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The role of pheromone receptors for communication and mating in *Hypocrea jecorina* (*Trichoderma reesei*)

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ABSTRACT

Discovery of sexual development in the ascomycete *Trichoderma reesei* (*Hypocrea jecorina*) as well as detection of a novel class of peptide pheromone precursors in this fungus indicates promising insights into its physiology and lifestyle. Here we investigated the role of the two pheromone receptors HPR1 and HPR2 in the *H. jecorina* pheromone-system.

We found that these pheromone receptors show an unexpectedly high genetic variability among *H. jecorina* strains. HPR1 and HPR2 confer female fertility in their cognate mating types (MAT1-1 or MAT1-2, respectively) and mediate induction of fruiting body development. One compatible pheromone precursor–pheromone receptor pair (*hpr1–hpp1* or *hpr2–ppg1*) in mating partners was sufficient for sexual development. Additionally, pheromone receptors were essential for ascospore development, hence indicating their involvement in post-fertilisation events.

Neither pheromone precursor genes nor pheromone receptor genes of *H. jecorina* were transcribed in a strictly mating type dependent manner, but showed enhanced expression levels in the cognate mating type. In the presence of a mating partner under conditions favoring sexual development, transcript levels of pheromone precursors were significantly increased, while those of pheromone receptor genes do not show this trend. In the female sterile *T. reesei* strain QM6a, transcriptional responses of pheromone precursor and pheromone receptor genes to a mating partner were clearly altered compared to the female fertile wild-type strain CBS999.97. Consequently, a delayed and inappropriate response to the mating partner may be one aspect causing female sterility in QM6a.

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1. Introduction

Communication in fungi is accomplished by the use of a complex chemical language, which serves to exchange information crucial for germination, growth, defence and development (Leeder et al., 2011). The decision whether to reproduce sexually or asexually is thereby crucially dependent on the signals emitted by a potential mate encountered in the environment as well as on the environmental conditions a fungus experiences (Debuchy et al., 2010; Dyer and O'Gorman, 2012). These environmental signals trigger a complex signal transduction network (Dyer and O'Gorman, 2012), which ultimately tips the balance between both ways of reproduction. In this network, peptide pheromones represent the signals characteristic for the presence of a mate and pheromone receptors initiate the physiological processes necessary for sexual reproduction upon reception of the pheromone signal (Bölker and Kahmann, 1993; Debuchy et al., 2010). In heterothallic ascomycetes mating involves the *MAT*α/MAT1-1 and *MATa*/MAT1-2 mating type loci encoding transcription factors that regulate pheromone precursor and receptor genes in a mating type specific manner (Bardwell, 2004; Lee et al., 2010). The *Saccharomyces cerevisiae* pheromones belong to two distinct classes of peptide pheromones found throughout ascomycetes. The 12 amino acid, hydrophobic **a**-factor is synthesised by *MATa* cells, cleaved from a precursor peptide and its CAAX domain is subsequently prenylated and methylated. The 13 amino acid, unmodified and hydrophilic α-factor of *MAT*α cells derives from a precursor produced by *MAT*α cells (Jones and Bennett, 2011). The pheromone receptors belong to the seven-transmembrane-helix G-protein coupled receptor (GPCR) superfamily. Despite a generally low sequence conservation, several characterised and functionally relevant residues are conserved among fungal pheromone receptors (Choi and Konopka, 2006; Eilers et al., 2005). In the seven transmembrane G-protein coupled pheromone receptor STE2p the helix forming part of the protein exerts multiple functions in ligand and G-protein binding, signal transmission and receptor oligomerisation (Celic et al., 2003; Dosil et al., 1998; Naider and Becker, 2004; Umanah et al., 2010; Wang and Konopka, 2009). The C-terminus in turn is

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thought to fulfil a role in signal adaptation through post-translational modifications such as phosphorylation and mono-ubiquitination (Hicke et al., 1998; Konopka et al., 1988). The loss of the C-terminus or mutation of specific phosphorylation sites results in pheromone hypersensitivity (Chen and Konopka, 1996; Konopka et al., 1988).

In heterothallic filamentous ascomycetes mating between cells of opposite mating type is accomplished by cells designated as male and female. Both mating types can act as males or females. The observed chemotrophy is mediated by pheromone–pheromone receptor pairs that share structural characteristics with their *S. cerevisiae* homologues (Debuchy et al., 2010). Peptide pheromone precursor genes have been reported and characterised from different ascomycetes including *Cryphonectria parasitica*, *Giberella zeae*, *Magnaporthe grisea*, *Neurospora crassa* and *Podospora anserina* (Bobrowicz et al., 2002; Coppin et al., 2005; Lee et al., 2008; Shen et al., 1999; Zhang et al., 1998). In the heterothallic ascomycetes *N. crassa* and *P. anserina* deletion of the mating type specific pheromone precursor genes causes male sterility (Coppin et al., 2005; Kim and Borkovich, 2006). By contrast, the deletion of the *N. crassa* pheromone receptor genes leads to female sterility and impaired directional growth of the trichogyne (Kim and Borkovich, 2006; Kim et al., 2012). In homothallic ascomycetes and basidiomycetes pheromone receptors were moreover shown to be required in post-fusion developmental stages such as crozier and clamp formation or nuclear migration (Casselton and Feldbrügge, 2010; Mayrhofer et al., 2006; O'Shea et al., 1998; Raudaskoski and Kothe, 2010; Vaillancourt et al., 1997; Wendland et al., 1995). For pheromone precursors ambiguous findings were reported in this context. The role of pheromones in *P. anserina* is reported to be restricted to fertilisation (male fertility) (Coppin et al., 2005).

Sexual development in the industrial cellulase producer *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) was only discovered recently (Seidl et al., 2009). Since for this fungus protoperithecia as major female reproductive structures were not observed so far, mating under laboratory conditions can only be achieved by confronting compatible strains on plates (Schmoll et al., 2010; Seidl et al., 2009). Only known from one single isolate, QM6a, the parental strain of all *T. reesei* strains used in research and industry so far, was characterised as MAT1-2 and found to be female sterile (Seidl et al., 2009). While this represents a serious drawback for application of crossing, the availability of a female sterile strain facilitates detailed investigation of male and female sterility despite the lack of protoperithecia and hence classical crossing techniques. Relevance of a gene for male fertility can thus be analysed in a female sterile background, where male sterility would abolish mating with a fertile partner. In contrast, when using such a female sterile strain as a mating partner for a mutant in the gene of interest, the ability to undergo sexual development will be informative of female fertility.

The characterisation of the two *H. jecorina* pheromone precursor genes led to the description of a new class of hybrid (h-type) pheromone precursor genes (Schmoll et al., 2010). The h-type pheromone precursor HPP1 exhibits characteristics of both a- and α-type pheromone precursors, with a threefold CAAX domain comprising motif. However, neither its two putative KEX protease cleavage sites nor its cAMP dependent phosphorylation site are needed for its function in sexual development. HPP1 is essential for male fertility and assumes a-type function. These peculiarities in the pheromone system of *H. jecorina* warrant further investigations as to its mechanism, which will also reveal whether there are functions of h-type peptide pheromone precursor not assumed by a-type pheromones. In *H. jecorina* both pheromone precursor genes are transcribed during development in both mating types, albeit at different levels (Schmoll et al., 2010; Seibel et al., 2012). Investigation of the influence of light and components of the light signalling machinery in regulation of the *H. jecorina* pheromone system revealed that BLR1, BLR2 and ENV1 act negatively on transcript levels of both precursor and receptor genes. Lack of ENV1 thereby causes de-regulation of the system, which results in female sterility of the respective strains in light – presumably due to a saturation of signal reception and loss of sexual identity (Seibel et al., 2012). In addition, the influence of nutrient sensing on the pheromone system of *H. jecorina* is reflected in the regulation of transcription of *hpp1* and the putative pheromone transporter gene *ste6* by the phosducin like protein PhLP1 and the G-protein beta and gamma subunits GNB1 and GNG1. This regulation obviously results in somewhat decreased fruiting body formation and ascospore discharge (Tisch et al., 2011b).

