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"Role of circadian regulation in the mycoparasitic interaction between *Botrytis cinerea* and *Trichoderma atroviride*"

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GLOSSARY

TaFRQ: *Trichoderma atroviride* Frequency
BLR-1: Blue light regulator 1
BLR-2: Blue light regulator 2
CWDE: Cell wall degrading enzymes
$_{nc}$*c-box*: clock box from *Neurospora crassa*
FRQ: Frequency
FWO: FRQ WCC Oscillator
BcFRQ: *Botrytis cinerea* Frequency
BcWCL1: *Botrytis cinerea* White-Collar 1
TTFL: transcriptional translational feedback loop
DD: constant darkness conditions
LL: constant light conditions
LD: light dark cycle
LP: light pulse
WT: wild-type
WC: White collar
WCC: White collar complex
PDA: potato dextrose agar
h: hours

RESUMEN

Aun cuando los genes que componen el oscilador circadiano se pueden encontrar en numerosos hongos, durante años, la investigación sobre relojes circadianos se ha centrado en *Neurospora crassa*, habiendo apenas escasa descripción fenomenológica de ritmos en otros hongos. Sin embargo, ninguno de esos estudios ha demostrado la presencia de un oscilador circadiano funcional, como en *N. crassa*, exceptuando Hevia et al., 2015, que mostró un reloj circadiano funcional en *Botrytis cinerea*, y su influencia en virulencia.

Las especies de *Trichoderma* son conocidas por su actividad de biocontrol contra patógenos de plantas, en los que varios mecanismos como la competencia de nutrientes, la secreción de CWDE, el metabolismo secundario y el micoparasitismo actúan en sinergia. *T. atroviride* también ha sido estudiado por sus respuestas a daño mecánico, lo que permitió establecer un modelo de regeneración en hongos, así como también por sus respuestas a luz azul y la descripción de BLR1 y BLR2: los fotorreceptores y factores de transcripción homologos a WC1 y WC2 de *N. crassa*, y que forman parte del oscilador central. Dado que el genoma de *T. atroviride* codifica para un homólogo de *frq* de *N. crassa*, *tafrq*, se ha sugerido la presencia de un reloj circadiano hipotético en *T. atroviride*. A pesar de que todavía no se han observado oscilaciones ni un fenotipo circadiano, la presencia de los genes para los componentes del reloj en *T. atroviride*, nos impulsó a evaluar si realmente existe un reloj circadiano funcional en este organismo, y que pudiera estar regulando varios de sus rasgos fenotípicos característicos, como el micoparasitismo, programas de desarrollo y metabolismo secundario. Así, en este estudio, utilizando reporteros de luciferasa, mostramos por primera vez oscilaciones circadianas en *T. atroviride*, con un período cercano a las 26 horas en la oscuridad constante.

Así también encontramos que los elementos centrales del reloj juegan un papel en la regulación de la conidiación, el metabolismo secundario y el micoparasitismo.

SUMMARY

For decades, research on circadian clocks in fungi have been mainly focused in the model *Neurospora crassa*, and there has been scarce description of phenotypic rhythms in other fungi, despite the fact genome analyses have revealed the presence of clock orthologues in many genus. Thus, there has been no studies functionally proving the existence of a functional circadian clock in fungi other than Neurospora, with the exception of Hevia et al., 2015, that characterized a fully functional circadian clock in the fungal phytopathogen *Botrytis cinerea*, showing that it also regulated its virulence.

Trichoderma species are well known for their biocontrol activity against plant pathogens, in which several mechanisms such as nutrient competition, secretion of cell wall degrading enzymes (CWDE), secondary metabolism, and mycoparasitism act in synergy. *Trichoderma atroviride* has also been studied for its ability to respond to mechanical injury that allowed to create a model for tissue regeneration in fungi. Likewise, responses to blue-light, mediated by the complex formed by the transcription factors known BLR1 and BLR2 homologs to WC1 and WC2 of *N. crassa*, have been characterized. Since the genome of *T. atroviride* encodes for a FREQUENCY homolog, *tafrq*, this observation suggests the presence of a putative circadian clock in *T. atroviride*. Even though no circadian oscillations nor a circadian phenotype have been observed yet in this fungus, the presence of the core clock components in *T. atroviride* led us to hypothesize if it is indeed a functional clock that oscillates and if it can regulate several of its attractive phenotypic traits, such as its biocontrol activity, development and secondary metabolism. In this thesis, using translational and transcriptional luciferase reporter genes, we show for the first-time evidence of circadian oscillations in *T, atroviride,* with a period close to 24 h in constant darkness and how core clock components play a role in regulation conidiation, secondary metabolism, and mycoparasitism.

