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ENVOY Is a Major Determinant in Regulation of Sexual Development in *Hypocrea jecorina* (*Trichoderma reesei*)

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Light is one crucial environmental signal which can determine whether a fungus reproduces asexually or initiates sexual development. Mating in the ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) occurs preferentially in light. We therefore investigated the relevance of the light response machinery for sexual development in *H. jecorina*. We found that the photoreceptors BLR1 and BLR2 and the light-regulatory protein ENV1 have no effect on male fertility, while ENV1 is essential for female fertility. BLR1 and BLR2 were found to impact fruiting body formation although they are not essential for mating. Quantitative reverse transcription-PCR (qRT-PCR) analyses revealed that BLR1, BLR2, and ENV1 negatively regulate transcript levels of both pheromone receptors as well as peptide pheromone precursors in light but not in darkness and in a mating type-dependent manner. The effect of BLR1 and BLR2 on regulation of pheromone precursor and receptor genes is less severe than that of ENV1 as strains lacking *env1* show 100-fold (for *ppg1*) to more than 100,000-fold (for *hpp1*) increased transcript levels of pheromone precursor genes as well as more than 20-fold increased levels of *hpr1*, the pheromone receptor receiving the HPP1 signal in a MAT1-1 strain. ENV1 likely integrates additional signals besides light, and our results indicate that its function is partially mediated via regulation of *mat1-2-1*. We conclude that ENV1 is essential for balancing the levels of genes regulated in a mating-type-dependent manner, which contributes to determination of sexual identity and fruiting body formation.

Sexual development is one of the most important evolutionary achievements in nature. "Nothing in biology makes sense unless it is in the light of reproduction" (14). This quotation briefly summarizes the relevance of sexual development for almost all living organisms and, hence, for its application in research and industry. Accordingly, a complex regulatory network of signaling and metabolic pathways is responsible for timing and efficiency of mating in fungi (2, 11). Peptide pheromones and their cognate receptors, which have been characterized in many fungi, are of crucial importance for communication of mating partners and subsequent sexual development (3, 11). In heterothallic fungi, only partners of different mating types can mate with each other. Thereby, the mating type is defined by a specific genomic locus, which contains one of two different sequences (often called idiomorphs) occupying the same chromosomal locus in the genome (12, 44). Fungi are usually hermaphroditic and can form both male and female reproductive structures, which are strongly dependent on environmental factors. At the molecular level, in most cases pheromone receptors are crucial for female fertility (33), while peptide pheromone precursors are reported to be essential for male fertility (9, 34, 54).

Sexual development remained undiscovered for decades in *Trichoderma reesei*, albeit *Hypocrea jecorina* could be identified as its teleomorph (36). Detection of this process in the industrial workhorse *H. jecorina* represents a major improvement in research with this organism. Since successful mating has been achieved (57), the mechanism and determinants of the underlying process have been subjected to elaborate analysis aiming at a more detailed understanding of physiology of *H. jecorina* as well as strategies for industrial strain improvement. The female sterile phenotype of heterothallic QM6a, however, is a drawback in this respect. At the same time the availability of a female sterile strain offers the possibility to investigate determinants of female fertility in mutants of sexually competent wild-type isolates. Sexual development in *H. jecorina* is favored in light and upon growth on rich medium (57), which is in contrast to many other fungi (11). Another peculiarity in *H. jecorina* is the composition of its pheromone system. *H. jecorina* has the usual types of pheromone receptors (50): one Ste3-type receptor termed HPR1 and one Ste2-type receptor (HPR2) as well as a normal alpha-type peptide pheromone precursor, PPG1. However, the peptide pheromone precursor HPP1 is the first member of the new class of h-type (hybrid type) peptide pheromone precursors, which assumed **a**-type function in *H. jecorina*. No classical **a**-type peptide pheromone precursor was found in the genome (54).

Light is a crucial environmental factor for fungi (10, 24) and also for the developmental decision whether to reproduce sexually or asexually. The perception of light and thus the physiological adaptation to light are evolutionarily old mechanisms found in zygomycetes (27), ascomycetes (26), basidiomycetes (60), and several other lineages (28). In many fungi, the light-induced changes in gene expression impact growth, the direction of growth, asexual and sexual reproduction, pigment formation, carbon metabolism, and circadian rhythms (63). In *Cryptococcus neoformans* an interesting connection between blue light perception, virulence, and mating inhibition was found: the deletion of the photoreceptor gene *bwc1* or *bwc2* results in reduced virulence and causes release of mating inhibition by light (26). Further connections between mating and the photoreceptors LreA and LreB were characterized in *Aspergillus nidulans*. Both proteins positively af-

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fect cleistothecium formation and secondary metabolism in *A. nidulans* (48).

The conserved *Neurospora crassa* blue light photoreceptor complex (White Collar complex [WCC]), consisting of White Collar 1 (WC-1) and WC-2, represents a central means of light perception and regulation of light-responsive genes (49).

Upon illumination, the WCC acts as a transcriptional activator of a third photoreceptor, VVD, which acts as a universal brake of light responses upon prolonged illumination and is essential for gating of light responses (7, 23). VVD hence also acts as negative regulator by inactivating the WCC and consequently its own expression (18, 23). This function in photoadaptation is achieved by physical interaction of VVD with the WCC (6, 25), and lack of VVD consequently causes increased effects of WCC.

The *H. jecorina* photoreceptor homologues of WC-1 and WC-2, denominated BLR1 (for blue light receptor 1) and BLR2, contain the characteristic PAS domains as well as zinc fingers, reflecting their function as transcription factors (5, 51). The PAS domain protein ENVOY (ENV1), representing the VVD homologue, is essential for light tolerance of *H. jecorina* (52). ENV1 represents an important node in the network connecting the light response pathway, heterotrimeric G-protein signaling, and the cyclic AMP (cAMP) pathway (61) and is induced by BLR1 and BLR2 (5). In particular, ENV1 acts negatively on transcription of the G-protein alpha subunit gene *gna3* (53) and interferes with the positive feedback of *gna1* upon activation of its encoded G-alpha subunit (61). In accordance with its function in regulation of heterotrimeric G-protein signaling, ENV1 also impacts light responsiveness of the class I phosducin-like protein-encoding gene *phlp1*. Due to the fact that PhLP1 is assumed to act as a cochaperone for G-protein beta and gamma subunit folding, this finding further supports the interrelationship of ENV1 with G-protein signaling (62). Since the heterotrimeric G-protein pathway is assumed to predominantly transmit nutrient signals besides the pheromone signals (39), ENV1 is likely to integrate these signals with the light response pathway.

In this study, we provide insights into the role of the blue light photoreceptors BLR1 and BLR2 and the regulatory protein ENVOY in regulation of sexual development, all of which contribute to the respective adjustment of signal transmission and reception. We further show that *env1* is essential for balanced regulation of pheromone precursor genes and receptor genes. Consequently, ENV1 is important for successful mating, and its regulatory function was found to be at least in part achieved by a negative effect on the mating type gene *mat1-2-1* in a MAT1-2 strain.