In this study we characterise the two *H. jecorina* pheromone receptor genes on the molecular level. We provide insights into the yet poorly understood pheromone mediated communication of the first member of the biotechnologically important *Hypocreaceae*. We show that the expression of pheromone precursors and pheromone receptors is controlled depending on the mating type and the presence of a compatible mating-partner. Moreover, we provide insights into the mating type dependent requirements of pheromone precursors and pheromone receptors for sexual development and ascosporogenesis.

2. Material and methods

2.1. Microbial strains and culture conditions

T. reesei wild-type strain QM6a (ATCC 13631), *T. reesei* QM9414 (ATCC 26921), *H. jecorina* CBS999.97 (CBS, Utrecht, The Netherlands, sampled in French Guiana) and Δ*hpp*1 (Schmoll et al., 2010) were used throughout this study. For CBS999.97 purified and confirmed MAT1-1 or MAT1-2 single spore isolates described by (Seidl et al., 2009) were used for this study. Wild-type and recombinant strains analysed in this study (Table 1) were propagated on 2% (w/v) malt extract agar plates at 22 °C in daylight to promote fruiting body formation. *H. jecorina* strains CPK158 (MAT1-2, sampled on Celebes, Indonesia), CPK170 (MAT1-1, sampled in French Guiana), CPK1127 (MAT1-2, sampled on New Caledonia), CPK1407 (MAT1-1, sampled on Papua New Guinea) (Druzhinina et al., 2010; Seidl et al., 2009) were obtained from the strain collection of the Institute of Chemical Engineering, Vienna University of Technology. Strains were inoculated on opposite sides of a malt extract agar plate and grown for 14 days in daylight at 22 °C. For analysis of fruiting body formation and ascosporogenesis, strains were cultivated as described previously (Seibel et al., 2012; Tisch et al., 2011b). All experiments were done at least in triplicates. For transcript analysis, plates were covered with cellophane to facilitate harvesting of mycelia. Mycelia within 5 mm of the growth front of at least three equally treated plates were harvested. To avoid a possible influence of circadian rhythms harvesting occurred at noon for each respective day. Thereby the width of 5 mm of the outer colony perimeter was harvested. For analysis of asexual growth, plates were inoculated with an agar-plug at one side of the Petri dish, mycelia were harvested four and 6 days after inoculation, corresponding to mycelial contact or onset of fruiting body formation upon growth in the presence of a mating partner. Mycelia at contact (initial contact of confronted colonies) stages of development were harvested (occurring three to 4 days after inoculation). Upon start of fruiting body development (occurring 6 days after inoculation) 5 mm of mycelium was harvested from each side of the confrontation line. *Escherichia coli* JM109 (Yanisch-Perron et al., 1985) was used for DNA manipulations.

Table 1
Strains used in this study.

Strain	Relevant genotype	Source and/or reference
QM6a	Wild-type MAT1-2	Martinez et al. (2008)
QM9414	QM6a derivative, MAT1-2	ATCC26921; Vitikainen et al. (2010)
QM9414Δ*hpr1*	Δ*hpr1::hph* + MAT1-2	This study
QM9414Δ*hpr2*	Δ*hpr2::hph* + MAT1-2	This study
QSGH4	Female sterile derivative of QM9414, MAT1-1	Schuster et al. (2012)
CBS999.97 MAT1-1	Wild-type MAT1-1	Seidl et al. (2009)
CBS999.97 MAT1-2	Wild-type MAT1-2	Seidl et al. (2009)
CBS999.97 MAT1-1Δ*hpr1*	Δ*hpr1::hph* + MAT1-1	This study
CBS999.97 MAT1-2Δ*hpr1*	Δ*hpr1::hph* + MAT1-2	This study
CBS999.97 MAT1-1Δ*hpr2*	Δ*hpr2::hph* + MAT1-1	This study
CBS999.97 MAT1-2Δ*hpr2*	Δ*hpr2::hph* + MAT1-2	This study
CBS999.97 MAT1-1Δ*hpp1*	Δ*hpp1::hph* + MAT1-1	Schmoll et al. (2010)
CBS999.97 MAT1-2Δ*hpp1*	Δ*hpp1::hph* + MAT1-2	Schmoll et al. (2010)
CBS999.97 MAT1-1Δ*hpr1*Δ*hpr2*	Δ*hpr1*Δ*hpr2::hph* + MAT1-1	This study
CBS999.97 MAT1-1Δ*hpr1*Δ*hpr2*	Δ*hpr1*Δ*hpr2::hph* + MAT1-2	This study
CBS999.97 MAT1-1Δ*hpr1*Δ*hpp1*	Δ*hpr1*Δ*hpp1::hph* + MAT1-1	This study
CBS999.97 MAT1-1Δ*hpr1*Δ*hpp1*	Δ*hpr1*Δ*hpp1::hph* + MAT1-2	This study
CBS999.97 MAT1-1Δ*hpr2*Δ*hpp1*	Δ*hpr2*Δ*hpp1::hph* + MAT1-1	This study
CBS999.97 MAT1-1Δ*hpr2*Δ*hpp1*	Δ*hpr2*Δ*hpp1::hph* + MAT1-2	This study
CPK158	Wild-type MAT1-2	Seidl et al. (2009)
CPK170	Wild-type MAT1-1	Seidl et al. (2009)
CPK1407	Wild-type MAT1-1	Seidl et al. (2009)

2.2. Nucleic acid isolation and manipulation

For screening of primary transformants strains were grown on malt-extract agar and DNA was isolated as described by Liu et al. (2000). For positive transformants DNA was isolated using phenol/chloroform (Schmoll et al., 2004). Total RNA was isolated as described previously (Tisch et al., 2011a).

2.3. cDNA preparation and qRT-PCR

1 µg of total RNA was treated with DNAse I (Thermo Fisher/Fermentas, St. Leon Rot, Germany) for 30 min. 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 (Thermo Fisher/Fermentas) was used for first strand cDNA synthesis according to the manufacturer's protocol. Primer pairs (sequences are given in Table 2) to be used in qRT-PCR were designed for *hpp1* (hpp1F, hpp1R), *ppg1* (ppg1F, ppg1R), *hpr1* (hpr1F, hpp1R) and *hpr2* (hpr2F, hpr1R) using Gene Runner (version 3.05, Hastings Software, Inc.). *rpl6e*, a gene encoding a ribosomal protein, was used as reference gene for the analysis, using primers RTL6eF1/RTL6eR1 (Tisch et al., 2011a). Transcript levels of the reference gene *rpl6e* was tested and compared to two different commonly used reference genes (*gpdh*, *tef1*) and was found to be best suitable as reference gene (Tisch et al., 2011a). Furthermore, this gene was also chosen as reference gene in qRT-PCR experiments in *N. crassa*, because it is neither clock-nor temperature regulated (Froehlich et al., 2005; Lee et al., 2003; Nowrousian et al., 2003). Transcript abundance of *rpl6e* was shown to be at constitutive levels also for experiments involving changes in the light conditions (Tisch et al., 2011a). Reliable constitutive expression of *rpl6e* is especially relevant for our experiments, because the pheromone expression was shown to be influenced by the light regulatory protein ENVOY and the blue light receptors BLR1 and BLR2 in *H. jecorina* (Seibel et al., 2012). The quantitative PCR reactions (25 µl) consisted of 1 µl diluted cDNA solution, gene specific primers, fluorescent dye SYBR GreenPCR Master Mix Kit (BioRad Laboratories GmbH, Hercules, CA, USA) protocol. Reactions were performed in an IQ5 Real-Time PCR Detection System (BioRad Laboratories GmbH). Melting curve analysis was performed after the PCR reaction to exclude primer dimers or unspecific amplification. Cycle threshold (Ct) values were determined for a minimum of three biological replicates and based on three technical replicates. All results shown represent transcript levels significantly above background noise. Primer efficiencies were determined for each primer pair and included in qRT-PCR data analysis using REST software (Pfaffl et al., 2002).

