Analysis of Circadian Rhythms in the Basal Filamentous Ascomycete *Pyronema confluens*

Stefanie Traeger and Minou Nowrousian
Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, 44780 Bochum, Germany

**ABSTRACT** Many organisms use circadian clocks to adapt to daily changes in the environment. Major insights into the molecular mechanisms of circadian oscillators have been gained through studies of the model organism *Neurospora crassa*; however, little is known about molecular components of circadian clocks in other fungi. An important part of the *N. crassa* circadian clock is the frequency (*frq*) gene, homologs of which can be found in Sordariomycetes, Dothideomycetes, and Leotiomycetes, but not Eurotiomycetes. Recently, we identified a *frq* homolog in *Pyronema confluens*, a member of the early-diverging Pezizomycotina lineage of filamentous ascomycetes. The *P. confluens* FRQ shares many conserved domains with the *N. crassa* FRQ. However, there is no known morphological phenotype showing overt circadian rhythmicity in *P. confluens*. To investigate whether a molecular clock is present, we analyzed *frq* transcription in constant darkness, and found circadian oscillation of *frq* with a peak in the subjective morning. This rhythm was temperature compensated. To identify additional clock-controlled genes, we performed RNA sequencing of two time points (subjective morning and evening). Circadian expression of two morning-specific genes was verified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) over a full time course, whereas expression of two putative morning-specific and five putative evening-specific genes could not be verified as circadian. *frq* expression was synchronized, but not entrained by light. In summary, we have found evidence for two of the three main properties of circadian rhythms (free-running rhythm, temperature compensation) in *P. confluens*, suggesting that a circadian clock with rhythmically expressed *frq* is present in this basal filamentous ascomycete.

Adaptation to daily changes in the environment, e.g., fluctuating temperatures and light levels, is a central feature in the life of most organisms. Circadian clocks aid with these adaptations by enabling cells and organisms to anticipate such changes instead of simply responding to them (Dunlap and Loros 2006; Fuller et al. 2014). Circadian systems can be found in prokaryotes and eukaryotes, and the molecular mechanisms generating these rhythms have been studied in model organisms as varied as the cyanobacterium *Synechococcus elongatus*, the plant *Arabidopsis thaliana*, the fungus *Neurospora crassa*, the fly *Drosophila melanogaster*, and mammals, e.g., mice (Bell-Pedersen et al. 2005; Salomé and McClung 2004; Sancar and Brunner 2014). Major contributions toward elucidating clock mechanisms have come from studies on circadian rhythms in the filamentous ascomycete *N. crassa* (Dunlap 2006; Heintzen and Liu 2007). In several other fungi, circadian rhythms have been described for different morphologic or metabolic outputs; however, little is known about molecular components of circadian oscillators in these species (Greene et al. 2003; Hurley et al. 2015; Loros and Dunlap 2001; Oliveira et al. 2015).

Circadian rhythms can be defined by three characteristic features, namely a period length of about 24 hr, the ability to be reset or entrained by periodic light or temperature cues, and the ability to run stably within the physiological range of changes in nutrients or temperature (temperature compensation) (Bell-Pedersen et al. 2005; Fuller et al. 2014). In *N. crassa*, an easily observable phenotypic output of the circadian clock is the rhythmic conidiation; and the molecular machinery driving circadian output is the FWO or FRQ/WCC (white collar complex) oscillator, which is named for three of its essential components, the
Figure 1 Continued.
Figure 1: Multiple alignment of FRQ homologs from different ascomycetes. Protein domains that were characterized in the N. crassa FRQ are indicated below the alignment: Coiled-coil domain (Cheng et al. 2001); FCD, FRQ-CK1a interaction domain (He et al. 2006; Querfurth et al. 2011); FFD, FRQ/FRH interaction domain (Guo et al. 2010); NLS, nuclear localization signal (Luo et al. 1998); PEST domains (Merrow and Dunlap 1994). Intrinsically unstructured regions were predicted with IUPred (Dosztányi et al. 2005) for the N. crassa and P. confluens FRQ proteins, indicated by dashed black and gray lines, respectively, above the sequence. The following sequences were used: A.c. Acremonium chrysogenum gb|KFH48196.1; A.o. Arthrobotrys oligospora gb|EGX51094.1; B.b. Beauveria bassiana ref|XP_008594847.1; B.c. Botrytis cinerea ref|XP_001547609.1; C.o. Colletotrichum orbiculare gb|ENH76664.1; C.p. Claviceps purpurea emb|CCE27108.1; F.g. Fusarium graminearum gb|ESU12098.1; L.m. Leptosphaeria maculans ref|XP_003845311.1; M.p. Macrophomina phaseolina gb|EKG17747.1; N.c. Neurospora crassa gb|AAA57121.1; P.t. Pyrenophora tritici-repentis ref|XP_001941961.1; P.c. Pyronema confluens PCON_09365; S.a. Scedosporium apiospermum gb|KEZ43196.1; S.m. Sordaria macrospora ref|XP_003351398.1. The alignment was visualized in Jalview using the ClustalX color scheme (Thompson et al. 1997; Waterhouse et al. 2009).
frequency (frq), white collar-1 (wc-1), and white collar-2 (wc-2) genes (Fuller et al. 2014). The FWO organizes a stable circadian rhythm via a transcriptional/translational feedback loop that requires two main protein complexes. One is the WCC consisting of the photoreceptor WC-1 and the WC-2 protein, and the second comprises FRQ and FRQ-interacting helicase (FRH) (Fuller et al. 2014). The WCC acts as the positive arm in the oscillator loop by binding the frq promoter and activating frq transcription (Froehlich et al. 2002, 2003). FRQ protein is made and interacts with FRH, and the resulting complex acts as the negative arm of the oscillator by binding to the WCC, leading to phosphorylation and inactivation of this complex (Cheng et al. 2005; He et al. 2006; Schafmeier et al. 2005). This, in turn, leads to reduced frq transcription and subsequently to a decrease in FRQ protein levels. Once FRQ/FRH-mediated inhibition of the WCC is reduced, the WCC resumes its activity and the cycle starts again.