MATERIALS AND METHODS

Microbial strains and culture conditions. *H. jecorina* (*T. reesei*) wild-type strain QM6a, its derivative QM9414 (ATCC 26921), and *H. jecorina* CBS999.97 MAT1-1 and MAT1-2 strains (40, 57) as well as *H. jecorina* (*T. reesei*) QM9414 strains lacking *blr1*, *blr2*, and *env1* (5) were used throughout this study (Table 1). Propagation of strains occurred on 2% (wt/vol) malt extract-agar plates. If not indicated otherwise, sexual development was analyzed at room temperature and under daylight conditions (cycles 12 h of light and 12 h of darkness [12:12]). For the analysis of fruiting body formation, strains of opposite mating types were inoculated on opposing sides of a petri dish and monitored over a period of 14 days (57). For the additional evaluation of mating success, spore solution (0.1% Triton X-100, mixture of equal amounts of conidia from strains of opposite mating type; 2×10^4 conidia per strain were applied) was inoculated at the center of a petri dish and monitored over 14 days at room temperature and under daylight conditions. For determination of stroma biomass, strains were inoculated accordingly. Per strain, stromata from five equally treated plates were harvested, cleaned from agar residues, dried overnight at 80°C, and weighed subsequently. For transcript analysis, malt extract-agar plates were covered with cellophane to facilitate harvesting of mycelia. Mycelia of at least three equally treated plates were harvested and pooled. To avoid a possible influence of circadian rhythms, harvesting occurred at noon for each respective day. Mycelia were harvested 3, 4, and 6 days after inoculation, corresponding, respectively, to stages of precontact, contact, and the beginning of fruiting body formation of sexually developing cultures.

TABLE 1 Strains used in this study

Strain	Relevant genotype	Source and/or reference
QM6a	Wild-type MAT1-2	43
QM9414	QM6a derivative, MAT1-2	ATCC 26921; 64
QM9414 Δ*env1*	Δ*env1*::*hph*$^+$ MAT1-2	5
QM9414 Δ*blr1*	Δ*blr1*::*hph*$^+$ MAT1-2	5
QM9414 Δ*blr2*	Δ*blr2*::*hph*$^+$ MAT1-2	5
CBS999.97 MAT1-1	Wild-type MAT1-1	57
CBS999.97 MAT1-2	Wild-type MAT1-2	57
CBS999.97 MAT1-1 Δ*env1*	Δ*env1*::*hph*$^+$ MAT1-1	This study
CBS999.97 MAT1-2 Δ*env1*	Δ*env1*::*hph*$^+$ MAT1-2	This study
CBS999.97 MAT1-1 Δ*blr1*	Δ*blr1*::*hph*$^+$ MAT1-1	This study
CBS999.97 MAT1-2 Δ*blr1*	Δ*blr1*::*hph*$^+$ MAT1-2	This study
CBS999.97 MAT1-1 Δ*blr2*	Δ*blr2*::*hph*$^+$ MAT1-1	This study
CBS999.97 MAT1-2 Δ*blr2*	Δ*blr2*::*hph*$^+$ MAT1-2	This study
CBS999.97 MAT1-1 Δ*blr1* Δ*blr2*	Δ*blr1blr2*::*hph*$^+$ MAT1-1	This study
CBS999.97 MAT1-2 Δ*blr1* Δ*blr2*	Δ*blr1blr2*::*hph*$^+$ MAT1-2	This study

Escherichia coli JM109 (65) was used for DNA manipulations.

Nucleic acid isolation and transcript analysis. For DNA isolation, strains were grown on malt extract-agar, and chromosomal DNA was isolated as described previously (8, 41).

RNA was isolated as described by Tisch et al. (61). For reverse transcription and quantitative PCR (RT-qPCR), 1 μg of total RNA was treated with DNase I (Fermentas, Vilnius, Lithuania) for 30 min. Termination of the DNase digest was accomplished by adding EDTA to a final concentration of 2.5 mM and incubation at 65°C for 10 min. A RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific/Fermentas, St. Leon-Rot, Germany) was used for first-strand synthesis according to the manufacturer's protocol. For qPCR cDNA was diluted 1:5, aliquoted, and stored at −80°C. For the quantification of *hpr1* (GenBank accession number for QM6a, JN787117; CBS999.97, JN684208), *hpr2* (GenBank accession number for QM6a, JN787118; CBS999.97, JN678730), *hpp1*, *ppg1*, and *mat1-2-1* transcript levels, the primers shown in Table 2 were used. As a reference gene, *rpl6e* encoding the ribosomal protein RPL6e was used. The expression of *rpl6e* was shown to be unaffected by light or light regulators (61). The quantitative PCRs were performed in an IQ5 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany) using the primer pairs hpp1F and hpp1R, ppg1F and ppg1R, hpr1F and hpr1R, and hpr2F and hpr2R. Melting curve analysis was performed after the PCR to confirm that the signal was the result of single-product amplification and not due to primer dimers or arbitrary amplification. Cycle threshold (C_T) values were determined for a minimum of three biological replicates and three technical replicates. Analysis of qPCR data was performed using REST software (46).

Preparation of deletion strains. To obtain deletion mutants of *blr1*, *blr2*, and *env1* (Table 1), strain CBS999.97 was transformed with plasmids pDELBLR1-C, pDELBLR2-C, and pDELENV1-C, respectively. In these

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
BLR1DEL3F	5′-ATGAATTCCTTGCTCATTTGATGCGAG-3′
BLR1DEL3R	5′-ATGGATCCAGCGTCCGATCGTATTCC-3′
BLR1DEL5F	5′-ATGGTACCACCAATTGTCCTCGTGAG-3′
BLR1DEL5R	5′-ATCTCGAGAAAGAATGAGGGAGAGGC-3′
BLR1c1F	5′-TGTGCCTTTGTCGTTTGTG-3′
BLR1c1R	5′-CAATCTCAGCCAGTCCGA-3′
HPHin1NF	5′-CGTTATGTTTATCGGCACTTTG-3′
BLR1ex5F	5′-ATTCTAGACTCGAGTTGCCAGGGACTAGGAAG-3′
BLR1ex3R	5′-AATTAACCCTCACTAAAGGG-3′
BLR1c3R	5′-ATACTAGTCCTTTGCCTCACCTCAACC-3′
BLR2DEL5F	5′-ATTCTAGACAGATACAAAGCCGAGGACC-3′
BLR2DEL5R	5′-ATAAGCTTGGTCGCGGTAGTATTGCTATAC-3′
BLR2DEL3F	5′-ATGTCGACATGGCGGAGAAAGAAAG-3′
BLR2DEL3R	5′-ATGGTACCTCCGATCCTGCACGATC-3′
BLR2cDF	5′-CATCGCATTTGCCTTCCAG-3′
BLR2cDR	5′-AAAGAATCGACAGCAACAATGG-3′
ENVDEL5F2	5′-ATGGTACCTACGATTATTGGCATTGC-3′
ENVDEL5R2	5′-ATCTCGAGTAAAGAAGAGGTCACAGCC-3′
ENVDEL3F	5′-AACCCGGGATAGATGCTAGGCGTACC-3′
ENVDEL3R	5′-ACGGATCCGAGAAGATTGCATTCATTAC-3′
ENV1F	5′-TCCCTGGATCTGGATACG-3′
ENV2R	5′-CTGGCGTGGTATTTCTCTGAC-3′
MATa1-F	5′-GCGCACCACGGTATTTCATTG-3′
MATa1-R	5′-ATTTGCGCGGCTTGTATTGG-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′

[a] Introduced restriction sites are underlined.