Table 2
Sequences of oligonucleotides used in this study. Restriction sites introduced to facilitate cloning are underlined.

Oligonucleotide	Sequence
DELHPR13F	5′-ATGTCGACATGGCCTCATCCTCCAAC-3′
DELHPR13R	5′-AT*GGTACC*TCGATAGCGTCGTTACCG-3′
DELHPR15F	5′-ATACTAGTTGTAAGCCGGATGTCGTG-3′
DELHPR15R	5′-ATAGATCTGGCTGGTGGTGTAGTACAAAG-3′
HPR1cDF	5′-ATGGTACCCTTTCCAGAGCATCACCG-3′
HPR1cDR	5′-ATGTCGACACGTCGTGAAGTTGCTCG-3′
DELHPR23F	5′-ATCTCGAGGCCAGCCTCTATTCAACAAC-3′
DELHPR23R	5′-ATGGTACCGTTGTGGACAGTGTCAGCAG-3′
DELHPR25F	5′-ATGCGGCCGCAATGAGAGAGGGAGGGTTTG-3′
DELHPR25R	5′-ATTCTAGAGTGTGGTGAAGCTGGATTTC-3′
HPR2cDF	5′-ATGGTACCTGTCAGCATCACCACTTATAC-3′
HPR2cDR	5′-ATCTCGAGATAGAGAGAGAGAGAGAGAGTTG-3′
HPR2K1F	5′-AGAGTGCGATGCTTCAGACG-3′
HPR2K1R	5′-TGTTGACGCCATGCTTCTTG-3′
hpp1cDNA2005F	5′-TGCTCGTAGCAGAACAATC-3′
hpp13R	5′-ATGGGCCCTTCGAATCTCCAGCCTCGACAAAG-3′
hpp1F	5′-ACAATCACCGTGGGACATCTG-3′
hpp1R	5′-TCCCTGCTGTTCCGCTGATG-3′
ppg1F	5′-TGGAGACGAAGGAGAAGACTG-3′
ppg1R	5′-GCGATGTGTGGTGATGGAG-3′
hpr1F	5′-TTGGCACCTTGATTGGCTG-3′
hpr1R	5′-CGGCGGGAGAATCACAAAG-3′
hpr2F	5′-TGGCACCACTTCATCAACTTC-3′
hpr2R	5′-GGAGTAGGAGGAGGATGTGTTG-3′
MATa-1F	5′-GCGCACCACGGTATTTCATTG-3′
MATa-1R	5′-ATTTGCGCGGCTTGTATTGG-3′

To evaluate overgrowth in confrontation samples ("contact" and "fruiting body" samples) relative abundance (ratios) of mating type genes in co-precipitated chromosomal DNA was determined in respective total RNA samples. To this end we quantified DNA originating from MAT1-1 and MAT1-2 by applying qPCR with the primer combinations matA1-fw/matA1-rv (which anneals to the *mat1-1-1* mating type transcription factor gene; (Seidl et al.,

2009)) and MATa-1F/MATa-1R (annealing to *mat1-2-1* transcription factor gene), respectively. Both primer pairs yielded almost identical reaction efficiencies (~100%) and hence allow for an estimation of confrontation partner contamination. Using this approach the fraction of undesired RNA originating from confrontation partners could be quantified to be 0.01–2.41% of total RNA.

2.4. Construction of hpr1 and hpr2 deletion strains

For the knock-out of *hpr1* and *hpr2* the deletion vectors pDELhpr1 and pDELhpr2 were created. In these plasmids the sequence spanning the mRNA of receptor genes as predicted by the *T. reesei* genome database is replaced by the *E. coli hph* gene under *T. reesei* expression signals (Mach et al., 1994). The sequences of the primers described in this section are given in Table 2.

The vector pDELhpr1 was constructed as follows: A 1162 bp fragment of the 3′-flanking region of *hpr1* was amplified by PCR using primers DELHPR13F and DELHPR13R, digested with *Sal*I-*Acc*65I (all restriction enzymes by Thermo Fisher/Fermentas, St. Leon Rot, Germany) and cloned into the *Xho*I-*Acc*65I sites of pBSXH (Schmoll et al., 2005), which contains the *pki1p:hph:cbh2t* cassette from pRLMex30. Thereafter, a 1299 bp fragment of the 5′ flanking sequence of *hpr1* was amplified by PCR using primers DELHPR15F and DELHPR15R, the amplicons were cleaved with *Spe*I and *Bgl*II and cloned into the *Spe*I-*Bam*HI sites of pBSXH::3′-*hpr1*. QM9414 chromosomal DNA was used as template for PCR amplification.

The resulting pDELhpr1 was cleaved with *Acc*65I and *Spe*I, and the excised fragment thus obtained was used for transformation of QM9414 and CBS999.97 (Gruber et al., 1990). Transformants were selected on plates containing 50 μg/ml hygromycin B (Calbiochem, Merck KGaA, Darmstadt, Germany). Fungal DNA was isolated and subjected to PCR analysis to verify replacement of the gene. PCR-verification was performed using the primers HPR1cDF and HPR1cDR. The presence of wild-type *hpr1* was indicated by a band at 1339 bp, the deletion construct yielded a band at 2912 bp. One deletion strain of QM9414 (Δ*hpr1*-17b) and one of CBS999.97 (MAT1-1) (Δ*hpr1*-33a1) were used.

Due to the higher sequence variability of *hpr2* between CBS999.97 and QM6a, we constructed the deletion vectors for these strains separately. The vector pDELhpr2 was constructed for the transformation of QM9414: A 1113 bp fragment of the 3′-flanking region of *hpr2* was amplified by PCR using primers DELHPR23F and DELHPR23R, digested with *Xho*I-*Acc*65I and cloned into the *Xho*I-*Acc*65I sites of pBSXH (Schmoll et al., 2005). Thereafter, a 1314 bp fragment of the 5′ flanking sequence of *hpr2* was amplified by PCR using primers DELHPR25F and DELHPR25R, the amplicons were cleaved with *Not*I and *Xba*I and cloned into the *Not*I–*Xba*I sites of pBSXH::3′-*hpr2*.

The resulting pDELhpr2 was cleaved with *Acc*65I and *Not*I, and the excised fragment obtained was used for transformation. PCR-verification was performed using the primers HPR2cDF and HPR2cDR. The presence of wild-type *hpr2* was indicated by a band at 1937 bp, the deletion construct yielded a band at 3326 bp.