Besides the FWO, other oscillators in N. crassa have been collectively described as FLOs or frq-less-oscillators (De Paula et al. 2006; Dragovic et al. 2002; Lakin-Thomas et al. 2011; Nsa et al. 2015). Molecular components of the FLOs that are not also part of the FWO were largely unknown until the recent characterization of an oscillator that does not require frq but the cryochrome-encoding cry gene and was therefore named cry-dependent oscillator (Nsa et al. 2015). However, most FLOs, including the cry-dependent oscillator, lack some of the defining properties of circadian rhythms, giving rise to the hypothesis that the FWO is the major oscillator in N. crassa with other oscillators being ancillary or active only under certain physiological conditions (Dunlap and Loros 2004).

The frq gene as a core component of the FWO has been studied intensively for more than 20 yr, and besides the aforementioned regulatory events, has been found to be regulated at every conceivable level of expression to fine-tune the N. crassa clock under a wide range of environmental conditions (Cha et al. 2015; Fuller et al. 2014). Interestingly though, FRQ is the least conserved among the known oscillator proteins in N. crassa. Whereas FRH is a conserved eukaryotic protein, and WC-1 and WC-2 are conserved widely in fungi from zygomycetes and WC-1 and WC-2 are conserved widely in fungi from zygomycetes and Dothideomycetes, and Eurotiomycetes (Traeger et al. 2013). This also implies that frq was probably lost in the ancestor of the Eurotiomycetes, which is the only lineage of filamentous ascomycetes where no frq homolog has been found to date (Salichos and Rokas 2010).

The P. confluens frq is light induced with blue light being the activating part of the visible spectrum, similar to its N. crassa counterpart (Crosthwaite et al. 1995; Froehlich et al. 2002; Traeger et al. 2013). Furthermore, analysis of the P. confluens genome as well as expression analyses revealed that homologs of other clock components, namely WC-1, WC-2, and the downstream transcription factor SUB-1, are present and expressed in P. confluens (Traeger et al. 2013). Therefore, we wondered whether a circadian clock was present in this fungus and whether other properties of the P. confluens frq besides light induction were also similar to N. crassa, namely a circadian regulation of transcript levels.

Here, we present evidence that P. confluens frq transcript levels are rhythmic and temperature-compensated. Furthermore, we performed RNA sequencing (RNA-seq) for two different time points during the circadian cycle and identified several putative clock-controlled genes (ccls). Circadian expression under free-running conditions could be verified for two morning-specific genes by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Our data suggest that a circadian clock with a rhythmically expressed frq gene is active in the basil filamentous ascomycete P. confluens.

MATERIALS AND METHODS

Strains and culture conditions

P. confluens CBS100304 was grown on minimal medium as described previously (Nowrousian and Kück 2006; Traeger et al. 2013). For analysis of circadian rhythms, P. confluens was grown in shaken cultures in minimal medium (7.5 mL in 100-mL Erlenmeyer flask) at 25°C or 30°C that were inoculated with mycelial plugs from 3-d cultures in liquid medium as described (Nowrousian and Kück 2006). Cultures were entrained for at least one day in constant white light (LL), shifted to dark (DD) in 4-hr intervals over 2 d (Supporting Information, Figure S1), and harvested under red light as described (Nowrousian and Kück 2006). Previous analyses had shown that P. confluens is not sensitive to red light (Nowrousian and Kück 2006; Traeger et al. 2013). For light entrainment analysis, 100-mL cultures inoculated with mycelial plugs were kept at 25°C in LL for at least 4 hr and were subsequently subjected to dark/light cycles (9 hr/9 hr, 12 hr/12 hr, or 14 hr/14 hr) and harvested under red light at defined intervals before and after “lights on” on the fourth day (Figure S2). For analyses of free-running rhythms as well as entrainment, cultures at the time of harvest had approximately the same age (72–80 hr). Under the shaking conditions used, mycelial plugs reached a stationary phase (cease of mycelial growth) after about 2.5 d, therefore cultures were in stationary phase at the time of harvest.

RNA extraction and RT-qPCR analysis

P. confluens RNA was prepared with the RNAeasy lipid tissue mini kit (QUIGEN, Hilden, Germany) with modifications as described (Nowrousian and Kück 2006). Reverse-transcription and RT-qPCR were performed as described previously (Gesing et al. 2013; Schindler and Nowrousian 2014). Oligonucleotide primers for RT-qPCR are given in Table S1. Statistical analysis of rhythmicity (period length 20–28 hr) was performed with JTK_CYCLE (Hughes et al. 2010) in the R computing environment (version 3.1.1) using the normalized expression values (greatest value set to 100%) for all replicates at 25°C and if performed) at 30°C for each gene for analysis as described in the JTK_CYCLE manual, with genes with P-values ≤ 0.01 regarded as rhythmic.

RNA-seq analysis

For Illumina/Solexa RNA-Seq analysis, RNA of three independent biological replicates of the subjective morning (DD36) and subjective evening (DD24) was sequenced at GATC Biotech (Konstanz, Germany). Raw data were analyzed and trimmed as described previously and mapped to the reference genome v01_2 of P. confluens (Teichert et al. 2012; Traeger et al. 2013). For quantitative analysis of gene expression, DESeq and LOX were used to calculate expression ratios (morning vs. evening) for all predicted genes (Anders and Huber 2010; Zhang et al. 2010).