plasmids the sequence spanning the mRNA of blue light regulator genes, as predicted in the Joint Genome Institute (JGI) *T. reesei* genome database, version 2.0, is replaced by the *E. coli hph* gene under *H. jecorina* expression signals (42). Primer sequences mentioned in the following are shown in Table 2. The vector pDELBLR1-C was constructed using *H. jecorina* CBS999.97 genomic DNA as the template for PCR amplifications. A 1,542-bp fragment of the 3′ flanking region of *blr1* was amplified by PCR using primers BLR1DEL3F and BLR1DEL3R. The amplicons were digested with EcoRI-BamHI (all restriction enzymes were from Thermo Fisher Scientific/Fermentas) and cloned into the EcoRI-BamHI sites of pBluescript (pBS) SK+. Thereafter, a 1,530-bp fragment of the 5′ flanking sequence of *blr1* was amplified by PCR using primers BLR1DEL5F and BLR1DEL5R. The fragment was digested using SalI (cleaving 22 bp from the 5′ flanking region) and the XhoI site, which was introduced by the primer BLR1DEL5R and cloned into the XhoI digested vector. After dephosphorylation of the resulting plasmid pBS::3′-*blr1*, the hygromycin resistance cassette excised from pRLMex30 (42) using XhoI and HindIII was filled in using Klenow polymerase to create blunt ends and integrated into EcoRV-digested and dephosphorylated pBS::3′,5′-*blr1*.

The resulting transformation cassette now carried in pDELBLR1-C was amplified by PCR using the primers BLR1ex5F and BLR1ex3R and used for transformation (20) of *H. jecorina* CBS999.97. Transformants were selected on plates containing 50 μg/ml hygromycin B (Calbiochem, Merck KGaA, Darmstadt, Germany). Fungal DNA was isolated (see above) and subjected to PCR analysis to verify replacement of the gene. To determine the presence of the wild-type fragment, PCR verification was performed using the primers BLR1c1F and BLR1c1R. The wild-type fragment yielded a band at 2,098 bp. The presence of the deletion construct was verified using the primers HPHin1NF and BLR1c3R, which bind within the *hph* marker and outside the cassette. The deletion construct yielded a band at 2,912 bp. Transformants underwent three rounds of single-spore isolation until no wild-type PCR product was detectable. Two deletion strains of CBS999.97 (MAT1-1) (Δ*blr1*5b1β and -66a1α) were used and displayed clear and similar phenotypes.

The vector pDELBLR2-C was constructed with *H. jecorina* CBS999.97 DNA as the template for PCR amplifications. A 1,222-bp fragment of the 3′ flanking region of *blr2* was amplified by PCR using primers BLR2DEL3F and BLR2DEL3R, digested with SalI-Acc65I and cloned into the XhoI-Acc65I sites of pBS SK+. Thereafter, a 1,300-bp fragment of the 5′ flanking sequence of *blr2* was amplified by PCR using primers BLR2DEL5F and BLR2DEL5R. The amplicons were cleaved with HindIII and XbaI and cloned into the HindIII-XbaI sites of pBS::3′-*blr2*.

The hygromycin resistance cassette was digested with XhoI/HindIII and integrated in SalI and HindIII sites of pBS::3′-5′-*blr2*. The resulting pDELBLR2-C was cleaved with Acc65I and NotI, and the excised fragment obtained was used for transformation of *H. jecorina* CBS999.97. PCR verification was performed using the primers BLR2cDF and BLR2cDR. The presence of wild-type *blr2* was indicated by a band at 2,345 bp, and the deletion construct yielded a band at 3,649 bp (data not shown). Positive transformants underwent at least three rounds of single-spore isolation until no wild-type background was detectable by PCR.

The vector pDELENV-C was constructed as follows. A 1,094-bp fragment of the 5′ flanking region of *env1* was amplified by PCR using primers ENVDEL5F2 and ENVDEL5R2, digested with XhoI-Acc65I, and cloned into the XhoI-Acc65I sites of pBSXH (52), which contains the *pki1p*::*hph*::*cbh2t* cassette from pRLMex30. Thereafter, a 986-bp fragment of the 3′ flanking sequence of *env1* was amplified by PCR using primers ENVDEL3F and ENVDEL3R; the amplicons were cleaved with XmaI and BamHI and cloned into the respective sites of pBSXH::5′-*env1*.

The resulting pDELENV-C was cleaved with Acc65I and NotI, and the excised fragment thus obtained was used for transformation of *H. jecorina* CBS999.97. PCR verification was performed using the primers ENV1F and ENV2R. The presence of wild-type *env1* was indicated by a band at 1,099 bp; the deletion construct yielded a band at 3,067 bp. Positive transformants underwent at least three rounds of single-spore isolation until no wild-type background was detectable by PCR.

In order to obtain deletion strains in both *H. jecorina* CBS999.97 mating types, primary transformants were crossed with the *H. jecorina* CBS999.97 wild-type strain. Resulting ascospores were cultivated on plates containing 50 μg/ml hygromycin B (Calbiochem, Merck KGaA, Darmstadt, Germany). Hygromycin-resistant progeny were PCR verified for deletion of *blr1*, *blr2*, and *env1*. In order to determine the mating type of ascospore clones, primer pairs binding within the *mat1-1* (57) or *mat1-2* (Table 1, MATa1F and MATa1R) mating type locus were chosen. Double mutants in *blr1* and *blr2* were constructed and verified accordingly.

RESULTS

Sexual development and the *H. jecorina* QM9414 light response machinery. Sexual development in *H. jecorina* preferentially occurs in light (57). In order to achieve a first insight into the role of the light response machinery in sexual development, we used the available *H. jecorina* deletion strains QM9414 Δ*blr1* and QM9414 Δ*blr2* (MAT1-2) (5) for crosses with the wild-type strain CBS999.97 (MAT1-1). Deletion of *env1* is known to enhance pheromone gene expression and sexual development with this strain (54). Accordingly, mating was successful using Δ*env1*, Δ*blr1*, and Δ*blr2* strains, and ascospore discharge was not abolished (see Fig. S1 in the supplemental material). However, since these strains are derivatives of the wild-type isolate QM6a, which is female sterile (57), any influence on female fertility would not be testable using this approach. Additionally, defects in gene regula-

tion of pheromone precursors or receptors due to female sterility could not be excluded. A defect in male fertility, however, would abolish mating in these crosses because this strain would then be both male and female sterile. Therefore, it can be concluded that deletion of *blr1*, *blr2*, and *env1* does not cause major defects in male fertility.

Photoreceptors BLR1, BLR2, and ENV1 in *H. jecorina* CBS999.97. In order to investigate an effect of the blue light regulators on sexual reproduction in a fully (male and female) fertile wild-type background, we used *H. jecorina* CBS999.97 (57). Sequence analysis of *blr1*, *blr2*, and *env1* in CBS999.97 revealed only minor differences to QM6a at the DNA (identities in *blr1*, 99%; *blr2*, 99%; *env1*, 96%) and protein (identities in BLR1, 99%; BLR2, 100%; ENV1, 99%) levels. *blr1*, *blr2*, and *env1* were thus deleted in *H. jecorina* CBS999.97 (MAT1-1). The same deletions in the respective MAT1-2 strain as well as Δ*blr1* Δ*blr2* double mutants in both mating types were constructed by crossing. Strains lacking *blr1* or *blr2* did not show a significant growth defect. In contrast, growth of the Δ*env1* strain was severely perturbed in light (Fig. 1A), hence confirming the phenotype reported for *H. jecorina* QM9414 Δ*env1* (52). As in the wild-type, conidiation is enhanced by light in all mutant strains. Lack of *blr1* or *blr2* caused a less distinct phenotype in light and darkness than observed for the wild type (Fig. 1B). Conidiation of the Δ*env1* strain was unaltered in darkness but dense in light, which contrasts with earlier data and may be due to slightly altered regulation of asexual development in QM6a and its derivatives in comparison to CBS999.97.