The vector to be used for deletion of *hpr2* in CBS999.97 was constructed using the same primers and restriction sites as used for the construction of pDELhpr2, but using DNA of CBS999.97 as template for PCR amplification of fragments. The resulting pDELhpr2-C wasexcised with *Not*I and *Sal*I. For verification of the knock-out the primers HPR2K1F and HPR2K1R were used. Several transformants of CBS999.97 (MAT1-2) were tested. All of them displayed the same phenotype in mating with the MAT1-1 transformant of QM6a. All deletion strains were subject to at least two rounds of single spore isolation until no wild-type background was detectable by PCR.

In order to obtain double mutants of *hpp1* with the pheromone receptor genes, a deletion mutant of *hpp1* in the fertile CBS999.97 strain (Schmoll et al., 2010) was used as crossing partner. Mutants of both mating types as well as double mutants were constructed by crossing and verified as described above, deletion of *hpp1 in Δhpr1Δhpp1* and Δ*hpr2Δhpp1* was determined using the primers hpp1cDNA2005F and hpp13R. In order to determine the mating type of ascospore clones, primer pairs binding within the *mat1-1* (Seidl et al., 2009) or *mat1-2* (MATa1F and MATa1R) mating type loci were chosen.

2.5. Microscopic analysis of stromata

Stromata preparations, sections and microscopic analyses were performed as described previously (Jaklitsch, 2009). Fully differentiated stromata were harvested 12–14 days after inoculation i.e. upon start of ascospore discharge. Thereafter stromata were dried over night at 80 °C and rehydrated for sectioning over night in a wet chamber on humid paper towels. Stromata were subsequently embedded in Tissue-Tek O.C.T. Compound 4583 (Sakura Finetek Inc., Torrance, CA, USA). Sections (10–12 μm) were performed in a Leica CM1900 cryostat (Leica Microsystems, Nussloch, Germany) at chamber and object temperature of −25 °C. Sections were micrographed in 3% (w/v) KOH using an Axioplan 2 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Stromata of pheromone receptor mutants were monitored for up to 2 months to exclude a possible delay in ascospore development.

2.6. Bioinformatic analyses

Analyses of *hpr1*, *hpr2* and the pheromone receptor genes of 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/fgi/). Blast searches were done with the NCBI Blast server (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequences were aligned with Clustal X 2.0.9 (Thompson et al., 1997) and then manually adjusted using GeneDoc (Nicholas and Nicholas, 1997). Sequence data of *hpr2* open reading frames from *H. jecorina* strains CPK 158 (Genbank accession number JN678727), CPK 170 (JN678728), CPK 1407 (JN678729) and CBS999.97 (JN678730) were obtained using PCR amplicons generated with the primer pair HPR2cDF and HPR2cDR. For the open reading frame of *hpr1* from *H. jecorina* CBS999.97 (JN684208) the primer pair HPR1cDF and HPR1cDR was used. In CBS999.97 we additionally used DELHPR15F and DELHPR15R for the 5′-flanking region of *hpr1* and DELHPR25F and DELHPR25R for the 5′-flanking region of *hpr2*. Primers used are shown in Table 2.

Prediction of transmembrane segments of the receptors was done using the TMHMM, TMAP (Persson and Argos, 1994) and MEMSAT3 (Nugent and Jones, 2009) software tools of the biology-workbench versus 3.2 available at http://seqtool.sdsc.edu/CGI/BW.cgi#! and http://bioinf.cs.ucl.ac.uk/web_servers/, respectively. Prediction of phosphorylation sites was done using the Netphos software (Blom et al., 1999) available at http://www.cbs.dtu.dk/services/NetPhos/.

3. Results

3.1. Characteristics of pheromone receptor genes in H. jecorina (T. reesei)

The *H. jecorina* QM6a genome contains two candidate pheromone receptor genes (Schmoll, 2008). HPR1 (*H. jecorina* Pheromone Receptor 1, STE3-like, JN787117) shares 54%, 33% and 28% identities with its characterised *G. zeae* (PRE-1, XP_387446.1), *N. crassa* (PRE-1, CAO78593.1) and *Aspergillus nidulans* (GPRB,

DAA01795.1) homologues (Kim and Borkovich, 2004; Lee et al., 2008; Seo et al., 2004). The 1326 bp ORF of *hpr1* is interrupted by a predicted 137 bp intron and encodes a 441 aa protein. HPR2 (*H. jecorina* Pheromone Receptor 2; JN787118) displays similarities with the already functionally characterised STE2p-like pheromone receptors of *G. zeae* (PRE-2, XP_382831.1), *N. crassa* (PRE-2, CAP17766.1) and *A. nidulans* (GPRA, DAA01796.1) (46%, 37% and 29% identities) (Lee et al., 2008; Pöggeler and Kück, 2001; Seo et al., 2004). The 1288 bp ORF is interrupted by a predicted 108 bp intron and encodes a 428 aa protein. Results from TMHMM, TAMP and MEMSAT3 prediction tools for transmembrane topology indicate seven transmembrane helices in both pheromone receptors. Within the promoter regions of both genes Pheromone Response Elements (PRE, 5′ CAAAG 3′) were detected (Supplementary Table 1). PREs were demonstrated to be necessary and sufficient for the induction of pheromone inducible genes in *S. cerevisiae* and *Ustilago maydis* (Hagen et al., 1991; Hartmann et al., 1996; Urban et al., 1996) and are specifically bound by the *N. crassa* mating type factor MTa1 *in vitro* (Philley and Staben, 1994).

3.2. Genetic variability of pheromone receptor genes in H. jecorina (T. reesei)

Due to the female sterility of *H. jecorina* QM6a a comparison of the candidate pheromone receptors of this strain to that of the fully mating competent *H. jecorina* CBS999.97 isolate was performed. For HPR1 we found 9 amino acids to be altered (98% identities) between CBS999.97 and QM6a (Fig. 1A). Sequences of HPR2 of QM6a and CBS999.97 differed in 20 amino acids (96% identities).

Most mutations between the pheromone receptors of the QM6a and CBS999.97 were found in the C-terminal part of the proteins. These mutations affect several phosphorylation sites (Supplementary Table 2; Fig. 1), which may result in differences in receptor internalisation and regulation of its activity (Hicke et al., 1998). Mutations in the N-terminal and helix-forming part of the protein are more abundantly found in HPR2. Affected residues however do not tamper with transmembrane topology (data not shown).

Due to its similarity to *S. cerevisiae* Ste2p, representing the pheromone receptor of the **a**-mating type receiving alpha-type pheromone signals, HPR2 is likely to represent the pheromone receptor of MAT1-2, which is the mating type of female sterile QM6a. We therefore additionally analysed the predicted HPR2 protein sequences of three more sexually competent *H. jecorina* natural isolates (CPK158, CPK170, CPK1407; (Druzhinina et al., 2010; Kuhls et al., 1996)). The high variability in HPR2 sequences was also observed in these strains (Fig. 1B). Amino-acid substitutions in QM6a versus CBS999.97 either occur in other fertile *H. jecorina* strains as well, are conservative or are unique to CBS999.97. Hence, these mutations rather reflect the variability found within the species than defects of QM6a. Additionally, these mutations do not include amino acid residues shown to be essential for pheromone receptor function in yeast (Supplementary Fig. 1; Supplementary Table 3) (Choi and Konopka, 2006; Eilers et al., 2005). Consequently, the female sterile phenotype of *T. reesei* QM6a is unlikely to be due to a mutation in the pheromone receptors.

