.B., Brutlag, D.L., Karp, P.D., Lathrop, R.H., and Sears, D.B. (eds). Menlo Park, CA: AAAI Press, pp. 28–36.

Bistis, G. (1981) Chemotropic interactions between trichogynes and conidia of the opposite mating-type in Neurospora crassa. Mycologia 73: 959–975.

Bistis, G. (1983) Evidence for diffusible, mating-type-specific trichogyne attractants in Neurospora crassa. Exp Mycol 7: 292–295.

Bobrowicz, P., Pawia, R., Correa, A., Bell-Pedersen, D., and Ebbole, D.J. (2002) The Neurospora crassa pheromone precursor genes are regulated by the mating type locus and the circadian clock. Mol Microbiol 45: 795–804.

Boeker, M., and Kahmann, R. (1993) Sexual pheromones and mating responses in fungi. Plant Cell 5: 1461–1469.

Chikashige, Y., Ding, D.Q., Imai, Y., Yamamoto, M., Haraguchi, T., and Hiraoka, Y. (1997) Meiotic nuclear reorganization: switching the position of centromeres and telomeres in the fission yeast Schizosaccharomyces pombe. EMBO J 16: 193–202.

Coppin, E., de Renty, C., and Debuchy, R. (2005) The function of the coding sequences for the putative pheromone precursors in Podospora anserina is restricted to fertilization. Eukaryot Cell 4: 407–420.

Davey, J. (1992) Mating pheromones of the fission yeast Schizosaccharomyces pombe: purification and structural characterization of M-factor and isolation and analysis of two genes encoding the pheromone. EMBO J 11: 951–960.

Debuchy, R. (1999) Internuclear recognition: a possible connection between euascomycetes and homobasidiomycetes. Fungal Genet Biol 27: 218–223.

Debuchy, R., and Turgeon, B.G. (2006) Mating-type structure, evolution and function in euascomycetes. In The Mycota I. Kues, U., and Fischer, R. (eds). Berlin: Springer-Verlag, pp. 293–323.

Debuchy, R., Bertaux-Lecellier, V., and Silar, P. (2010) Mating systems and sexual morphogenesis in ascomycetes. In Cellular and Molecular Biology of Fila-mentous Fungi. Borkovich, K.A., and Ebbole, D.J. (eds). Washington, DC: ASM Press, pp. 501–535.

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El-Gogary, S., Leite, A., Crivellaro, O., El-Dorry, H., and Eveleigh, D.E. (1989) *Trichoderma reesei* cellulase – from mutants to induction. In *Trichoderma Reeseei Cellulases: Biochemistry, Physiology, Genetics and Applications*. Kubicek, C.P., Eveleigh, D.E., Esterbauer, W., Steiner, W., and Kubicek-Pranz, E.M. (eds). London: Royal Society of Chemistry Press, pp. 200–211.

Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Protoc 2*: 953–971.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution 39*: 783–791.

Fuller, R.S., Sterne, R.E., and Thorner, J. (1988) Enzymes required for yeast prehormone processing. *Annu Rev Physiol 50*: 345–362.

Garnier, J., Osguthorpe, D.J., and Robson, B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol 120*: 97–120.

Glomset, J.A., Gelb, M.H., and Farnsworth, C.C. (1990) Prenyl proteins in eukaryotic cells: a new type of membrane anchor. *Trends Biochem Sci 15*: 139–142.

Gruber, F., Visser, J., Kubicek, C.P., and de Graaff, L.H. (1990) The development of a heterologous transformation system for the celluloletic fungus *Trichoderma reesei* based on a *pyrG*-negative mutant strain. *Curr Genet 18*: 71–76.

Hartmann, H.A., Kahmann, R., and Bolker, M. (1996) The pheromone response factor coordinates filamentous growth and pathogenicity in *Ustilago maydis*. *EMBO J 15*: 1632–1641.

Huyer, G., Kistler, A., Nouvet, F.J., George, C.M., Boyle, M.L., and Michaelis, S. (2006) *Saccharomyces cerevisiae* a-factor mutants reveal residues critical for processing, activity, and export. *Eukaryot Cell 5*: 1560–1570.

Huynen, M.A., Snel, B., and Bork, P. (2001) Inversions and the dynamics of eukaryotic gene order. *Trends Genet 17*: 304–306.

Imai, Y., and Yamamoto, M. (1994) The fission yeast mating pheromone P-factor: its molecular structure, gene structure, and ability to induce gene expression and G1 arrest in the mating partner. *Genes Dev 8*: 328–338.