CHAPTER 1

Theoretical framework

1.1 The relevance of circadian rhythms

Circadian rhythms are biological recurring cycles of *circa* ("around") 24 h that result from the synchronization between the organism's biology to the daily rhythms that occur as a result of the Earth's rotation around its axis. Throughout evolution, an endogenous mechanism called circadian clock has emerged independently several times, controlling the organism's responses to a changing/fluctuating environment, coordinating biological processes at large (Yerushalmi & Green, 2009). These clocks allow biological systems to keep track of time, anticipating and adapting to periodic and predictable changes such as exposure to UV radiation, temperature fluctuations, nutrients availability, among others; through the temporary coordination of gene expression, metabolic pathways, physiological responses and even behavior (Bell-Pedersen et al., 2005; De Paula et al., 2008). To classify a rhythm as circadian several conditions are normally required: 1.- be endogenous and persist in the absence of environmental signals with a period close to 24 h, 2.- be entrained by temperature and/or light cycles, and 3.- be compensated by temperature and nutrients, meaning that the period length does not change upon temperature or nutritional variations. These parameters allow distinguishing such oscillations from those that are merely regulated by the environment (Liu & Bell-Pedersen, 2006; Montenegro-Montero et al., 2015).

The appearance of circadian clocks independently in various lineages in the tree of life has highlighted the importance of circadian rhythms at the adaptive (organismal) level (Vaze

& Sharma, 2013; Yerushalmi & Green, 2009). Some studies, in organisms as diverse as fungi and cyanobacteria, account for these phenomena. For example, in the basidiomycete *Neonothopanus gardneri*, the production of biological light (bioluminescence) is under circadian control, with a maximum of light production during the night period of a 24 h day. This circadian control gives the fungus the advantage of attracting nocturnal insects that favor the dispersal of spores (Oliveira et al., 2015), a fundamental structure used by fungi to disseminate in nature. In the cyanobacteria *Synechococcus sp.*, synchronization of the internal clock with the environmental rhythm improves their *fitness*, and those that cannot, are displaced in a mixed population competition experiment (Woelfle et al., 2004). As well, in *Arabidopsis*, variation in period, phase and amplitude in 150 different *Arabidopsis* accessions was observed and a strong correlation between period and day length of the latitude of origin was observed, suggesting an adaptative advantage of correctly regulated circadian timing to the environment, that could have impact in flowering and its adaptation to season changes (Todd et al., 2003).

Recent findings have shown that interactions between organisms may also be subjected to circadian regulation (Hevia et al., 2015, 2016; Larrondo & Canessa, 2019)(Hevia et al., 2015, 2016; Larrondo L. & Canessa P., 2018)(Hevia et al., 2015, 2016; Larrondo L. & Canessa P., 2018). In mammals, disruption of the sleep-wake cycle transiently affects gut microbiome (Barik, 2019). Additionally, clinical, and experimental analysis have shown 24 h rhythms in patients' symptoms (such as fever and chills) during parasitic infection with malaria parasite *Plasmodium spp.* correlate with the 24 h parasite developmental stages (Carvalho et al., 2019). A more detailed circadian regulation of interactions has been described in plants. In this regard, the circadian clock of *Arabidopsis thaliana* controls jasmonate and salicylate phytohormones levels, both involved in plant defense mechanisms

including mainly, necrotrophic/herbivory phytopathogens and biotrophic ones, respectively. Under constant environmental conditions (e.g. 24 h of constant light; referred herein as LL), accumulation of each phytohormone have a peak during the middle of the subjective day and subjective night, respectively (Goodspeed et al., 2012). During herbivory, *Trichoplusia ni* displays a circadian feeding rhythm with a peak of activity during the late day. In constant darkness (referred herein as DD), when both *A. thaliana* and *T. ni* are interacting "*in phase"* - that is both organisms prior to the coincubation received the same light/dark entrainment cycle - the size of the plant tissue injury is smaller as the herbivore feeding hours matches with those of the plant jasmonate peak. On the contrary, during *antiphase* entrainment (prior the coincubation, both organisms received opposite light/dark cycles of entrainment), the insect that is in a "subjective day" displays a high feeding rate, while the plant in the "subjective night", with less jasmonate levels, results in significant tissue lesions (Goodspeed et al., 2012). Likewise, clock effects were reported by Hevia et al., 2015 in the circadian interaction between *A. thaliana* and the phytopathogenic fungus *Botrytis cinerea.* Firstly, they described a functional circadian clock in the fungus (discussed *in extenso* below) that controls the outcome of the pathogen-plant interaction. When the infection occurs at dusk, the observed lesion area was significantly bigger when compared to the lesion generated by diurnal (dawn) infection**.**