Blue light regulators are involved in sexual development of *H. jecorina* CBS999.97. Deletion mutants of *blr1*, *blr2*, and *env1* in the CBS999.97 background now allow for assessment of the influence of these genes also on female fertility. The relevance of the light response machinery for sexual development in the sexually competent *H. jecorina* CBS999.97 was evaluated by crosses of Δ*b1r1*, Δ*b1r2*, and Δ*env1* mutant strains of both mating types with the respective wild-type mating partner. As conditions for this assay, we chose daylight (12:12 cycles) and room temperature, which is favorable for sexual development in *H. jecorina*. Crosses of strains bearing similar mutations in *blr1*, *blr2*, or both genes clearly showed altered fruiting body formation compared to the wild type with fewer, but larger, fruiting bodies (Fig. 2A). This characteristic is also reflected by only a slight decrease in total dry mass of stromata (Fig. 2B). When Δ*env1* (MAT1-1) and Δ*env1* (MAT1-2) strains were used as partners, no fruiting body formation at all was apparent (Fig. 2A), thereby indicating a defect in sexual development caused by the lack of ENV1. Altered fruiting body formation was detected for crosses of mutant strains with a wild-type strain of the opposite mating type. However, the phenotypes were less severe than if both mating partners lacked components of the light signaling machinery (see Fig. S2 in the supplemental material). In crosses of Δ*env1* (MAT1-1 or MAT1-2) strains with the wild-type as partners, fruiting body formation was altered but not abolished.

Except for the crosses of two stains lacking *env1*, where no fruiting bodies are formed, mating of strains lacking *blr1*, *blr2*, or *env1* resulted in discharge of viable ascospores. Perithecium formation of these crosses did not reveal obvious defects (Fig. 2C to E).

In addition to analysis of mating efficiency of these strains, the female sterile strain QM6a and its derivative QM9414 enabled us to test whether BLR1, BLR2, or ENV1 would affect female fertility

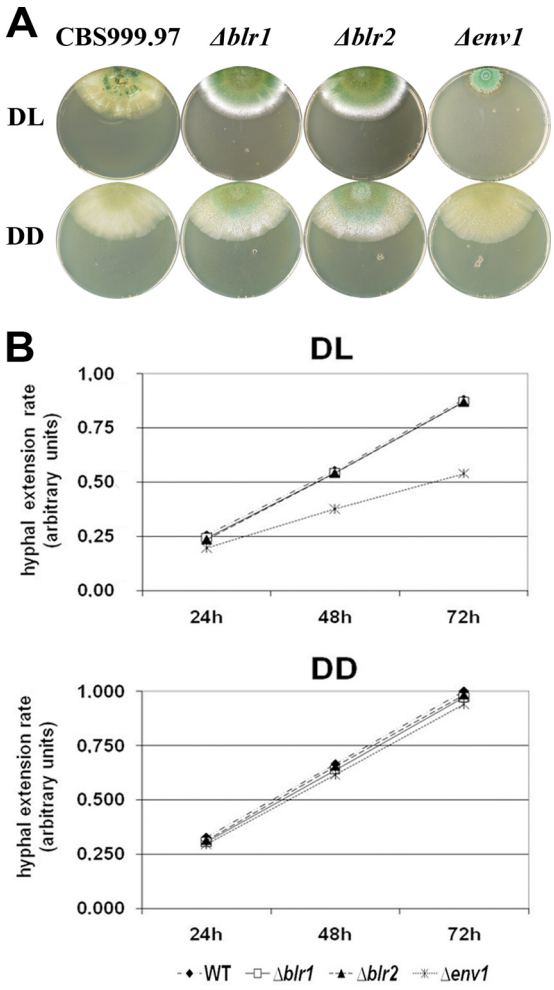

FIG 1 Phenotypes of CBS999.97 (MAT1-1) Δ*blr1*, Δ*blr2*, and Δ*env1* deletion strains. (A) Plates show growth characteristics and sporulation of the wild type (WT) and Δ*blr1*, Δ*blr2*, and Δ*env1* mutant strains in daylight (DL; 12:12) and constant darkness (DD) on malt extract-agar plates. One representative plate out of at least three replicates is shown. (B) Hyphal extension rates upon growth on malt extract-agar plates in daylight (DL; 12:12) and constant darkness (DD).

in the *H. jecorina* wild-type strain CBS999.97. We therefore mated the newly constructed Δ*env1*, Δ*blr1*, and Δ*blr2* strains with the CBS999.97 background of mating type MAT1-1 with the female sterile QM6a. In case of a defect in female fertility, in these strains sexual development would be abolished because of a lack of a female partner. While fruiting body formation still occurred with the Δ*blr1* or Δ*blr2* strain as a mating partner, sexual development was not observed with the Δ*env1* strain (Fig. 3). Together with the defect in fruiting body formation observed in a cross of two strains both lacking *env1* as described above, this indicates that ENV1 is essential for female fertility in *H. jecorina* CBS999.97.

Regulatory effects of the light signaling machinery. Peptide pheromone precursor genes and the heptahelical G-protein-coupled receptors of their matured gene product are major determinants of sexual development. Due to the clear effects of BLR1, BLR2, and ENV1 on sexual development, we investigated these factors as possible regulatory targets of the light response machinery on the molecular level. For ENV1, previous data indicate a function in regulation of pheromone levels (54).