Phylogenetic analysis

Multiple alignments were created in CLUSTALX (Thompson et al. 1997), manually adjusted, and visualized with Jalview (Waterhouse et al. 2009).
et al. 2009), and the same alignment was used for analysis by neighbor joining or maximum parsimony. Phylogenetic analyses were made with PAUP version 4.0b10 for Windows (D.L. Swofford, distributed by Sinauer Associates, copyright 2001 Smithsonian Institution). Neighbor joining and maximum parsimony analyses were performed as described using 1000 bootstrap replicates (Hall 2004). Consensus trees were graphically displayed with Dendroscope (Huson et al. 2007).

Data availability
The RNA-seq reads and derived expression ratios generated in this study were submitted to the GEO database (accession number GSE61263). The Supporting Information contains Figure S1, Figure S2, Figure S3, and Figure S4, as well as Table S1 and Table S3. Table S2 is presented in a separate Excel file. Figure S1, growth regime for analysis of free-running rhythms in P. confluens. Figure S2, growth regime for analysis of light entrainment in P. confluens. Figure S3, phylogenetic analysis of FRQ homologs from different ascomycetes. Figure S4, corrected genomic sequence of the S. macrospora frq homolog (SMAC_03705). Table S1, oblignucleotides used in the study. Table S2, analysis of differential gene expression at two different time points (DD24, DD36) in P. confluens by RNQ-seq. Table S3, short term light induction of putative clock-controlled genes.

RESULTS

P. confluens FRQ comprises conserved domains, and frq expression is rhythmic and temperature-compensated

Circadian clocks are characterized by three characteristics: (1) They sustain a near-24-hr rhythm under constant conditions; (2) the rhythm is stable under a wide range of conditions, e.g., at different temperatures; and (3) the rhythm can be entrained, i.e., it can adjust to environmental cues like day–night cycles (Dunlap et al. 2007). In N. crassa, an easily visible phenotypic output from the clock is the rhythmic banding of conidiation on race tubes in constant darkness (DD); P. confluens, however, does not produce any conidia. Fruiting body formation in P. confluens is strictly light-dependent (Claussen 1912; Gwynne-Vaughan and Williamson 1931; Traeger et al. 2013), therefore cannot be observed in DD; and in our analyses, fruiting body formation was not rhythmic in the light (data not shown). Thus, at present there is no morphologic phenotype that shows circadian rhythmicity in P. confluens; however, the presence of an frq gene opens the possibility that this gene might be associated with circadian rhythmicity and that a circadian clock might be active at the molecular level in this fungus.

The P. confluens frq gene was the first to be discovered in the early-diverging Pezizomycete lineage, pushing the evolutionary origin of frq back to (at least) the last common ancestor of filamentous ascomycetes (Traeger et al. 2013). This is also supported by the presence of a frq homolog in Arthrobotrys oligospora, a species that belongs to the Orbiliomycetes, a sister group of the Pezizomycetes (Traeger et al. 2013; Yang et al. 2011) (Figure 1, Figure S3). The FRQ homologs from these two species are moderately conserved across the full length of the predicted proteins with several highly conserved interspersed regions, and cluster together in a phylogenetic analysis of FRQ proteins from different ascomycete groups as expected (Figure 1, Figure S3).

Figure 2 Expression of P. confluens frq and two putative ccgs is rhythmic under free-running conditions. Transcript levels were determined by RT-qPCR after the indicated times in constant darkness (DD). The growth regime is shown in Figure S1. Means and standard errors from three independent replicates at 25°C and two independent replicates at 30°C, respectively, are shown. The greatest value in each time course was set to 100%. Standard errors are shown in one direction only (up or down) for better visualization. Statistical analysis of circadian expression was performed using JTK_CYCLE (Hughes et al. 2010), P-values for an analysis including all replicates at both temperatures are given after the corresponding gene names.
### Table 1: Overview of RNA-seq experiments for two time points (DD24 and DD36)

| Sample Name | Sample | No. of Reads | No. of Trimmed Reads | No. of Mapped Reads | % of Reads That Map |
|-------------|--------|--------------|----------------------|---------------------|---------------------|
| Pcon13      | DD24_a | 18,998,703   | 18,618,092           | 17,530,162          | 94.2                |
| Pcon14      | DD24_b | 31,096,297   | 30,465,329           | 28,232,290          | 92.7                |
| Pcon15      | DD24_c | 21,487,791   | 21,050,367           | 19,623,940          | 93.2                |
| Pcon16      | DD36_a | 23,668,267   | 23,190,410           | 21,286,467          | 91.8                |
| Pcon17      | DD36_b | 28,208,438   | 27,626,313           | 25,926,388          | 93.8                |
| Pcon18      | DD36_c | 24,131,812   | 23,625,427           | 22,375,339          | 94.7                |

Three independent biological replicates (a-c) were analyzed for each time point. RNA-seq, RNA sequencing.