Ivey, F.D., Hodge, P.N., Turnier, G.E., and Borkovich, K.A. (1996) The G alpha i homologe gna-1 controls multiple differentiation pathways in *Neurospora crassa*. *Mol Biol Cell 7*: 1283–1297.

Kim, H., and Borkovich, K.A. (2004) A pheromone receptor gene, *pre-1*, is essential for mating type-specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa*. *Mol Microbiol 52*: 1781–1798.

Kim, H., and Borkovich, K.A. (2006) Pheromones are essential for male fertility and sufficient to direct chemotropic polarized growth of trichogynes during mating in *Neurospora crassa*. *Eukaryot Cell 5*: 544–554.

Kim, H., Metzenberg, R.L., and Nelson, M.A. (2002) Multiple functions of *mfa-1*, a putative pheromone precursor gene of *Neurospora crassa*. *Eukaryot Cell 1*: 987–999.

Kim, H.K., Lee, T., and Yun, S.H. (2008) A putative pheromone signaling pathway is dispensable for self-fertility in the homothallic ascomycete *Gibberella zeae*. *Fungal Genet Biol 45*: 1188–1196.

Kothe, E. (1999) Mating types and pheromone recognition in the Homobasidiomycete *Schizophyllum commune*. *Fungal Genet Biol 27*: 146–152.

Kozak, M. (1996) Interpreting cDNA sequences: some insights from studies on translation. *Mamm Genome 7*: 563–574.

Kozak, M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. *Gene 299*: 1–34.

Krystofova, S., and Borkovich, K.A. (2005) The heterotrimeric G-protein subunits GNG-1 and GNB-1 form a Gbeta-gamma dimer required for normal female fertility, asexual development, and galpha protein levels in *Neurospora crassa*. *Eukaryot Cell 4*: 365–378.

Krystofova, S., and Borkovich, K.A. (2006) The predicted G-protein-coupled receptor GPR-1 is required for female sexual development in the multicellular fungus *Neurospora crassa*. *Eukaryot Cell 5*: 1503–1516.

Kubicek, C.P., Mikus, M., Schuster, A., Schmoll, M., and Seiboth, B. (2009) Metabolic engineering strategies for improvement of cellulase production by *Hypocrea jecorina*. *Biotechnol Biofuels 2*: 19.

Kuhls, K., Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., et al. (1996) Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc Natl Acad Sci USA 93*: 7755–7760.

Kurjan, J. (1993) The pheromone response pathway in *Saccharomyces cerevisiae*. *Annu Rev Genet 27*: 147–179.

Lee, J., Leslie, J.F., and Bowden, R.L. (2008) Expression and function of sex pheromones and receptors in the homothallic ascomycete *Gibberella zeae*. *Eukaryot Cell 7*: 1211–1221.

Leibowitz, M.J., and Wickner, R.B. (1976) A chromosomal gene required for killer plasmid expression, mating, and spore maturation in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA 73*: 2061–2065.

Lieckfeldt, E., Kullning, C.M., Samuels, G.J., and Kubicek, C.P. (2000) Sexually competent, sucrose- and nitrate- assimilating strains of *Hypocrea jecorina* (*Trichoderma reesei*) from South American soils. *Mycolologia 92*: 374–380.

Mach, R.L., Schindler, M., and Kubicek, C.P. (1994) Transformation of *Trichoderma reesei* based on hygromycin B resistance using homologous expression signals. *Curr Genet 25*: 567–570.

Martinez, D., Berka, R.M., Henrisat, B., Saloheimo, M., Arvas, M., Baker, S.E., et al. (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat Biotechnol 26*: 553–560.

Michaelis, S., and Herskowitz, I. (1988) The a-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. *Mol Cell Biol 8*: 1309–1316.

Moores, S.L., Schaber, M.D., Mosser, S.D., Rands, E., O’Hara, M.B., Garsky, V.M., et al. (1991) Sequence dependence of protein isoprenylation. *J Biol Chem 266*: 14603–14610.

Nicholas, K., and Nicholas, H. (1997) GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the authors [WWW document]. URL http://www.psc.edu/biomed/genedoc.

Olesnicky, N.S., Brown, A.J., Dowell, S.J., and Casselton, L.A. (1999) A constitutively active G-protein-coupled recep-
tor causes mating self-compatibility in the mushroom Coprinus. *EMBO J* 18: 2756–2763.

Pettila, M., Nevalainen, H., Ratto, M., Salminen, E., and Knowles, J. (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61: 155–164.

Poggeler, S. (2000) Two pheromone precursor genes are transcriptionally expressed in the homothallic ascomycete *Sordaria macrospora*. *Curr Genet* 37: 403–411.