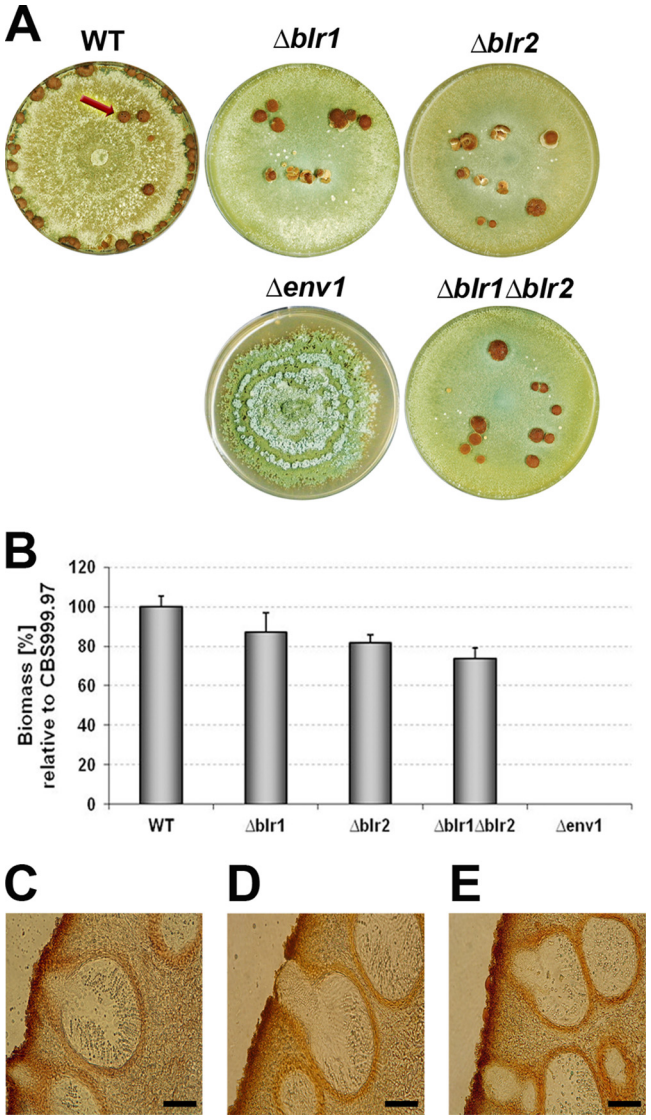

FIG 2 Effect of deletion of *blr1*, *blr2*, and *env1* on stroma formation. (A) Sexual development of *H. jecorina* CBS999.97 wild type (WT) and Δ*blr1*, Δ*blr2*, Δ*blr1* Δ*blr2*, and Δ*env1* mutant strains. Equal amounts of conidia from strains of opposite mating types (deletions in both mating partners) were inoculated in the center of a petri dish and cultivated under mating conditions (DL; 22°C on malt extract-agar plates). Crosses were performed between strains carrying the same wild-type or mutant allele (WT × WT, Δ*blr1* × Δ*blr1*, Δ*blr2* × Δ*blr2*, Δ*blr1* Δ*blr2* × Δ*blr1* Δ*blr2*, Δ*env1* × Δ*env1*). For the wild type, sexual structures are indicated by a red arrow. (B) Average dry weight of stromata (fruiting bodies) from wild-type or mutant crosses. At least five biological replicates were considered. (C to E) Microscopic analysis of stromata from representative wild type (C), Δ*blr1* Δ*blr2* double mutant (D), and wild-type × Δ*env1* (E) strains. Cross-sections of perithecia are shown for strains grown in light-dark cycles (DL; 22°C on malt-agar plates). Scale bar, 25 μm.

We analyzed transcript patterns of peptide pheromone precursor genes (*hpp1* and *ppg1*) as well as pheromone receptor genes (*hpr1* and *hpr2*) in mutant strains of both mating types. Strains were therefore harvested at time points when parallel mating control cultures were in the stage of precontact (3 days), contact of hyphae (4 days), or at the onset of fruiting body formation (6 days).

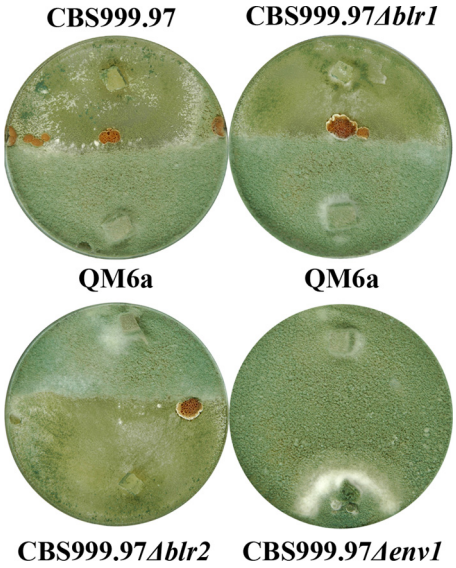

FIG 3 Effect of *blr1*, *blr2*, and *env1* deletion on female fertility. *H. jecorina* CBS999.97 (MAT1-1) wild type and Δ*blr1*, Δ*blr2*, and Δ*env1* mutant strains in confrontation with female sterile *H. jecorina* QM6a (MAT1-2). The cross between CBS999.97 Δ*env1* × QM6a did not result in fruiting body formation. Strains were grown under daylight conditions and at room temperature.

Deletion of *blr1*, *blr2*, or *env1* consistently enhanced transcription of pheromone genes, albeit to different extents (Fig. 4). With respect to the h-type peptide pheromone precursor gene *hpp1*, the effect was clearly mating type dependent in Δ*blr1*, Δ*blr2* (Fig. 4A), and Δ*env1* strains (Fig. 4B). The effect of the blue light photoreceptors BLR1 and BLR2 on *hpp1* occurred only in a MAT1-1 strain, while hardly any difference relative to the wild type was observed in the MAT1-2 strain. In the Δ*env1* strain, transcription of *hpp1* was strongly upregulated, even in a MAT1-1 background, reaching several thousand-fold in a MAT1-2 strain. For the alpha-type peptide pheromone precursor gene *ppg1*, the mating type dependence of gene regulation was less intense for *blr1* and *blr2*, but again the effect of their deletion was clearly positive (Fig. 4C). Lack of *env1*, however, again caused a strong and clearly mating-type-dependent upregulation of *ppg1*, albeit to a lesser extent than seen for *hpp1* (Fig. 4D).

In case of the *ste3*-type pheromone receptor *hpr1*, deletion of *blr2* has a clearly positive effect on transcript levels in a MAT1-1 strain (Fig. 5A). In contrast, the effect of *blr1* is considerably weaker, and transcript levels of *hpr1* fall below wild-type levels upon prolonged cultivation (6 days). Also, lack of ENV1 caused considerably increased levels of *hpr1* in the MAT1-1 strain (Fig. 5B). In the MAT1-2 strain, however, no effect of ENV1 or BLR1 or BLR2 on *hpr1* transcription was detected.

As already observed for the alpha-type peptide pheromone precursor gene *ppg1*, the putative cognate receptor gene *hpr2* also shows less strict mating-type-dependent regulation by BLR1, BLR2, and ENV1. The regulation of *hpr2* by BLR1 and BLR2 strongly resembles the situation of *hpr1*, with BLR2 having the strongest effect and levels of *hpr2* falling below wild-type levels upon prolonged cultivation in the case of a lack of *blr1* in a MAT1-1 strain (Fig. 5C). In a Δ*env1* strain, *hpr2* was clearly upregulated in a MAT1-2 strain, while in a MAT1-1 strain initial upregulation was followed subsequently by levels lower than those