---

The *N. crassa* FRQ protein comprises several protein domains that were shown to be involved in clock functions. These domains show varying degrees of conservation in *P. confluens* (Figure 1). Of the two PEST domains, both of which are phosphorylated and necessary for rhythmicity in *N. crassa* (Göril et al. 2001; Merrow and Dunlap 1994; Schafmeier et al. 2006), the second shows stronger conservation than the first (Figure 1). In *N. crassa*, the two PEST domains have distinct functions, with PEST1 involved in determining period length, and PEST2 involved in cytoplasmic accumulation of the WCC (Göril et al. 2001; Schafmeier et al. 2006). The greater conservation of PEST2 could indicate a more conserved function for this domain, or alternatively the function of PEST1 might be less dependent on actual sequence and rather on the ability to be phosphorylated. A coiled-coil domain in the *N. crassa* FRQ was shown to be essential for rhythmic conidiation and for the interaction of FRQ with itself (Cheng et al. 2001). The coiled-coil domain is conserved in most FRQ homologs, including two leucine residues (positions 165 and 169) that are important for FRQ-FRQ interaction in *N. crassa* (Cheng et al. 2001). Two FRQ-CK1α interaction domains (FCD1 and FCD2) are strongly conserved in *P. confluens* (Figure 1). In *N. crassa*, the domains are important for the interaction of casein kinase 1α with FRQ and its subsequent phosphorylation (He et al. 2006; Querfurth et al. 2011). In contrast, the FRQ/FRH interaction domain (FFD) is only moderately conserved in *P. confluens*. In *N. crassa*, only the FRQ–FRH complex can interact with the WCC and sustain stable rhythmicity (Guo et al. 2010). Whether the partially conserved FFD might support interaction with the *P. confluens* FRH homolog (PCON_11360) remains to be elucidated. The nuclear localization signal present in the *N. crassa* FRQ (Luo et al. 1998) is not conserved in *P. confluens*; however, PSORT (Nakai and Horton 1999) predicts the *P. confluens* FRQ as a nuclear protein with several putative nuclear localization signals located elsewhere in the protein (data not shown). Apart from the presence of several functional domains, it was shown that the *N. crassa* FRQ is an intrinsically disordered protein that contains large regions of low structural complexity (Hurley et al. 2013; Querfurth et al. 2011). Using IUPred (Dosztányi et al. 2005), a similar tendency was observed in the *P. confluens* FRQ (Figure 1). Overall, the conservation of many functionally important domains combined with large regions of low structural complexity indicates that major features of FRQ that were identified in *N. crassa* are conserved in *P. confluens* and other fungal species and therefore might have been already present in the last common ancestor of filamentous ascomycetes. In addition, there are several regions conserved across FRQ homologs from filamentous ascomycetes for which no molecular roles have been assigned yet (Figure 1). These might be potential regions of interest for future functional studies.

In *N. crassa*, *frq* transcript levels are rhythmic in DD, and this rhythm is temperature-compensated within the physiological temperature range for rhythmicity (Aronson et al. 1994; Liu et al. 1998). To check whether expression of the *P. confluens* *frq* is also rhythmic and temperature-compensated, we analyzed transcript levels by RT-qPCR during a 48-hr time course in DD at two different temperatures (Figure 2). *frq* expression peaked in the subjective morning, i.e., ~12 and ~36 hr after transfer to DD, with a ~24-hr rhythm that was sustained at 25 and 30°C, indicating temperature-compensation. Statistical analysis with *ttK_CYCLE* (Hughes et al. 2010) confirmed rhythmicity, despite high variability of individual time courses leading to large error bars in Figure 2 (see Discussion). Thus, *frq* in *P. confluens* shows properties of a clock-controlled gene (ccg). Whether it is part of a circadian oscillator machinery cannot be concluded from these data.

### Identification of ccgs by RNA-seq

To search for ccgs besides *frq*, we performed RNA-seq experiments with samples from two different time points during the circadian day representing the subjective evening and subjective morning. RNA was extracted from samples 24 hr (subjective evening) and 36 hr (subjective morning) after transfer to DD. RNA-seq was performed on three independent biological replicates per time point (Table 1). Differential gene expression analysis to identify genes that were up-regulated during the subjective morning or subjective evening was performed with DESeq and LOX (Anders and Huber 2010; Zhang et al. 2010). Expression data for all *P. confluens* genes as well as for the 80 most significantly regulated genes are presented in Table S2. Interestingly, of the 80 most significantly regulated genes, only eight were up-regulated in DD36 vs. DD24 and thus putative morning-specific ccgs, whereas the others were down-regulated and thus putative evening-specific ccgs (Table S2). To test whether putative ccgs identified by RNA-seq showed truly circadian expression patterns, four putative morning-specific and five evening-specific genes chosen from the 80 most significantly regulated genes were analyzed over a 48-hr time course by RT-qPCR (Figure 2, Figure 3, and Table 2). However, only expression of two of the morning-specific genes could be verified as circadian at 25°C and 30°C, whereas the five evening-specific genes were arrhythmic when tested at 25°C. The two remaining morning-specific genes were also arrhythmic, but with better P-values in a *ttK_CYCLE* test for circadian rhythmicity (Hughes et al. 2010), suggesting that they might be under weak clock control (Figure 3). Overall, two morning-specific genes were verified as ccgs with rhythmic and temperature-compensated expression.

The two novel ccgs encode a protein of unknown function (PCON_04080), and a WD40-repeat protein (PCON_09334) that is a 90S pre-ribosomal component required for proper cell cycle and bud morphogenesis in *Saccharomyces cerevisiae* (Dosil and Bustelo 2014), and a putative D-galactonate dehydratase (*PCON_04507*) that might be involved in sugar acid metabolism (Motter et al. 2014), and a putative protease (*PCON_13506*).
RNA-seq results (Table S2). One possibility for this might be that only two time points rather than a full time course were chosen for RNA-seq analysis, and therefore slight shifts of peak times might lead to lower signal to noise ratios.

Some ccgs are also light-regulated
In *N. crassa*, light signaling and the circadian clock share several components, and light is one of the factors that can entrain the circadian rhythm (Dunlap et al. 2007; Fuller et al. 2014). *P. confluens* shows...
strong reactions to light (including sexual development, which is completely light-dependent), and many genes, including frq, are light-induced (Traeger et al. 2013). Therefore, we tested whether the novel ccgs also might be controlled by light. We analyzed transcript levels of the two ccgs after long-term light induction (4 d), as well as induction after short light pulses from 5 to 60 min (Figure 4). The long-term light induction was chosen, because this is a biologically relevant condition under which P. confluens is able to develop fruiting bodies, whereas it is sterile in complete darkness. Under long-term light induction, PCON_04080 is strongly up-regulated, and PCON_09934 is marginally upregulated (Figure 4A). In the short-term light induction experiments, PCON_04080 is also up-regulated, and PCON_09934 is not differentially expressed (Figure 4B and Table S3). Thus, the two ccgs do not show a uniform reaction to light, but reveal distinct expression profiles under different light conditions. The two probably weakly clock-controlled genes, PCON_04507 and PCON_13506, are not differentially expressed under long-term light induction, whereas PCON_04507 is down-regulated in the short-term light induction experiments (Figure 4). 