At the DNA level, sequence conservation between QM6a and CBS999.97 is only 97% for *hpr2*. Consequently, the variability of the *hpr2* gene sequences could be used for phylogenetic strain analysis of closely related isolates.

3.3. hpr1 and hpr2 impact female fertility and ascospore development

To determine the role of *hpr1* and *hpr2* for sexual development of *H. jecorina* we generated *hpr1* and *hpr2* deletion strains in both the fully sexually competent *H. jecorina* CBS999.97 and the female sterile *H. jecorina* QM9414. For *H. jecorina*, so far only crossing by confrontation of compatible mating partners on plates was shown to be successful because no protoperithecia, which could be fertilised, were observed (Schmoll et al., 2010; Seidl et al., 2009). If in such a confrontation the female fertility of one mating partner is abolished by the deletion of the mating type specific receptor, this strain can still act as male and thus undergo sexual development with the wild-type due to the presence of the second pheromone–pheromone receptor pair. We therefore used the female sterile but male fertile strains QM9414 and QM6a for investigation of the relevance of pheromone receptor genes for male and female fertility.

Lack of *hpr1* or *hpr2* does not perturb growth or conidiation in CBS999.97 or QM9414. Crossing of CBS999.97 deletion mutants in *hpr1* or *hpr2* or both in MAT1-1 or MAT1-2 did not abolish mating with a wild-type mating partner (Fig. 2). The deletion of *hpr1* and *hpr2* in the female sterile background of QM9414 (MAT1-2) did not cause sterility upon crossing with CBS999.97 (MAT1-1). Consequently, *hpr1* and *hpr2* are not essential for male fertility (data not shown).

For evaluation of the relevance of *hpr1* and *hpr2* in female fertility we used the female sterile strain QM6a as mating partner. Deletion of *hpr1* in the CBS999.97 MAT1-1 mating type completely abolished mating with QM6a (MAT1-2) indicating female sterility (Fig. 2A). Accordingly, deletion of *hpr2* in CBS999.97 MAT1-2 abolished mating with a female sterile MAT1-1 derivative of QM9414 (Schuster et al., 2012) (Fig. 2B). In contrast, lack of *hpr1* in CBS999.97 MAT1-2 or of *hpr2* in CBS999.97 MAT1-1 was not relevant for mating with QM6a (Fig. 2A). HPR2 therefore appears to be crucial for female fertility in CBS999.97 MAT1-2, while HPR1 assumes this task in CBS999.97 MAT1-1. These findings are in agreement with a reported female sterility in *N. crassa* (*mat A*) Δ*pre1* strains (Kim and Borkovich, 2004).

Based on these results a complete abolishment of mating would be expected for a cross between CBS999.97Δ*hpr2* (MAT1-2) and CBS999.97Δ*hpr1* (MAT1-1). Surprisingly, stromata formation is not impaired in this cross (Fig. 3A). Moreover, the dried biomass of fruiting bodies produced in these crosses is not significantly different from wild-type crosses (WT: 50 mg ± 9 mg; Δ*hpr1* (MAT1-1) X Δ*hpr2* (MAT1-2): 47 mg ± 10 mg). Nevertheless, although stromata macroscopically appear normal, no ascospores were found and microscopic analysis confirmed that mutant stromata contain perithecia that fail to develop ascospores (Fig. 3B–F) resulting in sterility. Hence, pheromone receptors are essential for fertility in their cognate mating type, but the remaining two receptors may be sufficient to initiate fruiting body formation. In agreement with this hypothesis, fruiting body formation does not occur in a cross between the pheromone receptor double mutants (Δ*hpr1*Δ*hpr2*) (Fig. 3A).

3.4. Compatible pheromone-pheromone receptor pairs are essential for mating

The pheromone system of *H. jecorina* is distinct from that of other ascomycetes due to the unusual structure of the h-type pheromone precursor HPP1, which assumes a-type function (Schmoll et al., 2010). In order to evaluate its role in pheromone response together with the pheromone receptors, we constructed double mutants lacking one receptor and *hpp1*.

Because of its function as a-type pheromone, HPP1 should be recognised by HPR1. We thus analysed double mutants of both mating types lacking HPP1 and its cognate receptor. Crossing Δ*hpr1*Δ*hpp1* (MAT1-1) and Δ*hpr1*Δ*hpp1* (MAT1-2) still resulted in fruiting body formation and ascospore discharge (Fig. 4). This finding can be explained by the availability of the other pheromone–pheromone receptor pair (PPG1/HPR2).