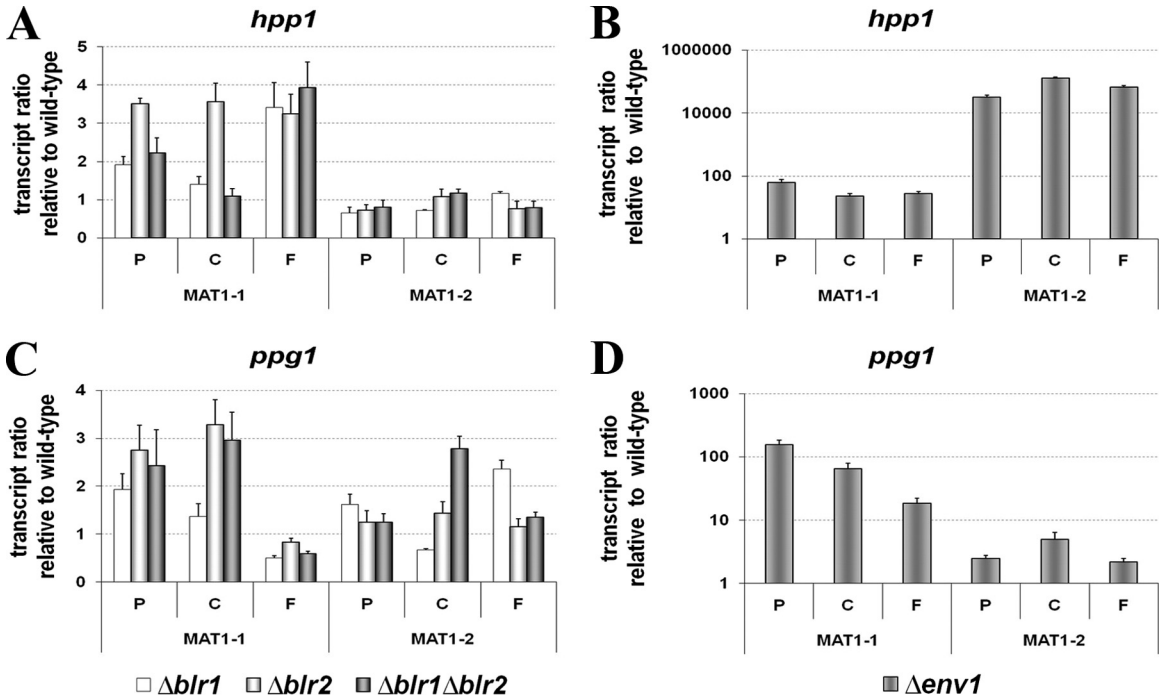

FIG 4 Transcription analysis of peptide pheromone precursor genes, as indicated, in *H. jecorina* CBS999.97 in Δ*blr1*, Δ*blr2*, Δ*blr1* Δ*blr2*, and Δ*env1* strains compared to the wild type in both mating types. Strains were cultivated in daylight at 22°C on malt extract-agar plates, i.e., conditions corresponding to mating assays. At indicated time points of 3, 4, and 6 days, confronted wild-type strains of opposite mating types undergo stages of precontact (P), initial contact between the colonies (C), and the macroscopically visible start of fruiting body formation (F), respectively. RNA was isolated at these time points after inoculation of the indicated strain from mycelia harvested from the growth front of the hyphae.

of the wild type (Fig. 5D). Again, as observed for *ppg1*, the regulatory effect of ENV1 on *hpr2* is moderate compared to the strong effect on *hpr1* at early time points in the MAT1-1 background.

In general, the effect of BLR2 on transcript levels of pheromones and their receptors appears stronger, but since the effect seen in the double mutant in almost all cases resembles that of BLR1, we conclude that regulation by BLR1 is pivotal in this mechanism.

In order to evaluate if these regulatory patterns also occur on contact with a mating partner, we analyzed transcription of peptide pheromone precursor genes in wild-type and mutant strains of mating type MAT1-1, which showed clear regulatory effects of BLR1, BLR2, and ENV1, in the presence of a wild-type MAT1-2 mating partner. Since to some extent blending of mating partners has to be expected upon fruiting body formation, this time point, corresponding to 6 days, has to be treated with caution. Indeed, comparison of transcript levels of mutant crosses with those of wild-type crosses in the respective same mating type showed that the negative effect of BLR1, BLR2, and ENV1 was also observed under conditions of sexual development for both *ppg1* and *hpp1* strains (see Fig. S3A and B in the supplemental material). Additionally, the wild-type mating partner (CBS999.97 MAT1-2) showed an enhanced response upon encountering the mutants, especially in case of *ppg1* strains (see Fig. 3B).

Light- and mating-type-specific effects of the light response machinery. Our studies of regulation of sexual development by the light signaling machinery were performed in daylight. We were thus interested whether this effect would be specific for light or if a regulatory function of ENV1, BLR1, or BLR2 would also be detected in darkness, which would indicate a function of these components independent of light.

We therefore analyzed regulation of *hpp1*, *ppg1*, *hpr1*, and *hpr2* in the same strains and under the same conditions as described above except that we cultivated the strains in constant darkness. The "contact" time point was used as a test case. Transcript analysis under these conditions did not show the regulatory and mating-type-dependent effects as seen in light (see Fig. S4 and S5 in the supplemental material). In contrast, we found that in darkness gene transcription of *hpp1*, *ppg1*, *hpr1*, and *hpr2* is more or less similar to that of the wild type (P values of >0.1). Only for a Δ*env1* strain did we detect a minor regulation of *hpr1* and *ppg1* under these conditions (about 1.5-fold). These data indicate that the function of ENV1, BLR1, and BLR2 in regulation of sexual development is specific to light.

As described above, we did not observe the strong regulation of pheromone precursor and receptor genes by ENV1 in darkness. If this strong regulation, indeed, interferes with sexual development and abolishes female fertility, this defect should be restricted to light conditions, and sexual development should be possible in darkness. In order to evaluate this hypothesis, we first analyzed whether sexual development is possible with these strains in darkness. Indeed, fruiting body formation eventually occurs in crossings with all strains in darkness (albeit with considerable delay compared to strains under light conditions), and if both *blr1* and *blr2* are absent, the strains behave as in light (see Fig. S6 in the supplemental material).

We also found that fruiting body formation was delayed in darkness both in the wild-type and in strains lacking *env1*. In a

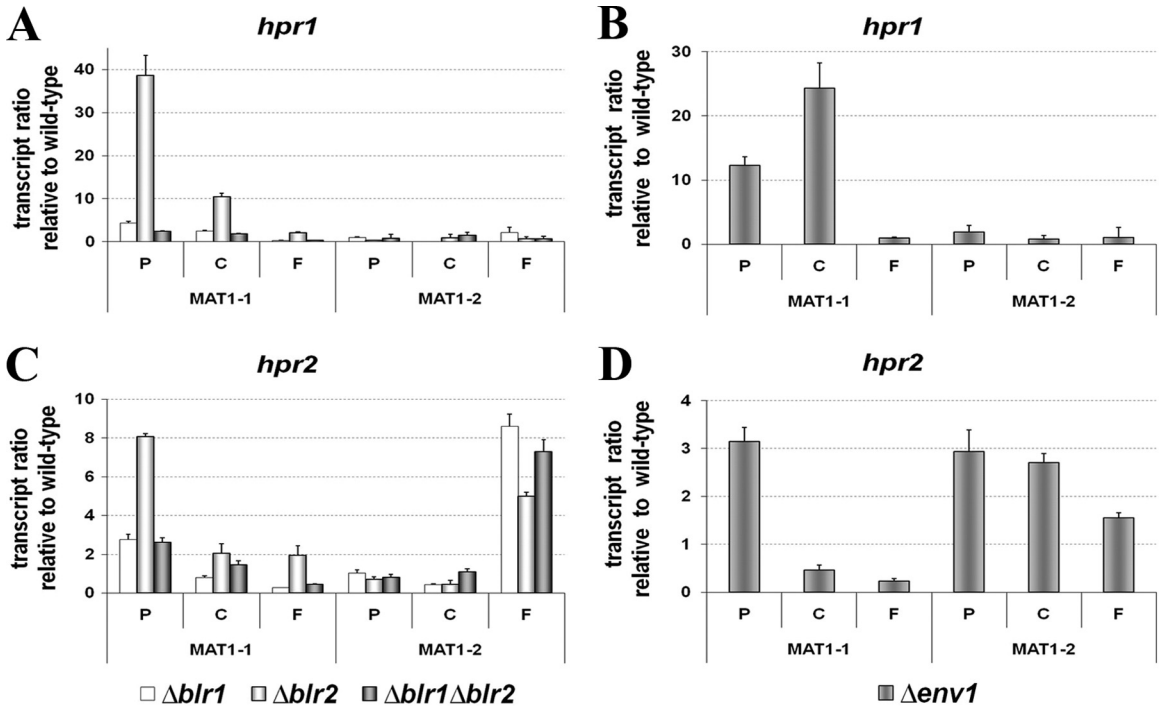

FIG 5 Transcription analysis of pheromone receptor genes *hpr1* and *hpr2* in Δ*blr1*, Δ*blr2*, Δ*blr1* Δ*blr2* and Δ*env1* strains compared to the wild type. Strains were cultivated in daylight at 22°C on malt-agar plates, i.e., conditions corresponding to mating assays. At indicated time points of 3, 4, and 6 days, confronted wild-type strains of opposite mating types undergo stages of precontact (P), initial contact between the colonies (C), and the macroscopically visible start of fruiting body formation (F), respectively. RNA was isolated at these time points after inoculation of the indicated strain from mycelia harvested from the growth front of the hyphae.