1.2 *Neurospora crassa*: a model in chronobiology

The first thorough description of circadian rhythms in fungi were conducted in the ascomycete *N. crassa*. This fungus displays a circadian conidiation phenotype that occurs at dawn, that persists in constant darkness conditions (Baker et al., 2012). Since these

fundamental observations, *N. crassa* has become one of the leading models in chronobiology, and its core circadian clock components and functions have been dissected after decades of research. Conceptually, the circadian clock can be seen as a molecular device composed of three parts or theoretical components. The first part is the so called "input pathways". These entrain the circadian clock since it allows perception and processing of environmental signals, such as light. The second component, the central oscillator, fluctuates with an oscillation close to 24 h, and controls the third component or "output pathways" that regulate in a temporal fashion different cellular and molecular processes (Montenegro-Montero et al., 2015). In *N. crassa*, the central oscillator is called FWO (Frequency White-Collar Oscillator) and comprises a negative transcriptional-translational feedback loop (TTFL), that is shared by almost all circadian clocks described to date (Ode & Ueda, 2018). The positive element of the TTFL is known as the White Collar Complex (WCC) and is formed by the physical interaction of the blue light receptor and transcription factor (TF) White Collar 1 (WC-1), and the TF White Collar 2 (WC-2). Both WC proteins correspond to GATA-type zinc TFs and possess a PAS (PER-ARNT-SIM) dimerization domains (Dunlap, 1999; Talora et al., 1999). WC-1 possess 2 additional PAS domains, and one of them is a specialized PAS called LOV (Light-Oxygen-Voltage) domain, that binds a flavin-adenine dinucleotide (FAD) chromophore, responsible of light activation of WCC (Dunlap & Loros, 2017; Montenegro-Montero et al., 2015) thus explaining, at least in part, the input pathway.

The WCC activates the expression of light-responsive and clock-controlled genes (*ccgs*), including the negative element of the TTFL, known as *frequency* (*frq*). The FRQ protein negatively regulates its own expression by recruiting kinases that phosphorylate the WCC, causing its inactivation. In this process, FRQ is progressively phosphorylated until reaching its maximum phosphorylation state at night, losing its affinity to WCC, which in turn,

activates *frq* expression again. The remaining FRQ is removed from the system via ubiquitin-proteasome degradation. In this process, *frq* mRNA and FRQ protein levels oscillate with a period close to 22.5 h, which correlates with the rhythmic conidiation determined in *Neurospora* (Figure 1.1) (Larrondo et al., 2015; Montenegro-Montero et al., 2015).

The WCC recognizes two *cis*-elements in the *frq* promoter. These are the *clock-box* (known as *c-box*), composed of a short sequence that are necessary and sufficient for the rhythmic expression of *frq* even in DD, and the second element, the Light Regulatory Element (LRE) which has a role in circadian entrainment and light regulation of *frq* (Froehlich et al., 2002, 2003).

As a result of the global circadian regulation, between 20-40% of *N. crassa's* genes have a rhythmic pattern of expression (Hurley et al., 2014) regulating critical biological processes such as DNA repair, sexual/asexual development, and stress responses, among others. Some *ccgs* have an expression peak before dawn, suggesting a previous preparation (anticipatory behavior) of the fungus for the stress caused by sunlight exposure (De Paula et al., 2008; Montenegro-Montero et al., 2015; Vitalini et al., 2007). The expression of *ccgs* is a complex process in which more than one pathway could be involved and be activated or repressed by light, stress, among other environmental signals, independently or in addition of circadian regulation. Indeed, MAPKs pathways, an important signaling component in fungi and other organisms, control several *ccgs* in *N. crassa* such as *ccg-4* (encodes a mating pheromone), *ccg-9* (encodes a trehalose synthase) and *pck-1* (which encodes for a phosphoenolpyruvate carboxykinase) (De Paula et al., 2008). The osmosensing pathway (OS) is a MAPK pathway in *N. crassa* and it is necessary for osmotic stress responses, but also a circadian clock's output pathway (Lamb et al., 2012). OS-2, a kinase in the OS MAPK cascade, has rhythmic phosphorylation oscillations in DD that are regulated by the circadian clock with a peak in

the subjective morning. However, the absence of a functional circadian clock in *N. crassa* does not affect the OS-2 responses to osmotic stress and the signal from the clock that actives OS can be overridden by intense osmotic stress. This suggest that the OS pathway receives information from the circadian clock and the environment (De Paula et al., 2008). The circadian regulation of OS and other signaling pathways may provide significative advantages to *N. crassa*, allowing to anticipate the occurrence of stressful events, rather than just respond to them.