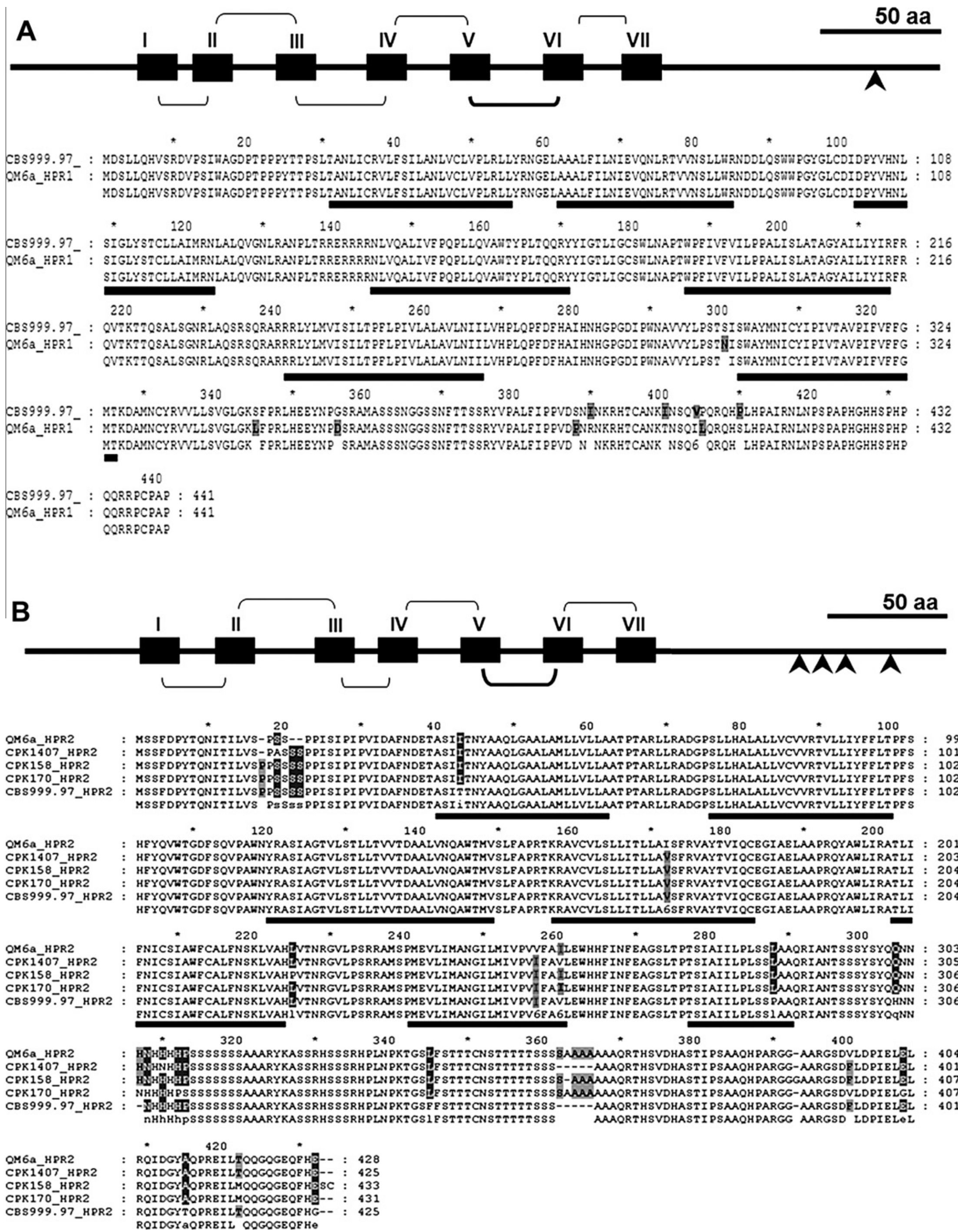

Fig. 1. Sequence analysis of *H. jecorina* pheromone receptors. (A) HPR1 characteristics and alignment of HPR1 sequences from *H. jecorina* strains QM6a and CBS999.97. (B) HPR2 characteristics and alignment of HPR2 sequences from QM6a, CBS999.97 and CPK-158, -170, -1407. Black bars indicate predicted transmembrane segments in sequence alignments from A and B. Schematic illustrations of HPR1 and HPR2: Brackets and black bars indicate approximate organisation of loop and transmembrane helix structures. Arrow heads indicate approximate position of phosphorylation sites diverging between CBS999.97 and QM6a.

Therefore we evaluated sexual development in strains lacking one component of each compatible pair – HPR2, which perceives the PPG1 signal and HPP1, which would be recognised by HPR1. Hence none of the two compatible pairs (HPR1/HPP1 or HPR2/PPG1) pair is available in this cross. Accordingly, crossing the Δ*hpr2*Δ*hpp1* double mutants of both mating types is not successful, hence confirming the requirement for one pheromone precursor and its cognate receptor (Fig. 4). These findings are in agreement with data for *Sordaria macrospora* (Mayrhofer et al., 2006) and *N. crassa* (Kim et al., 2012), which showed that one compatible pheromone precursor–pheromone receptor pair is necessary and sufficient for sexual development.

3.5. Expression levels of pheromones and their receptors respond to mating type and developmental stage

In *H. jecorina*, peptide pheromone precursor genes are not transcribed in a strictly mating type dependent manner (Schmoll et al.,

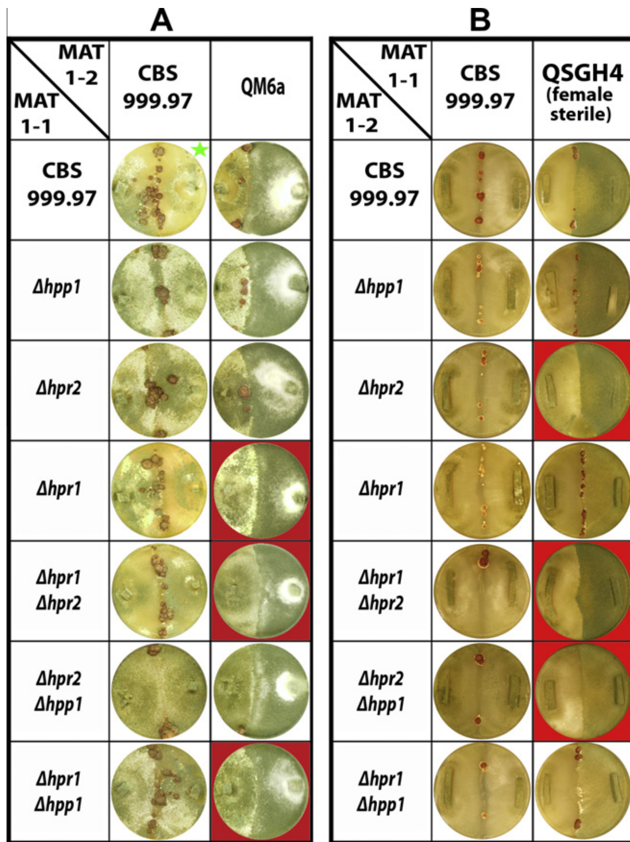

Fig. 2. Analysis of fertility of mutant strains with wild-type *H. jecorina* CBS999.97. The pheromone receptors *hrp1*, *hrp2* and all possible double-knock-out combinations in both mating types were tested for impaired fruiting body formation. Mating assays with female sterile *H. jecorina* QM6a and QSGH4, respectively, were performed to test for female fertility of CBS999.97 mutant strains. Red background highlights a complete abolishment of fruiting body formation.

2010). We therefore aimed to investigate regulation of pheromone precursor and receptor gene expression under conditions of sexual development and in the absence of a mating partner (asexual development). Cultures for investigation of asexual development were grown in parallel to those for analysis of sexual development under equal conditions and harvested at time points corresponding to contact of mycelia and the onset of fruiting body formation.

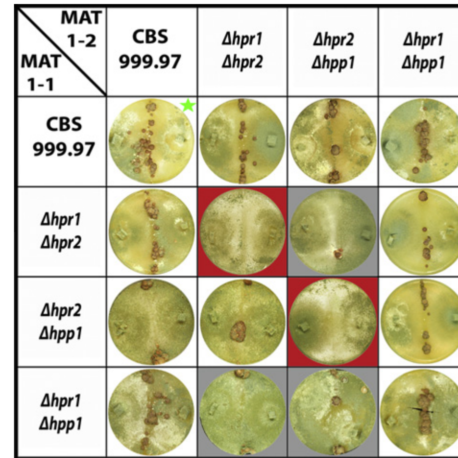

Fig. 4. Analysis of fertility of mutant strains lacking pheromone receptors *hpr1* or *hpr2* or both and/or the pheromone precursor gene *hpp1* in both mating types. Red background highlights a complete abolishment of fruiting body formation; grey background highlights sterile fruiting bodies (no ascospore discharge).

Our results on transcript abundance of pheromone precursor genes showed that transcript levels of *hpp1* in the absence of a mating partner are higher in MAT1-2 and those of *ppg1* are higher in MAT1-1, albeit the difference between mating types is more extensive for *hpp1* (Fig. 5A and B). Comparison with transcript levels of peptide pheromone precursor genes under conditions of sexual development indicate increased levels of both genes under these conditions. A more than hundred fold increase was observed for *hpp1* in MAT1-2 and only roughly threefold enhancement of transcription for *ppg1* in MAT1-2. Interestingly, we also found clearly increased transcription of *hpp1* in MAT1-1 and of *ppg1* in MAT1-2 in the presence of a mating partner (Fig. 5A and B).

Transcript levels on pheromone receptor genes *hpr1* and *hpr2* are in accordance with their mating type dependent function as described above and similar to the pheromone precursor genes. Moreover, *hpr1* and *hpr2* are not transcribed in a strictly mating type dependent manner (Fig. 5C and D). While *hpr1* is considerably more strongly transcribed in MAT1-1, *hpr2* transcription is enhanced in MAT1-2, albeit to a lesser extent. Encounter of a mating partner (sexual development) resulted in increased transcript levels of *hpr1* and *hpr2* in their cognate mating type upon fruiting body formation.