Δ*env1* MAT1-1 strain, which has strongly increased levels of both pheromone precursor and receptor genes, fruiting body formation started earlier and more vigorously than in the wild type (Fig. 6A). Fruiting body formation eventually also occurs in the Δ*env1* MAT1-2 strain, but the extremely strong upregulation of *hpp1* in this strain (>100,000-fold) may here interfere with proper regulation of this process—even upon crossing with the wild type. Crossing of strains lacking *env1* in both mating partners did not lead to earlier fruiting body formation in darkness (Fig. 6A). However, in accordance with our hypothesis, sexual development was possible in darkness between two strains lacking *env1* (Fig. 6B). Accordingly, transcript levels of both pheromone precursor genes and pheromone receptor genes are at wild-type levels under these conditions.

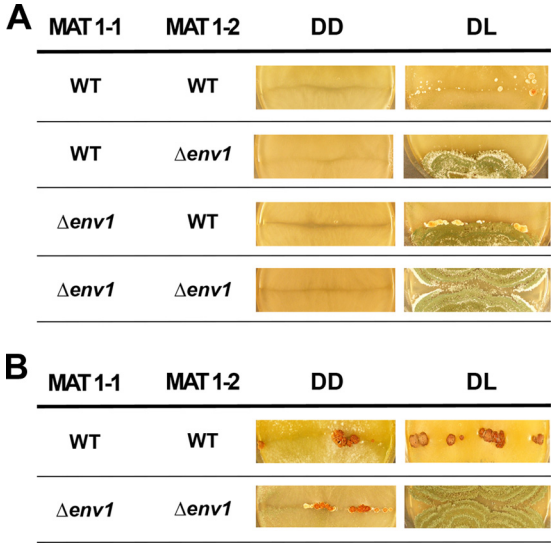

FIG 6 Fruiting body formation in wild-type (WT) and mutant crosses in daylight and darkness. (A) After 8 days of incubation, no fruiting body development is visible in darkness in all strains. (B) After 11 days of incubation in darkness in the wild-type as well as in the Δ*env1* mutant strains, fruiting bodies could be detected. Strains were cultivated either in constant darkness (DD) at 22°C, or they were kept under daylight conditions (12:12 cycles; DL).

ENVOY impacts transcription of the mating type gene *mat1-2-1*. The transcript analysis described above showed a mating-type-dependent effect for both BLR1/BLR2 and especially for ENV1 biased toward a MAT1-2 strain. Consequently, it is reasonable to assume that the regulatory output of BLR1, BLR2, and ENV1 is at least in part due to regulation of mating type genes. We used *mat1-2-1* in MAT1-2 strains as a test case for mating-type-specific regulation by the light response machinery. Indeed, we detected high transcript levels of *mat1-2-1* in strains lacking *blr1*, *blr2*, or both genes. Similar to the findings for pheromone and receptor genes, the effect was much stronger in the Δ*env1* strain (Fig. 7). As a consistently negative effect of BLR1, BLR2, and ENV1 was found, the effect on *mat1-2-1* is likely to be exerted by the photoreceptors via regulation of ENV1. The divergent transcript patterns for *hpp1*, *ppg1*, *hpr1*, and *hpr2* indicate that the mating-type-dependent involvement of BLR1, BLR2, and ENV1 in regulation of sexual development is not solely exerted via regulation of mating type genes but additionally uses other pathways.

Since the growth phenotype of *H. jecorina* QM9414 Δ*env1* (5,

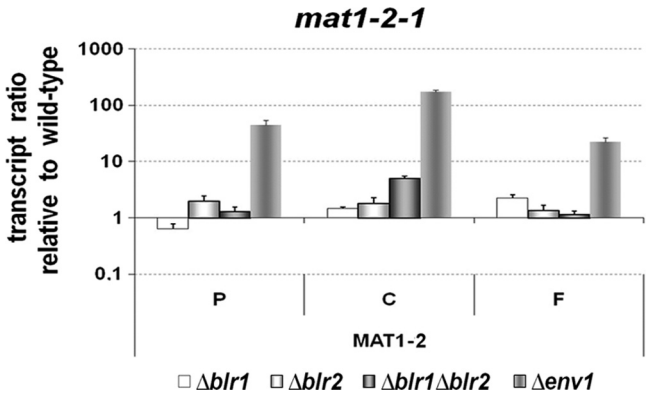

FIG 7 Transcript analysis of the mating type gene *mat1-2-1* in Δ*blr1*, Δ*blr2*, Δ*blr1* Δ*blr2*, and Δ*env1* strains compared to the wild type. Strains were cultivated in daylight at 22°C on malt-agar plates, i.e., conditions corresponding to mating assays. At indicated time points of 3, 4, and 6 days, confronted wild-type strains of opposite mating types undergo stages of precontact (P), initial contact between the colonies (C), and the macroscopically visible start of fruiting body formation (F), respectively. RNA was isolated at these time points after inoculation of the indicated strain from mycelia harvested from the growth front of the hyphae.

52) could not be rescued by deletion of *hpp1* in this strain (data not shown), it was concluded that pheromone-induced growth arrest potentially caused by the abnormally high levels of *hpp1* in the Δ*env1* strain (54) is not the reason for the strongly retarded growth of QM9414 Δ*env1* in light. Strong upregulation of mating type genes was, however, shown to result in suppression of vegetative growth and stimulation of sexual development in *A. nidulans* (45).

DISCUSSION

In several fungi blue light spectra modulate crucial developmental processes by the cooperative action of White Collar (4, 26, 37, 38, 58) and Vivid-like blue light regulators (52, 56). Recently, the effects of BLR1, BLR2, and ENV1 on conidiation and cellulase expression were described to be restricted to blue light in *H. jecorina* (5).

Both light induction of protoperithecium formation and phototropism of perithecia are subject to blue light induction and are impaired in *N. crassa* White Collar mutant strains (21, 29). Since neither phototropism nor protoperithecia formation has been observed so far in *H. jecorina* (54, 57), we could not evaluate if there is an influence on these phenomena.

We found altered sexual development in *H. jecorina* mutants lacking either BLR1, BLR2, or both photoreceptors in daylight. For *C. neoformans*, regulation of one pheromone gene and one homeodomain gene by BWC1 and BWC2 was observed (26). In *N. crassa*, an influence of circadian rhythms, which are subject to regulation by WC-1 and WC-2, on the pheromone precursor genes *ccg-4* and *mfa-1* is in accordance with these results (1). Together with the regulation of *hpp1* by ENV1 (54), these findings suggest an involvement of the light response machinery in regulation of genes important for mating (Fig. 8).

In this study, we show an involvement of BLR1, BLR2, and ENV1 in fruiting body formation and regulation of pheromone precursor and receptor genes as well as the mating type gene *mat1-2-1*. Interestingly, the effect of BLR1, BLR2, and ENV1 is mating type dependent. In *N. crassa* or *Sordaria macrospora*, regulation of pheromone receptor genes is mating type dependent and influenced by mating type genes (31, 35, 47). This regulation is considerably less distinct in *H. jecorina*, where both pheromone precursor genes were detected in both mating types (54).