1.3 The circadian clock in other fungi

Homologs of *wc-1*, *wc-2*, and *frq* genes have been found in several fungi (Montenegro-Montero et al., 2015; Salichos & Rokas, 2010) as well as the existence of some circadian phenotypes. For instance, rhythms in ascospores production have been reported in *Sordaria fimicola* (Austin, 1968), circadian rhythm of sclerotia formation in *Aspergillus flavus*, whereas in *A. nidulans* the *gpdh* gene oscillates in DD with a period between 28-32 h (Greene et al., 2003). Other examples include rhythmic melanization in *Cercospora kikuchii*, and mycelium banding in *Sclerotia sclerotium* (Bluhm et al., 2010) and *Aureobasidium pullullans* (Franco et al., 2017), which even persists in constant darkness. Hints of *frq* circadian expression have been observed in the latter fungus as well as in *Pyronema confluens* where even some putative *ccgs* have been described (Traeger & Nowrousian, 2015). Nevertheless, none of these studies have clearly proven in these fungi the existence of a central oscillator as the one described first in *N. crassa*. Indeed, the only study, describing clear oscillations of *frq* expression under different environmental conditions, and the importance of *frq* in daily phenotypes, in a fungus other than *N. crassa*, has been in *Botrytis cinerea* (Hevia et al., 2015).

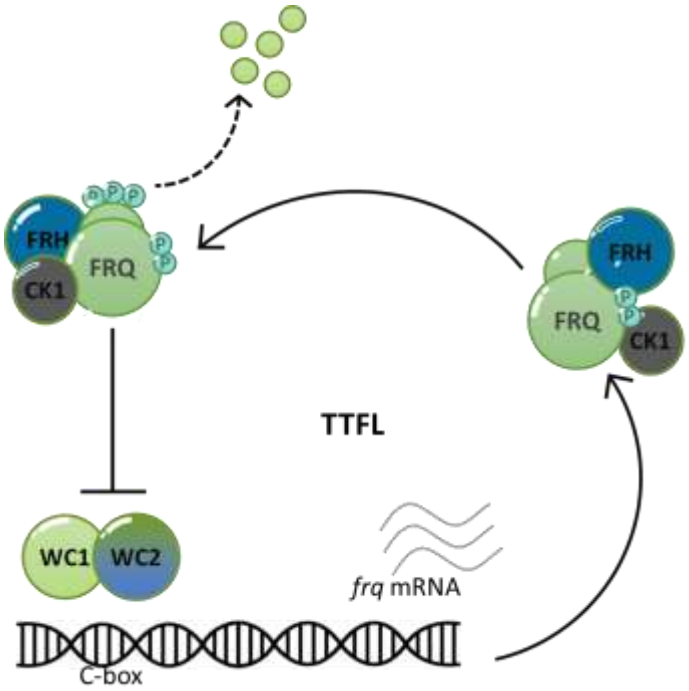

Figure 1.1. Schematic representation of *N. crassa's* circadian clock.
The expression of the negative element of the TTFL, frequency (*frq*)**,** is under the control of the positive element, the transcriptional complex WCC. In DD, in the late subjective night, WCC activates *frq* expression binding to a specific sequence in the *frq* promoter known as the *clock-box* (*c-box*), which is both necessary and sufficient for *frq* rhythmic expression (Froehlich et al., 2003). The FRQ is produced, dimerizes and associates with a RNA-helicase known as FRH, recruiting kinases such as CK1, which catalyzes a series of phosphorylation events on FRQ, moving from a hypo- phosphorylated to a hyperphosphorylated state. This complex promotes the phosphorylation of WCC, reducing the affinity of the WCC to the *c-box*, reducing the expression of *frq*. When FRQ reaches the hyperphosphorylation state, the ability to interact with CK1 and WCC decreases, restarting the cycle. Later, FRQ is recognized by FWD-1 which lead to FRQ ubiquitylaton and degradation in the proteasome (Larrondo et al., 2015; Montenegro-Montero et al., 2015).