Analysis of light-entrainment of ccgs 

An important property of circadian clocks is the ability to become entrained to external stimuli (“zeitgeber”), e.g., to daily cycles of light and temperature. The result of entrainment is that the period of the rhythm becomes equal to that of the entraining stimuli, and that a stable phase relationship is established between the external stimuli and the entrained rhythm (Johnson et al. 2003). In true entrainment, this means that the phase relationship depends on the period length of the zeitgeber and can be tested by using zeitgeber cycles that are shorter and temperature. The result of entrainment is that the period of the rhythm becomes equal to that of the entraining stimuli, and that a stable phase relationship is established between the external stimuli and the entrained rhythm (Johnson et al. 2003). In true entrainment, this means that the phase relationship depends on the period length of the zeitgeber and can be tested by using zeitgeber cycles that are shorter or longer than 24 h. If the phase angle of the resulting rhythm is the same phase relationship to the zeitgeber regardless of zeitgeber period lengths, this is a good indication of entrainment. However, if the resulting rhythm shows the same phase relationship to the zeitgeber regardless of zeitgeber period lengths, it is likely to be driven, not entrained (Johnson et al. 2003; Nsa et al. 2015). Thus, for a ~24-hr, free-running rhythm and a morning-specific gene, in case of light entrainment one would expect transcript levels to rise before the “lights on” with zeitgeber period lengths longer than 24 hr (e.g., in 14-hr DD / 14-hr LL cycles), and after “lights on” with zeitgeber period lengths shorter than 24 hr (e.g., in 9-hr DD / 9-hr LL cycles).

We tested whether the putative ccgs including frq can be light-entrained in P. confluens. The putative ccgs are morning-specific under free-running conditions, and therefore would be expected to increase expression around the time of “lights on” in dark/light cycles. We therefore analyzed expression at six time points covering a time frame from 4 hr before to 4 hr after “lights on” in three different dark/light regimes (9 hr/9 hr, 12 hr/12 hr, 14 hr/14 hr) (Figure 5). Light induction

---

Table 2 RNA-seq results for putative clock-controlled genes (ccgs) that were tested by RT-qPCR

| Gene                  | LOX | DESeq | p/padj | Product; Acc. No. of Homolog With Predicted or Described Function                                      |
|-----------------------|-----|-------|--------|--------------------------------------------------------------------------------------------------------|
| Putative morning-specific ccgs |
| PCON_04080            | 1.09| 0.82  | 0.00/0.04| Protein of unknown function                                                                            |
| PCON_04507            | 1.12| 0.94  | 0.00/0.04| D-galactonate dehydratase; EHA19069.1                                                                 |
| PCON_09334            | 1.29| 1.11  | 0.00/0.20| periodic tryptophan 2; P25635.2                                                                           |
| PCON_13506            | 1.32| 1.04  | 0.00/0.00| subtilisin-like protease; C5PCX1                                                                         |
| Putative evening-specific ccgs |
| PCON_01669            | -0.70| -0.88 | 0.00/0.03| mannitol-1-phosphate 5-dehydrogenase; Q9CLY7                                                             |
| PCON_03866            | -0.60| -0.79 | 0.00/0.13| trehalose phosphorylase; A6YRN9                                                                          |
| PCON_08647            | -1.25| -1.39 | 0.00/0.03| UPP0591 membrane protein C15E1.02c; Q9UT19                                                              |
| PCON_09693            | -2.19| -2.45 | 0.00/0.00| fibrinogen alpha chain; P02672                                                                           |
| PCON_12782            | -1.96| -1.76 | 0.00/0.00| protein of unknown function                                                                               |

Log2 values of ratios calculated with LOX or DESeq are given, as well as P-values and padj values for DESeq. RNA-seq, RNA sequencing; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

---

Figure 4 Analysis of light-dependent expression of two putative ccgs and two putative weakly clock-controlled genes by RT-qPCR. (A) Gene expression after growth for 4 d in constant light (LL) vs. constant darkness (DD). Mean ratios and standard deviations of two independent biological replicates are shown. A red dashed line indicates twofold up-regulation. (B) Gene expression ratios after growth for 4 d in constant darkness and subsequent light induction for 5–60 min. Mean ratios of two independent biological replicates are shown. Standard deviations are left out for clarity, values including standard deviations are given in Table S3. Dashed red lines indicate twofold up- or down-regulation.
Figure 5  Analysis of light-entrainment for three morning-specific genes by RT-qPCR. Cultures were subjected to dark/light cycles (9 hr/9 hr, 12 hr/12 hr, and 14 hr/14 hr). Samples were harvested at specific time points before or after “lights on” (time 0, dashed vertical line) on day 4 (for entrainment schedule, see Figure S2). Analyses were performed for two independent replicates for each light regime, the greatest value in each experiment was set to 100%, and the mean of two experiments is shown. Standard errors are shown in one direction only (up or down) for better visualization. was observed for frq but not consistently for the other ccgs. For PCON_04080, a short-term increase was observed in transcript levels 0.25 hr after “lights on,” which reflects the light induction that was also observed in the short-term light experiments (Figure 4B). In the case of frq, the increase in transcript levels coincides with “lights on” regardless of zeitgeber lengths, which is not consistent with entrainment, but rather with a driven (i.e., synchronized) rhythm. For the putatively weakly rhythmic genes PCON_04507 and PCON_13506, no light entrainment was found either (data not shown). Therefore, at present there is no evidence for light entrainment of P. confluens circadian rhythms.