Powers, S. (1991) Protein prenylation: a modification that sticks. *Curr Biol* 1: 114–116.

Reiss, Y., Stradley, S.J., Giersch, L.M., Brown, M.S., and Goldstein, J.L. (1991) Sequence requirement for peptide recognition by rat brain p21ras protein farnesyltransferase. *Proc Natl Acad Sci USA* 88: 732–736.

Rockwell, N.C., Wang, G.T., Krafft, G.A., and Fuller, R.S. (1997) Internally consistent libraries of fluorogenic substrates demonstrate that Kex2 protease specificity is generated by multiple mechanisms. *Biochemistry* 36: 1912–1917.

Rockwell, N.C., Krysan, D.J., Korniyama, T., and Fuller, R.S. (2002) Pheromone processing by kex2/furin proteases. *Chem Rev* 102: 4525–4548.

Sakagami, Y., Yoshida, M., Isogai, A., and Suzuki, A. (1981) Peptidal sex hormones inducing conjugation tube formation in compatible mating-type cells of *Tremella mesenterica*. *Science* 212: 1525–1527.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.

Schmoll, M., Zeilinger, S., Mach, R.L., and Kubicek, C.P. (2004) Cloning of genes expressed early during cellulase induction in *Hypocrea jecorina* by a rapid subtraction hybridization approach. *Fungal Genet Biol* 41: 877–887.

Schmoll, M., Franchi, L., and Kubicek, C.P. (2005) Envoy, a PAS/LOV domain protein of *Hypocrea jecorina* (Anamorph *Trichoderma reesei*), modulates cellulase gene transcription in response to light. *Eukaryot Cell* 4: 1998–2007.

Schuster, A., and Schmoll, M. (2010) Biology and biotechnology of *Trichoderma*. *Appl Microbiol Biotechnol* 87: 787–799.

Schuster, A., Kubicek, C.P., Friedl, M.A., Druzhinina, I.S., and Schmoll, M. (2007) Impact of light on *Hypocrea jecorina* and the multiple cellular roles of ENVOY in this process. *BMC Genomics* 8: 449.

Seiboth, B., Hakola, S., Mach, R.L., Suominen, P.L., and Kubicek, C.P. (1997) Role of four major cellulases in triggering of cellulase gene expression by cellulose in *Trichoderma reesei*. *J Bacteriol* 179: 5318–5320.

Seidl, V., Seibel, C., Kubicek, C.P., and Schmoll, M. (2009) Sexual development in the industrial workhorse *Trichoderma reesei*. *Proc Natl Acad Sci USA* 106: 13909–13914.

Seoighe, C., Federスピル, N., Jones, T., Hansen, N., Bivolarovic, V., Surzycki, R., et al. (2000) Prevalence of small inversions in yeast gene order evolution. *Proc Natl Acad Sci USA* 97: 14433–14437.

Shen, W.C., Bobrowicz, P., and Ebbole, D.J. (1999) Identification of a novel class of peptide pheromone precursors in hypovirulent strains of *Hypocrea jecorina*. *Fungal Genet Biol* 15: 31–37.

Tillier, E.R., and Collins, R.A. (2000) Genome rearrangement by replication-directed translocation. *Nat Genet* 26: 195–197.

Turina, M., Prodi, A., and Alfen, N.K. (2003) Role of the M11-1 pheromone precursor gene of the filamentous ascomycete *Cryptonectria parasitica*. *Fungal Genet Biol* 40: 242–251.

Urban, M., Kahmann, R., and Bolker, M. (1996) Identification of the molecular response element in *Ustilago maydis*. *Mol Gen Genet* 251: 31–37.

Wang, Y., and Dohlman, H.G. (2004) Pheromone signaling mechanisms in yeast: a prototypical sex machine. *Science* 306: 1508–1509.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33: 103–119.

Yun, S.H., Arie, T., Kaneko, I., Yoder, O.C., and Turgeon, B.G. (2000) Molecular organization of mating type loci in heterothallic, homothallic, and asexual fungi: reproduction, speciation and classification. *Annu Rev Phytopathol* 38: 97–126.

Zhang, L., Churchill, A.C., Kazmierczak, P., Kim, D.H., and Van Alfen, N.K. (1998) Viral infection in compatible mating-type cells of *Tremella mesenterica*. *Eukaryot Cell* 7: 1501–1509.

Zhang, L., Baasiri, R.A., and Van Alfen, N.K. (1993) Hypovirulence-associated traits released by multiple mechanisms. *Mol Biol Evol* 10: 4964–4970.

Zhang, L., and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876–4882.

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