B. cinerea is a necrotrophic phytopathogen fungus that causes grey rot in numerous economically significant plant species. To extract nutrients from its host, this fungus destroys the plant tissue cells by producing phytotoxic compounds and cell wall degrading enzymes, among several other molecular strategies to cause disease. The sexual/asexual development of *B. cinerea* is regulated by light, where the *B. cinerea* WCC (termed BcWCC) of the fungus mediates the transcription of genes in response to light, regulating processes such as inhibition of conidiation, resistance to oxidative stress, and also virulence (Canessa et al., 2013). As in *N. crassa*, the BcWCC is part of a functional circadian clock, that controls *bcfrq1* circadian expression. Indeed, *bcfrq1* mRNA levels display a rhythmic behavior both in constant darkness (DD), as well as Light: Dark cycles (LD), rhythms that depend on the BcWCC. Moreover, this study also confirmed daily oscillations of BcFRQ1 protein, by utilizing a luciferase translational reporter (Hevia et al., 2015). Interestingly, BcFRQ1 was found to have extra-circadian roles, since its absence alters the development of the mutant strain, growing as an "always sclerotia" mutant strain in LL (a condition where clock-function is impaired), meanwhile, the B05.10 wildtype strain develops asexual conidia under the same culture conditions. Interestingly, phenotypes caused by the absence of *bcfrq1* in *B. cinerea* can be reversed by supplementing the culture medium with primary nitrogen sources (Hevia et al., 2015). Equally relevant, this study showed for the first time that the outcome of a plant-fungal interaction could depend on the time of day when it occurs and that the *B. cinerea's* clock is key to maximizing the pathogen attack, independent of the plant's internal clock, developing more significant lesions in infections performed at dusk than at dawn (Hevia et al., 2015). The previous becomes more evident when both *B. cinerea* and the plant are entrained out of phase, that is, when during the inoculation the fungus experiences

"subjective night" is when lesions appear bigger 72 h latter, independent on whether the plant was in "subjective dawn" or "subjective night" when both organisms first interacted.

These findings raise important new questions about the relevance of fungal circadian clocks and their function in organismal interactions, which could shed light on how they help anticipate rhythms in biotic interactions between organisms (Hevia et al., 2016). Above all, the fact that BcFRQ1 presents new functions in *B. cinerea* (which have not been observed in *N. crassa*) leads to the question of whether those functions were acquired in *B. cinerea* or correspond to characteristics that have been lost in *N. crassa*. One way to address this question is to molecularly examine the clock in other ascomycetes, understand the roles involved, and the impact of circadian regulation on relevant biological traits and interactions.

1.4 *Trichoderma* species

The genus *Trichoderma* (*Hypocreales, Ascomycota*) is considered a cosmopolitan group of soil fungi and the most predominant component of the mycoflora in numerous soils, with a wide range of adaptability to different environmental conditions (Carreras-Villaseñor et al., 2012). They are also found in decaying wood, on the fruiting bodies of other fungi and associated with plant-roots (to both dicots and monocots), leaves, and seeds (Kubicek et al., 2019; Vos et al., 2015). The genus comprises 100 identified species, some of which are economically important producers of enzymes (e.g., cellulolytic enzymes by *Trichoderma reesei*), antibiotics (peptaibols) or are used as a biocontrol agent against phytopathogens in agriculture (e.g., *Trichoderma harzianum, Trichoderma atroviride*) (Druzhinina & Kubicek, 2005; Druzhinina et al., 2006).

Members of this genus are morphologically distinguished from other taxon based on their rapid growth, conidia size (less than 5 µm long and wide), conidia morphology (globose, ellipsoidal) and pigmentation ranging from deep green to nearly grey (Carreras-Villaseñor et al., 2012). Nevertheless, the nomenclature of this group is complicated as they can exist in two different morphological and physiological stages: sexual (known as *Hypocrea*) and asexual (known as *Trichoderma*). Several *Trichoderma* species have lost their ability to reproduce sexually, but some species like *T. reesei* can be isolated both in their sexual and asexual stages (Dattenböck et al., 2018; Druzhinina et al., 2011; Schmoll & Wang, 2016).