DISCUSSION

In this study, we have shown that the frq gene in the basal filamentous ascomycete P. confluens shows two of the three main properties that characterize a circadian rhythm (~24-hr free-running period length, temperature compensation). Furthermore, we identified two additional ccgs by RNA-seq that also show these two properties. Thus, a circadian clock seems to be active in P. confluens at the molecular level, even though at present no morphologic output is known. A similar finding was made in Aspergillus nidulans, where no developmental rhythms were observed, but expression of the gpdA gene was shown to be under circadian control (Greene et al. 2003). Entrainment of gpdA in A. nidulans required the combined input of light and temperature cycles, because neither light nor temperature alone was sufficient for entrainment (Greene et al. 2003). A similar phenomenon might be at work in P. confluens, where we found that light is able to drive, but not entrain frq rhythms (Figure 5).

The surprisingly high conservation of several domains of FRQ in P. confluens might lead to the hypothesis that the P. confluens frq is part of a circadian oscillator itself. If frq were part of the oscillator that would suggest that the FWO is an evolutionary conserved molecular mechanism that was already present in the ancestor of filamentous ascomycetes. However, frq might very well have been part of the clock-controlled output first, being recruited into the core oscillator only in selected groups like the Sordariomycetes later during evolution. Our data do not allow conclusions on whether frq is part of the oscillator, or part of the output controlled by a circadian clock in P. confluens.

One point to note is the high variability in replicate time course experiments for ccg expression including frq (large error bars in Figure 2). One possible explanation might be that the P. confluens circadian rhythm did not evolve to the same level of robustness as found in N. crassa, and in the recently described circadian clock in B. cinerea (Hevia et al. 2015; Hurley et al. 2015). The last common ancestor of the lineages leading to P. confluens and the other filamentous ascomycetes, respectively, was estimated to have lived 260–413 million years ago (Traeger et al. 2013). Therefore, most likely the evolution of circadian rhythms in the early diverging Pezizomycetes vs. the other filamentous ascomycetes including N. crassa and B. cinerea was largely independent. Other possible explanations include the growth conditions or the genetic background in the experiments (see below).

Using RNA-seq, we identified a number of potential ccgs. Surprisingly, the number of putative evening-specific genes was much greater among the significantly differentially regulated genes than the number of morning-specific genes (Table S2). However, none of the putative evening-specific genes that were tested could be verified as circadian, whereas two of four morning-specific genes that were tested showed properties of circadian regulation. One reason for this finding might be that the analysis of only two time points is not specific enough to suppress false-positives, and that full time courses are needed (Li et al. 2015). In N. crassa, the search for ccgs started more than 20 years...
ACKNOWLEDGMENTS
We thank Svenja Ellßel for excellent technical assistance and Prof. Dr. Ulrich Kück (Bochum) for continuing support. This work was supported by the German Research Foundation (DFG, grant NO407/4-1).