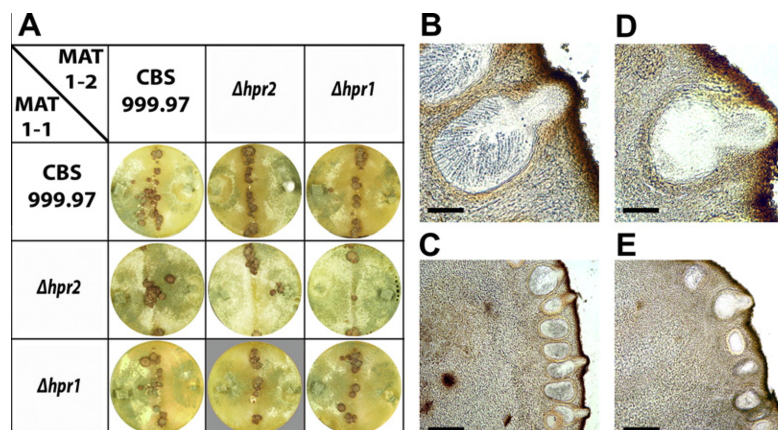

Fig. 3. Analysis of mating and stromata morphology in mutant strains lacking pheromone receptors. (A) The strains lacking pheromone receptors *hrp1*, *hrp2* in both mating types were tested for impaired fruiting body formation. Grey background highlights sterile fruiting bodies (no ascospore discharge). (B and C) Representative stromata and perithecia from CBS999.97wild-type and (D and E) CBS999.97Δ*hpr1* (MAT1-1) X CBS999.97Δ*hpr2* (MAT1-2) mutant stromata. No ascospores are discharged in the mutant confrontation. Scale bars: (B and D) 125 μm. (C) 2.5 mm; E: 5 mm.

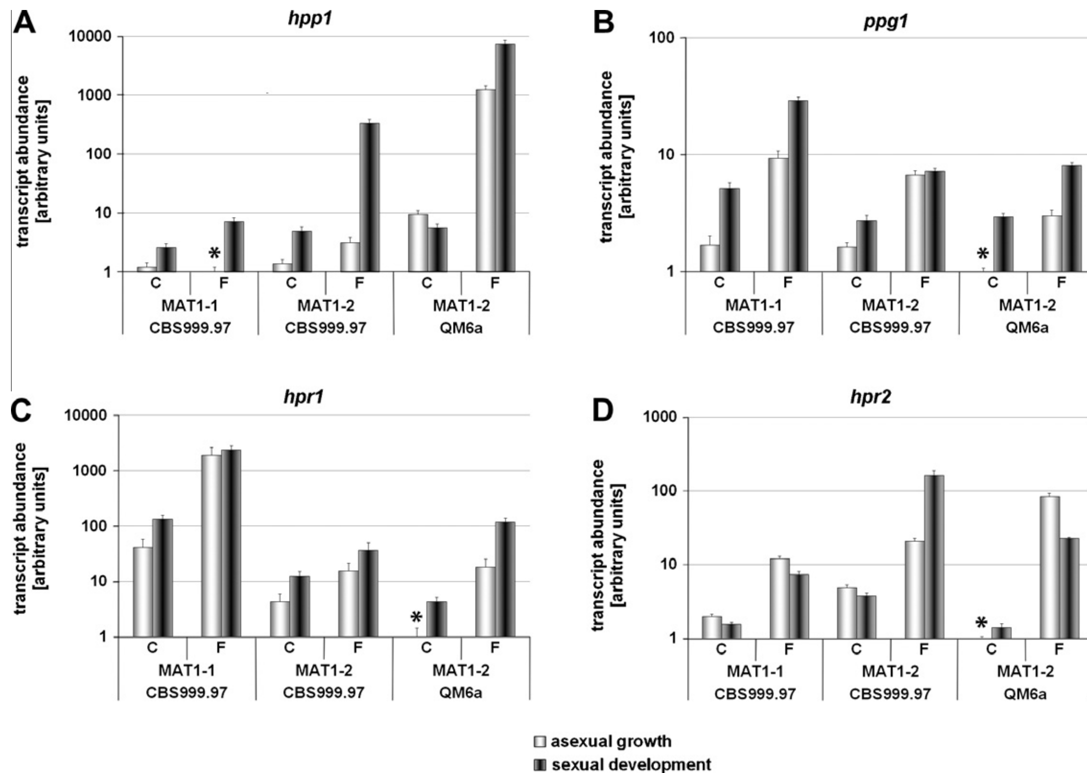

Fig. 5. Transcript analyses of *H. jecorina* pheromone precursor and pheromone receptor genes expression under asexual growth conditions (light bars) or upon sexual development (dark bars). For each gene transcript abundance from MAT1-1 and MAT1-2 samples (CBS999.97 MAT1-1, MAT1-2; QM6a MAT1-1) was related to the sample with the lowest expression level (as indicated by an asterisk). At time points of 4 and 6 days confronted wild-type strains of opposite mating type undergo stages of *initial contact between the colonies* (C) and *macroscopically visible start of fruiting body formation* (F). At sampling time points (4 days (C) and 6 days (F) after inoculation) RNA was isolated from mycelia harvested from the growth front of the hyphae. (A and B): Mating type dependent regulation of pheromone precursor genes *hpp1* (A) and *ppg1* (B) in asexually and sexually growing *H. jecorina* strains. (C and D): Mating type dependent regulation of pheromone receptor genes *hpr1* (C) and *hpr2* (D) in asexually and sexually growing *H. jecorina* strains.

We conclude that the pheromone–receptor pair HPP1-HPR1 is significantly more tightly regulated in the different mating types, especially under conditions of sexual development than PPG1-HPR2. While pheromone precursor genes are responsive to the presence of a mating partner, this effect is less obvious for pheromone receptor regulation.

3.6. Communication related to sexual development is altered in QM6a

The defects causing the female sterile phenotype of QM6a (MAT1-2) may lead to altered communication during sexual development. We therefore analysed how this strain would behave in the presence of a mating partner in terms of pheromone precursor and pheromone receptor gene regulation.

We therefore cultivated QM6a under equal conditions as for the mating experiments of the wild-type CBS999.97 described above and investigated the transcriptional response to the presence of a mating partner in this cross. Despite certain alterations in transcript levels, we found that the patterns for QM6a still resemble MAT1-2 data observed for the wild-type strain. Consequently, mating type dependent regulation of these genes does not appear to be abolished.

Nevertheless, while *hpp1* is up-regulated considerably in QM6a compared to CBS999.97 MAT1-2, transcription of *ppg1* is unaltered under conditions of sexual development and down-regulated in the absence of a mating partner (Fig. 5A and B). Transcription of pheromone receptor genes upon contact of mating partners and also at the corresponding time point in the absence of a mating partner are decreased compared to wild-type, but increase upon fruiting body formation (Fig. 5C and D). Hence, a delayed and probably inappropriate (too strong pheromone transcription along with decreased receptor transcript abundance) response to a mating partner, especially reflected in the altered transcript levels of *hpr2*, the cognate pheromone receptor of MAT1-2 may be caused by the defect of QM6a (MAT1-2) leading to female sterility.

4. Discussion

Sexual development in fungal species happens between two morphologically similar, yet genetically different individuals, which communicate in a chemical language and mutually stimulate each other (Ni et al., 2011). In *H. jecorina*, one of the components of this mutual signalling, the h-type peptide pheromone HPP1 was found to be distinct from pheromones of other ascomycetes. In addition, the pheromone receptor and pheromone precursor genes are not as specifically regulated according to the mating type as it would be expected for a heterothallic fungus (Kim et al., 2012; Schmoll et al., 2010). Our results now provide insight into the characteristics and peculiarities of this communication system.