As a group, *Trichoderma* species play a significant role in the carbon/nitrogen balance in different soil ecosystems, establishing various successful heterotrophic interactions, including decomposition of soil litter, parasitism over other fungi, and opportunistic endophytism with plants (Druzhinina et al., 2006). As plant symbionts, they decompose organic matter found in the soil, allowing plants better nutrient assimilation and, therefore, more biomass production. Also, they induce systemic and localized resistance in plants against phytopathogens, and stimulate plant growth and development (Harman et al., 2004). On the other hand, *Trichoderma* species are well-known mycoparasites as they can overgrow and feed from other fungi found in the soil. *Trichoderma* can degrade the cell wall of the parasitized fungi through the secretion of cell wall degrading enzymes (CWDE) and inhibit their growth secreting antifungal secondary metabolites (Druzhinina et al., 2011; Steyaert et al., 2003). This is a complex process (discussed below) that involves a series of steps that can be observed at the microscopic level, in which *Trichoderma* hyphae is coiling around its prey (Figure 1.2A)or at a macroscopic level in confrontation assays (Figure 1.2B) in which *Trichoderma* overgrows its prey.

1.5 Mycoparasitism in *Trichoderma*

In the 1930s, Weindling described for the first time the antifungal benefits of *Trichoderma* species. He observed the secretion of a "lethal principle" at the edge of the *T. lignorum* colony (Topolovec-Pintaric, 2019). Since then, *Trichoderma* species have been used as biocontrol agents of phytopathogens in agriculture due to their ability to antagonize these fungi. The fast growth characteristics of *Trichoderma* species exert a competition for nutrient availability with other microbes, allowing the colonization of diverse ecological niches at the expense of other microorganisms. In this process, *Trichoderma* secretes secondary metabolites with antifungal activity and CWDE such as chitinases, cellulases, and glucanases, among others, that confer a significative advantage over other microbes present in the soil (Bélanger et al., 1995). These processes form part of its mycoparasitic lifestyle, in which *Trichoderma* can also feed from a fungal prey (Mukherjee et al., 2012). This behavior is present in several *Trichoderma* species, which have been described as the ancestral lifestyle in this group (Kubicek et al., 2011).

The mycoparasite interaction between *Trichoderma* and its prey is not specific and comprises a series of steps. Firstly, prior contact between fungi, *Trichoderma* species constitutive secrete CWDE at low levels. Whole-genome transcriptomics analysis have shown that several protease and oligopeptide encoding genes are expressed before and during the contact between *Trichoderma* and its prey (Atanasova et al., 2013; Seidl et al., 2009; Vos et al., 2015). These enzymes degrade the prey's hyphae, and the small molecules that are released as a result of each enzyme activity can function as signals in *Trichoderma*, which are transduced to the necessary regulatory targets, allowing recognition of the prey (Zeilinger et al., 2005). After recognition, *Trichoderma* directs its growth towards the prey, and its

hyphae start coiling around the host mycelium, producing physical strangulation. Thereafter, more CWDE are produced, and the prey's cell wall is degraded, allowing *Trichoderma's* hyphae to penetrate the lumen of the prey (Bélanger et al., 1995). After achieving this, the prey activates different defense strategies to respond to the attack by *Trichoderma*, producing Reactive Oxygen Species (ROS) and/or antifungal secondary metabolites to counteract the attack. In turn, *Trichoderma* activates its own defense responses, also involving the expression of genes related to ROS, heat shock proteins, and detoxification response genes (e.g. ABC and multidrug resistance transporters) (Contreras-Cornejo et al., 2020; Steindorff et al., 2014). Finally, *Trichoderma* kills its prey by the synergistic action of antifungal secondary metabolites and the secretion of a high amounts of CWDE (Druzhinina et al., 2011; Sharma et al., 2011; Steyaert et al., 2003) (Figure 1.2 A).

Signaling pathways and protein receptors are crucial in transducing different signals generated during a mycoparasite interaction. In *T. atroviride* heterotrimeric G protein signaling has been described as crucial for its role in transducing the mycoparasitism related signals. This system comprises G protein-coupled receptors (GPCRs), the heterotrimeric G protein (α, β, γ), and downstream effectors (Brunner et al., 2008).