LITERATURE CITED
Anders, S., and W. Huber, 2010 Differential expression analysis for sequence count data. Genome Biol. 11: R106.
Aronson, B., K. Johnson, J. J. Loros, and J. C. Dunlap, 1994 Negative feedback defining a circadian clock: autoregulation in the clock gene frequency. Science 263: 1578–1584.
Belden, W. J., L. F. Larrondo, A. C. Froehlich, M. Shi, C.-H. Chen et al., 2007 The band mutation in Neurospora crassa is a dominant allele of ras-l implicating RAS signaling in circadian output. Genes Dev. 21: 1494–1505.
Bell-Pedersen, D., M. L. Shinohara, J. J. Loros, and J. C. Dunlap, 1996 Circadian clock-controlled genes isolated from Neurospora crassa are late night to early morning specific. Proc. Natl. Acad. Sci. USA 93: 13096–13101.
Bell-Pedersen, D., V. M. Cassone, D. J. Earnest, S. S. Golden, P. E. Hardin et al., 2005 Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. 6: 544–556.
Bennett, L. D., P. Beremand, T. L. Thomas, and D. Bell-Pedersen, 2013 Circadian activation of the mitogen-activated protein kinase MAK-1 facilitates rhythms in clock-controlled genes in Neurospora crassa. Eukaryot. Cell 12: 59–69.
Cha, J., M. Zhou, and Y. Liu, 2015 Mechanism of the Neurospora circadian clock, a FRQUENCY-centric view. Biochemistry 54: 150–156.
Cheng, P., Y. Yang, C. Heinzen, and Y. Liu, 2001 Coiled-coil domain-mediated FRQ-FRQ interaction is essential for its circadian clock function in Neurospora. EMBO J. 20: 101–108.
Cheng, P., Q. He, Q. He, L. Wang, and Y. Liu, 2005 Regulation of the Neurospora circadian clock by an RNA helicase. Genes Dev. 19: 234–241.
Clausen, P., 1912 Zur Entwicklungsgeschichte der Ascomyceten. Pyronema confluens. Zeitschr. f. Bot. 4: 1–63.
Correa, A. Z. A. Lewis, A. V. Greene, J. J. March, R. H. Gomer et al., 2003 Multiple oscillators regulate circadian gene expression in Neurospora. Proc. Natl. Acad. Sci. USA 100: 13597–13602.
Covington, M., J. Maloof, M. Straume, S. Kay, and S. Harmer, 2008 Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol. 9: R130.
Crosthwate, S. C., J. J. Loros, and J. C. Dunlap, 1995 Light-Induced re-setting of a circadian clock is mediated by a rapid increase in frequency transcript. Cell 81: 1003–1012.
De Paula, R. M., Z. A. Lewis, A. V. Greene, K. S. Seo, L. W. Morgan et al., 2006 Two circadian timing circuits in Neurospora crassa cells share components and regulate distinct rhythmic processes. J. Biol. Rhythms 21: 159–168.
Dong, W., X. Tang, Y. Yu, R. Nilsen, R. Kim et al., 2008 Systems biology of the clock in Neurospora crassa. PLoS One 3: e3105.
Dosil, M., and X. R. Bustelo, 2004 Functional characterization of Pwp2, a WD family protein essential for the assembly of the 90 S pre-ribosomal particle. J. Biol. Chem. 279: 37385–37397.
Dosztányi, Z., V. Csizmók, P. Tompa, and I. Simon, 2005 IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. Bioinformatics 21: 3433–3434.
Dragovic, Z., Y. Tan, M. Görl, T. Roenneberg, and M. Merrow, 2002 Light reception and circadian behavior in ‘blind’ and ‘clock-less’ mutants of Neurospora crassa. EMBO J. 21: 3643–3651.
Dunlap, J. C., 2006 Proteins in the Neurospora circadian clockworks. J. Biol. Chem. 281: 28489–28493.
Dunlap, J. C., and J. J. Loros, 2004 The Neurospora circadian system. J. Biol. Rhythms 19: 414–424.
Dunlap, J. C., and J. J. Loros, 2006 How fungi keep time: circadian system in Neurospora and other fungi. Curr. Opin. Microbiol. 9: 579–587.
Dunlap, J. C., J. J. Loros, H. Y. Colot, A. Mehra, W. J. Belden et al., 2007 A circadian clock in Neurospora: how genes and proteins cooperate to produce a sustained, entrainable, and compensated biological oscillator with a period of about a day. Cold Spring Harb. Symp. Quant. Biol. 72: 57–68.
Froehlich, A. C., Y. Liu, J. J. Loros, and J. C. Dunlap, 2002 White Collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. Science 297: 815–819.
Froehlich, A. C., J. J. Loros, and J. C. Dunlap, 2003 Rhythmic binding of a WHITE COLLAR-containing complex to the frequency promoter is inhibited by FREQUENCY. Proc. Natl. Acad. Sci. USA 100: 5914–5919.
Fuller, K. K., J. M. Hurley, J. J. Loros, and J. C. Dunlap, 2014 Photobiology and circadian clocks in Neurospora, pp. 121–148 in The Mycota vol. XIII. Fungal Genomics, edited by Nowrousian, M. Springer, Berlin, Heidelberg.
Gesing, S., D. Schindler, and M. Nowrousian, 2013 Suppression subtractive hybridization and comparative expression analysis to identify developmentally regulated genes in filamentous fungi. J. Basic Microbiol. 53: 742–751.
Motter, F. A., J. Kuivanen, H. Keränen, S. Hilditch, M. Penttilä, 2003 A circadian oscillator in Aspergillus spp. regulates daily development and gene expression. Eukaryot. Cell 2: 231–237.

Guo, J., P. Cheng, and Y. Liu, 2010 Functional significance of FRH in regulating the phosphorylation and stability of Neurospora circadian clock protein FRQ. J. Biol. Chem. 285: 11508–11515.

Gwynne-Vaughan, H. C. I., and H. S. Williamson, 1931 Contributions to the study of Pyronema confluens. Ann. Bot. (Lond.) 45: 355–371.

Hall, B. G., 2004 Phylogenetic Trees Made Easy, Sinauer Associates, Sunderland, MA.

He, Q. G., Q. He, H. C. Lee, Y. Yang et al., 2006 CKI and CKII mediate the FREQUENCY-dependent phosphorylation of the WHITE COLLAR complex to close the Neurospora circadian negative feedback loop. Genes Dev. 20: 2552–2565.

Heintzen, C., and Y. Liu, 2007 The Neurospora crassa circadian clock. Adv. Genet. 58: 25–66.

Hevia, M. A., P. Canessa, H. Müller-Esparza, and L. F. Larrondo, 2015 A circadian oscillator in the fungus Botrytis cinerea regulates virulence when infecting Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. 112: 8747–8749.

Hong, C., J. Zámbrorský, M. Back, L. Labiscak, K. Ja et al., 2014 Circadian rhythms synchronize mitosis in Neurospora crassa. Proc. Natl. Acad. Sci. USA 111: 1397–1402.

Hughes, M. E., L. Ditacchio, K. R. Hayes, C. Vollmers, S. Pulivarthi et al., 2009 Harmonics of circadian transcription in mammals. PLoS Genet. 5: e1000442.

Hughes, M. E., J. B. Hogenesch, and K. Kornacker, 2010 JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J. Biol. Rhythms 25: 372–380.

Hurley, J. M., L. F. Larrondo, J. J. Loros, and J. C. Dunlap, 2013 Conserved RNA helicase FRH acts nonenzymatically to support the intrinsically disordered Neurospora clock protein FRQ. Mol. Cell 52: 832–843.

Hurley, J. M., A. Dasgupta, J. M. Emerson, X. Zhou, C. S. Ringelberg et al., 2014 Analysis of clock-regulated genes in Neurospora reveals widespread posttranscriptional control of metabolic potential. Proc. Natl. Acad. Sci. USA 111: 16995–17002.

Hurley, J., J. J. Loros, and J. C. Dunlap, 2015 Dissecting the mechanisms of the clock in Neurospora. Methods Enzymol. 551: 29–92.

Huson, D. H., D. C. Richter, C. Rausch, T. Denzilian, M. Franz et al., 2007 Dendroscope: an interactive viewer for large phylogenetic trees. BMC Bioinformatics 8: 460.

Johnson, C. H., J. A. Elliott, and R. Foster, 2003 Entrainment of circadian programs. Chronobiol. Int. 20: 741–774.