Consistent with a previously reported high evolutionary speed of sex genes in *Neurospora* (Karlsson et al., 2008; Pöggeler and Kück, 2001) both pheromone receptor genes displayed a relatively high rate of mutations between QM6a and other *H. jecorina* strains. In agreement with earlier findings (Karlsson et al., 2008), the intracellular C-terminal tail of the receptors appears to constitute the rapidly evolving domain. On the basis of detailed *in silico* analyses no hints for a severe impairment of basic HPR2 signal transducing capability are obvious in QM6a. More evidence for HPR2 (QM6a)

being active in signalling is provided by our expression data showing differential patterns between asexually and sexually growing cultures. The mutations in *hpr2* are hence unlikely to be the cause of female sterility in QM6a. Considering the changes in promoter motifs and C-terminal phosphorylation sites it cannot be excluded that these mutations affect expression levels or activity and hence signalling as well as mating success. Such theory finds support from studies on *Neurospora*, in which the fast evolving C-termini of pheromone receptors were proposed to contribute to reproductive isolation (Karlsson et al., 2008).

While the significance of the relatively high sequence variability in *hpr2* of *H. jecorina* remains to be explored, this characteristic may offer novel perspectives for evaluation of nature isolates: In *Microbotryum* pheromone receptor genes were used to analyse hybridisation between species (Devier et al., 2010) which might be interesting also for *H. jecorina*. Also, a high number of isolates of *Hypocrea* species exist, which cannot easily be distinguished using common phylogenetic markers. Often even closely related members of these species vary significantly in their cellulolytic or biocontrol potentials (Druzhinina et al., 2010). The data shown here render the pheromone receptor genes in *Hypocrea* an excellent marker to distinguish individuals on strain level, which could be used as a molecular identification tool for closely related strains. *Hypocrea* pheromone receptor genes could thus help in characterisation of new strains for use in biotechnology.

Our results revealed the function of pheromone receptor genes not to be limited to signal recognition and female fertility, but also to extend to ascosporogenesis. In mating confrontations using *H. jecorina* mutants and the female sterile *T. reesei* QM6a both *hpr1* and *hpr2* were shown to be mating type specifically required for mating. Several lines of evidence point an interaction of the h-type pheromone HPP1 with the **a**-factor receptor homologue HPR1 in *H. jecorina*. The female sterile phenotype of CBS999.97Δ*hpr1* (MAT1-1) and the normal sexual phenotype of CBS999.97Δ*hpr2* (MAT1-1) consequently hint at a HPP1-HPR1 interaction in mating with QM6a. This assumption is supported by correlating expression patterns of *hpp1* and *hpr1* and moreover by results from crosses between wild-type and different mutants of CBS999.97. The mating type dependent role of pheromone receptors is consistent with findings from *N. crassa*. In *N. crassa* the deletion of *pre-1* caused a complete inability of *mat A* trichogynae to recognise *mat a* hyphae and to initiate fertilisation (Kim and Borkovich, 2004). Essentially the same phenomenon became obvious for *pre-2* and *ccg-4* (Kim et al., 2012).

However, despite their inability to undergo sexual development with a female sterile partner, a cross between CBS999.97Δ*hpr1* (MAT1-1) and CBS999.97Δ*hpr2* (MAT1-2) resulted in fruiting body formation. Nevertheless, although this initial stage of sexual development could be achieved – presumably due to certain levels of interaction between the pheromone and its receptor in their non-cognate mating type – no ascospores were formed. Hence appropriate completion of the sexual cycle requires a functional, mating-type specifically regulated pheromone system. Similar requirements were shown for *N. crassa* using combinations of misexpressed pheromone receptor and pheromone precursor genes in different mating type backgrounds (Kim and Borkovich, 2006; Kim et al., 2012).

At the molecular level, we found that both pheromone precursor genes as well as pheromone receptor genes show elevated transcript levels in their cognate mating type, but are also transcribed in the respective other mating type. Expression of pheromone precursor genes appears clearly mating type specific in ascomycetes like *C. parasitica*, *M. grisea*, *N. crassa* and *P. anserina* while pheromone receptor genes show comparatively lower mating type specificity in *N. crassa* (Coppin et al., 2005; Karlsson et al., 2008; Kim and Borkovich, 2006; Pöggeler and Kück, 2001; Shen et al., 1999; Zhang et al., 1998). Although the regulation of pheromone precursors and pheromone receptors does not seem strictly mating type dependent on the transcriptional level, the role of posttranscriptional regulation as shown for *P. anserina* (Bidard et al., 2011; Coppin et al., 2005) remains to be determined. However, our results would at some stages be in agreement with the presence of pheromone precursors and receptors also in their non-cognate mating type. Also the fact that lack of one pheromone receptor influences transcription of the other (Kim et al., 2012), suggests that they do not function completely independently and that the availability of a pheromone receptor in its non-cognate mating type is physiologically relevant.

While transcript abundance of pheromone precursors clearly increases in their cognate mating type upon sexual development versus vegetative growth, a comparative response is only obvious for *hpr2* in MAT1-2, but not for *hpr1*. *N. crassa* responds to the presence of a mating partner with an immediate and strong MAT-specific upregulation of pheromone precursor genes and a moderate transcriptional response for receptor genes (Karlsson et al., 2008). A comparable induction is also found in homothallic *G. zeae* after fertilisation of cultures with spermatia (Lee et al., 2008).

The formation of fruiting bodies in a cross between strains lacking their cognate pheromone receptors, but having the second receptor available, pointed at an involvement of both pheromone receptors and precursors in every strain for efficient sexual development in *H. jecorina*. This conclusion is also in accordance with findings that in *N. crassa* lack of either pheromone receptor causes reduced expression of both pheromones and the respective other pheromone receptor (Kim and Borkovich, 2004; Kim et al., 2012). Consequently, lack of one receptor also impacts efficiency of the other pheromone receptor pair. Functions of pheromone receptors beyond recognition of the pheromone signal have been observed also for other fungi: The pheromone systems of the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium*, were shown to mediate clamp cell fusion and nuclear migration (Erdmann et al., 2012; James et al., 2011; O'Shea et al., 1998; Vaillancourt et al., 1997; Wendland et al., 1995). A similar mechanism was hypothesised for crozier formation in filamentous ascomycetes (Debuchy, 1999). Recently, evidence for a role of pheromones and the G-protein alpha subunit GNA1 also in post fertilisation events in *N. crassa* was shown (Kim et al., 2012). For *P. anserina* no involvement of pheromones after fertilisation could be shown (Coppin et al., 2005). Using the *H. jecorina* system we were able to provide evidence for a role of pheromone receptors for ascospore formation in a heterothallic species.

5. Conclusions

In summary, we provide a functional characterisation of the pheromone – receptor system of *H. jecorina*. In addition to the function of the pheromone precursors in male fertility, we identified the pheromone receptors to be essential for female fertility in their cognate mating type. Crosses of strains lacking the mating-type specific pheromone receptor still resulted in formation of fruiting bodies as female reproductive structures. While ascosporogenesis was not observed in these crosses, this finding hints at an involvement of pheromone receptors – even if not in the cognate mating type – in fruiting body formation. The presence of transcripts of pheromone precursor and pheromone receptor genes in both mating types, albeit at different levels, may explain such an involvement. Consequently, the pheromone system of *H. jecorina* essentially resembles those of other heterothallic ascomycetes, but also shows intriguing new aspects, which warrant further investigation.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.07.004.

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