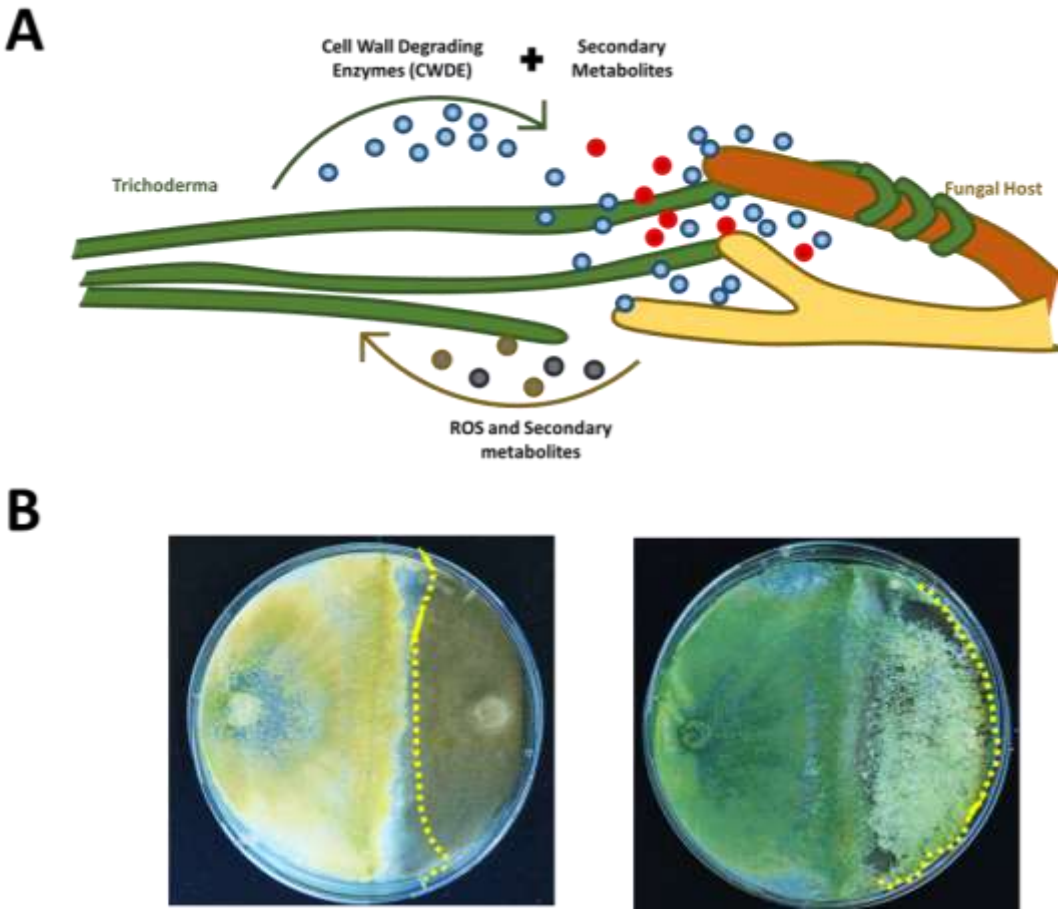

Figure 1.2. Microscopic and macroscopic mycoparasitism in *T. atroviride*. A) *Trichoderma* constitutively secretes proteases and CDWE at low levels. Before the contact with the pathogenic fungus, these enzymes reach the prey, releasing small molecules from the prey's cell wall that can be recognized by *Trichoderma.* These small molecules bind to G protein-coupled receptors (such as Gpr1) or nitrogen-sensing receptors on the *Trichoderma* surface, activating a MAPK signaling pathway, that ultimately enhances the biosynthesis of secondary metabolites and CWDE. The fungal prey responds by secreting secondary metabolites and ROS that activate a stress response and detoxification in *Trichoderma* (Druzhinina et al. 2011). **B)** Confrontation assay of *T. atroviride* against *B. cinerea*. Left panel: Trichoderma cannot overgrow *B. cinerea*. Right panel: a different *Trichoderma* strain that can fully overgrow *B. cinerea* is shown. In each case, a yellow dot-line indicates the edge of the *Trichoderma* growth front.

The class IV G protein-coupled receptors (GPCRs) have been found in many *Trichoderma* species, and it is believed that it is responsible for signal transduction in mycoparasitism interaction. In *T. atroviride*, four receptor encoding genes (*gpr1-4*) are expressed upon carbon starvation. The *gpr3* gene responds to the presence of fungal hyphae and when the *gpr1* in silenced, fungi cannot overgrow its prey and the production of secondary metabolites such as the volatile antifungal compound known as 6-pentyl pyrone (6-PP) is reduced (Omann et al., 2012). Also, in *T. atroviride*, two Gα subunits have been described as relevant in mycoparasitism: Tga1 and Tga3. The absence of *tga1* reduces the mycoparasite coiling of *Trichoderma* over its prey along with a substantial reduction of chitinase activities and decreased production of the antifungal compound 6-PP (Druzhinina et al., 2011; Rocha-Ramírez et al., 2002). Strains deficient in *tga3* are avirulent in confrontation assays against *Rhizoctonia solani* and *B. cinerea* (Zeilinger et al., 2005).