Lakin-Thomas, P. L., D. Bell-Pedersen, and S. Brody, 2011 The genetics of circadian rhythms in Neurospora. Adv. Genet. 74: 55–103.

Li, J. G. R. Grant, J. B. Hogenesch, and M. E. Hughes, 2015 Considerations for RNA-seq analysis of circadian rhythms. Methods Enzymol. 551: 349–367.

Liu, Y., M. Merrow, I. J. Loros, and J. C. Dunlap, 1998 How temperature changes reset a circadian oscillator. Science 281: 825–829.

Loros, J. J., and J. C. Dunlap, 2001 Genetic and molecular analysis of circadian rhythms in Neurospora. Annu. Rev. Physiol. 63: 757–794.

Loros, J. J., S. A. Denome, and J. C. Dunlap, 1989 Molecular cloning of genes under the control of the circadian clock in Neurospora. Science 243: 385–388.

Luo, C., J. J. Loros, and J. C. Dunlap, 1998 Nuclear localization is required for function of the essential clock protein FREQUENCY. EMBO J. 17: 1228–1235.

Merrow, M., and J. C. Dunlap, 1994 Intergeneric complementation of a circadian rhythmicity defect: Phylogenetic conservation of the 989 amino acid open reading frame in the clock gene frequency. EMBO J. 13: 2257–2266.

Motter, F. A., J. Kuivanen, H. Keränen, S. Håldtch, M. Penttilä et al., 2014 Categorisation of sugar acid dehydratases in Aspergillus niger. Fungal Genet. Biol. 64: 67–72.

Nakai, K., and P. Horton, 1999 PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. 24: 24–26.

Nowrouzian, M., and U. Kück, 2006 Comparative gene expression analysis of fruiting body development in two filamentous fungi. FEMS Microbiol. Lett. 257: 328–335.

Nowrouzian, M., G. E. Duffield, J. J. Loros, and J. C. Dunlap, 2003 The frequency gene is required for temperature-dependent regulation of many clock-controlled genes in Neurospora crassa. Genetics 164: 923–933.

Nsa, I. Y., N. Karunaratna, X. Liu, H. Huang, B. Boettger et al., 2015 A novel cryptochrome-dependent oscillator in Neurospora crassa. Genetics 199: 233–245.

Oliveira, A. G., C. V. Stevani, H. E. Waldenmaier, V. Viviani, J. M. Emerson et al., 2015 Circadian control sheds light on fungal bioluminescence. Curr. Biol. 25: 964–968.

Querfurth, C., A. C. Diernfellner, E. Gin, E. Malzahn, T. Höfer et al., 2011 Circadian conformational change of the Neurospora clock protein FREQUENCY triggered by clustered hyperphosphorylation of a basic domain. Mol. Cell 43: 713–722.

Salichos, L., and A. Rokas, 2010 The diversity and evolution of circadian clock proteins in fungi. Mycologia 102: 269–278.

Salomé, P. A., and C. R. Mcclung, 2004 The Arabidopsis thaliana clock. J. Biol. Rhythms 19: 425–435.

Sancar, G., and M. Brunner, 2014 Circadian clocks and energy metabolism. Cell. Mol. Life Sci. 71: 2667–2680.

Schafmeier, T., A. Haase, K. Káldi, J. Scholz, M. Fuchs et al., 2005 Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell 122: 235–246.

Schafmeier, T., K. Káldi, A. Diernfellner, C. Mohr, and M. Brunner, 2006 Phosphorylation-dependent maturation of Neurospora circadian clock protein from a nuclear repressor toward a cytoplasmic activator. Genes Dev. 20: 297–306.

Schindler, D., and M. Nowrouzian, 2014 The polykytosine synthase gene pks4 is essential for sexual development and regulates fruiting body morphology in Sordaria macrospora. Fungal Genet. Biol. 68: 48–59.

Seaver, F. J., 1909 Studies in pyrophilous fungi - I. The occurrence and cultivation of Pyronema. Mycologia 1: 131–139.

Shaafatian, R. M. A. Payton, and J. D. Reid, 1996 PWP2, a member of the WD-repeat family of proteins, is an essential Saccharomyces cerevisiae gene involved in cell separation. Mol. Gen. Genet. 252: 101–114.

Teichert, I., G. Wolff, U. Kück, and M. Nowrouzian, 2012 Combining laser microdissection and RNA-seq to chart the transcriptional landscape of fungal development. BMC Genomics 13: 511.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, 1997 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 26: 4876–4882.

Træger, S., F. Altegoer, M. Freitag, T. Gabaldon, F. Kempen et al., 2013 The genome and development-dependent transcriptomes of Pyronema confluens: a window into fungal evolution. PLoS Genet. 9: e1003820.

Waterhouse, A. M., J. B. Procter, D. M. A. Martin, M. Clamp, and G. J. Barton, 2009 Jalview Version 2: a multiple sequence alignment editor and analysis workbench. Bioinformatics 25: 1189–1191.

Yang, J., L. Wang, X. Ji, Y. Feng, X. Li et al., 2011 Genomic and proteomic analyses of the fungus Arabobryotis oligospora provide insights into nematode-trap formation. PLoS Pathog. 7: e1002179.

Zhang, Z., F. López-Giráldez, and J. P. Townsend, 2010 LOX: inferring Level Of eXpression from diverse methods of census sequencing. Bioinf. 26: 1918–1919.

Zhu, H., M. Nowrousian, D. Kupfer, H. V. Colot, G. Berrocal-Tito et al., 2001 Analysis of expressed sequence tags from two starvation, time of day-specific libraries of Neurospora crassa reveals novel clock-controlled genes. Genetics 157: 1057–1065.

Communicating editor: J. C. Dunlap