The transcript profiles of *hpp1*, *ppg1*, *hpr1*, *hpr2*, and *mat1-2-1* in Δ*blr1* and Δ*blr2* strains suggest that BLR1 and BLR2 predominantly act as a complex and show a consistently negative effect on transcription of these genes. Lack of ENV1 was found to have a

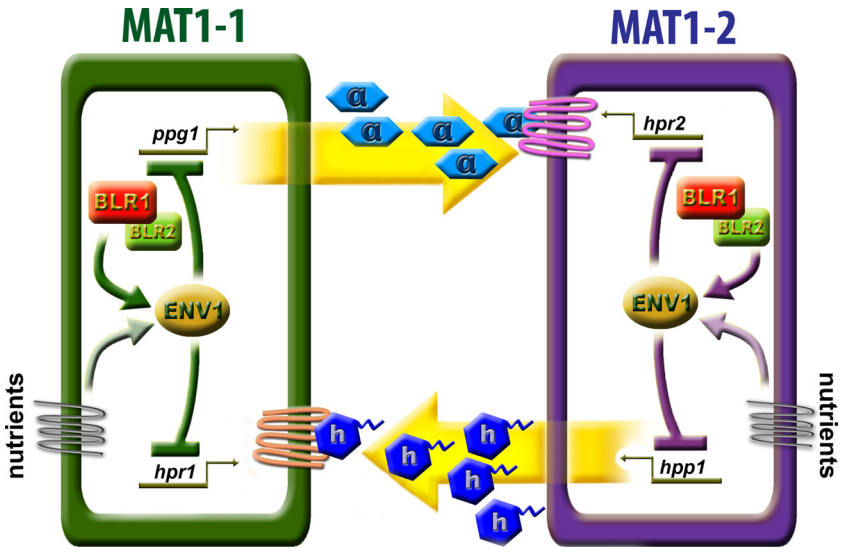

FIG 8 Schematic representation of the regulatory role of the light signaling machinery in MAT1-1 and MAT1-2 strains. Both pheromone precursors and receptors are influenced by the photoreceptors BLR1 and BLR2, likely via their impact on ENV1. The impact of the light response machinery is to a certain extent mating type dependent. In the MAT1-1 strain, the light signaling machinery predominantly acts on *ppg1* transcription and on transcript levels of the pheromone receptor presumably receiving the HPP1 (MAT1-2 mating type) signal, *hpr1*. In contrast, BLR1, BLR2, and ENV1 in the MAT1-2 background more strongly act on transcript levels of *hpp1* and the cognate receptor of the MAT1-1 pheromone precursor PPG1, *hpr2*. This strong effect on *hpp1* in this mating type is also reflected in earlier and more vigorous fruiting body formation of the Δ*env1* strain with the wild-type strain. The strong effect of ENV1 indicates that this factor may act as a node integrating mating signals with the nutrient signaling pathway. The pale arrows indicate this hypothesis, which remains to be proven.

considerably stronger effect than that of BLR1 or BLR2. This finding suggests that the effect of BLR1 and BLR2 is mediated by ENV1. Hence, ENV—as a node between pheromone and nutrient signals—may be responsible for cross talk between the respective pathways. The expression patterns in *env1* deletion strains, which are partially diverging from a *blr1* or *blr2* mutant, moreover, indicate that additional signals are integrated at this stage. We conclude that the signal introduced by light via photoreceptors is only one determinant for sexual development as regulated by this cascade. The involvement of ENV1 in the regulatory network of heterotrimeric G-protein signaling (53, 61, 62), which also transmits nutritional signals (39), supports this hypothesis.

Although BLR1 and BLR2 can be expected to act as a complex, our data indicate that they also have individual functions. The effect in mutants lacking *blr2* is in most cases stronger than that in mutants lacking *blr1*. In this respect a constitutive expression of BLR2 and thus higher abundance than BLR1, as suggested for their homologues in *N. crassa*, could be assumed. Together with the predicted importance of WC-2 for the interaction of WC-1 with FRQ (13), this could explain the more severe effect of deletion of *blr2* for regulation of sexual development. In *N. crassa*, neither the pheromone receptor genes nor the pheromone precursor genes or mating type genes are targets of the White Collar complex (59). Moreover, these genes also do not seem to be light induced in *N. crassa* (7). Consequently, we assume that in *H. jecorina* the homologues of these genes are likely to be indirect targets of BLR1 and BLR2. A flat hierarchical network (59) of transcription factors, as suggested for *N. crassa*, could be targeted by BLR1 and BLR2 in *H. jecorina*. However, since photoreceptors were shown to have a role in regulating carbon sensing and utilization (17), it cannot be excluded that altered cultivation conditions would also show binding to promoters of genes involved in sexual development.

While some evidence for an involvement of photoreceptor genes in regulation of sexual development in fungi was available, the possible relevance of genes homologous to *env1* for this process was suggested only in one previous report on *H. jecorina* (54). Our data suggest that the strongly negative effect of ENV1 on pheromone precursor and receptor genes and the mating type gene *mat1-2-1* are likely to be essential for proper regulation of sexual development in *H. jecorina*. The considerable deregulation of genes crucial for sexual development in a Δ*env1* strain can obviously be compensated by a wild-type mating partner or at least one that does not lack *env1* (such as a Δ*blr1* or Δ*blr2* strain). In this case the overexpression of pheromone and receptor genes results in earlier and enhanced sexual development (54; also this study). Such an effect can also be observed for Δ*blr1* and Δ*blr2* strains, which show certain upregulation of these genes but do not reach levels as high as in a Δ*env1* strain. Upon combination of equal amounts of spores, fruiting body formation seems to be initiated earlier and more vigorously than in the wild type as they are formed closer to the center of the petri dish (Fig. 2). In a Δ*env1* strain, however, signals specific to both mating types likely exceed saturation levels and consequently inhibit appropriate coordination of developmental programs, which abolishes fruiting body formation.

Since ENV1 is not a transcription factor, its effect is exerted by a downstream signal transduction cascade. This cascade has been shown to involve the heterotrimeric G-protein pathway as well as cAMP signaling in *H. jecorina* (53, 55, 61, 62). Considering an effect of *env1* on G-protein signaling, mate recognition, cell fusion and postfusion processes like ascospore or fruiting body development could be impaired, as shown for *N. crassa* G-alpha subunits (30, 32). In *Saccharomyces cerevisiae* or *Ustilago maydis*, cell fusion in mating is a process that requires pheromone signaling for the polarization of the cytoskeleton, polarisome, and Spitzenkörper (15, 19). Additionally, the pheromone-regulated plasma membrane merger protein PRM1 is required for cell fusion in sexual development of *S. cerevisiae* and *N. crassa* (16, 22). Hence, the mating defect of strains lacking *env1* may involve perturbed cell fusion.

In summary, we show that the light response machinery with its major constituents BLR1, BLR2, and ENV1 plays an important role in sexual development of *H. jecorina*. BLR1, BLR2, and ENV1 negatively influence expression of pheromone precursor and receptor genes. Alleviation of the strong repression exerted by ENV1 even disables fruiting body formation, likely due to signal strengths exceeding saturation levels and/or defects in cell fusion. The mating-type-dependent effects of these factors suggest a contribution to determination of sexual identity signaling in this heterothallic, hermaphroditic organism.

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