Another major signaling transduction pathway involved in mycoparasitism is the Mitogen-Activated Protein Kinase (MAPK). The pathway contains 3 MAPK, and in *T. atroviride*, one of them, Tmk1, plays a role in radial growth, conidiation, and formation of the infective structures over the prey (Reithner et al., 2007). Likewise, transcriptomic analysis of *T. atroviride* in response to *R. solani* or *B. cinerea* have shown the overexpression of the signal transduction kinase *tmk3* (Seidl et al., 2009). Tmk3 has been related to signal transduction of stress, and its expression is also light-induced (Esquivel-Naranjo et al., 2016). In *T. harzianum*, the mutant of the Th*hog1* gene (homolog to *tmk3*) cannot overgrow its prey, and it has been proposed to be necessary to neutralize stress agents produced by the prey such as ROS (Delgado-Jarana et al., 2006).

Another crucial aspect of the mycoparasite interaction is the secretion of CWDE and secondary metabolites. Both have a synergistic effect during the mycoparasite interaction

(Contreras-Cornejo et al., 2020; Druzhinina et al., 2011; Howell, 1998). Comparison of *T. virens* and *T. atroviride* genomes with the non-mycoparasite *T. reesei* have revealed a large amount of chitinases, glycoside hydrolases and β-1,3 endoglucanases and proteases encoding genes in *T. virens* and *T. atroviride*, but not in *T. ressei.* This observation highlights the relative high importance of CWDE in the mycoparasitism, and how it has been lost in the non-mycoparasite fungi such as *T. ressei* which displays a reduced number of CWDE genes (Kubicek et al., 2011). Likewise, secondary metabolite related genes, such as non-ribosomal peptide synthetases and polyketide synthases, are over-represented in *T. virens* and *T. atroviride* compared to *T. ressei* (Mukherjee et al., 2012). Some of these genes have an active role in the mycoparasite interaction, like the anthraquinone pachybasin of *T. harzianum*, that increases the numbers of coils around the prey (Lin et al., 2012). Additionally, production of compounds that inhibits mitochondria metabolism of the prey have also been reported (Contreras-Cornejo et al., 2020).

Due to the importance of secondary metabolism in *Trichoderma* mycoparasitism, a significant number of studies have focused on describing the secondary metabolites produced by this fungus. Over 100 secondary metabolites have been reported, including volatile and non-volatile compounds with different molecular weights (MW) (Reino et al., 2008; F. Vinale et al., 2008). Pyrones, terpenoids, steroids, and polyketides are among the low MW non-polar compounds. In contrast, high MW derived from the activity of non-ribosomal peptide synthases like peptaibols and siderophores (Mukherjee et al., 2012). Other antimicrobial compounds include viridins (steroidal), azaphilones, nitrogen heterocyclic compounds, butenolides, hydroxylactones, and diketopiperazines, among others (Vinale et al., 2014).

One of the best-studied secondary metabolites from *Trichoderma* is the volatile compound known as 6-PP which is the major signature of these species (Mukherjee et al., 2012). 6-PP is responsible for *Trichoderma* characteristic "coconut aroma" which have been exploited in the food industry (Serrano-Carreon et al., 1993). This compound corresponds to an unsaturated lactone that possesses antifungal and plant-growth-promoting activities (Stoppacher et al., 2010) and there is a strong correlation between the production of this pyrone and *Trichoderma* ability to antagonize different fungal preys (Reino et al., 2008). Despite the relevance of 6-PP in *Trichoderma*, little is known about the regulation of its production and biosynthetic pathway. So far, it is known that 6-PP production is regulated by the G protein Tga1 and affected in *tmk* mutants in *T. atroviride* (Atriztán-Hernández et al., 2019), and by the transcription factor Thtfl in *T. harzianum.* Also, the biosynthesis of 6-PP derives from lipid metabolism, specifically from linoleic acid, and it has been proposed that the lipoxygenases activity is the limiting step in 6-PP formation from linoleic acid (Serrano-Carreon et al., 1993). However, recently, it was found that a mutant lacking lipoxygenase gene *lox1* in *T. atroviride* P1 strain, can still produce 6-PP, therefore is not involved in its biosynthesis (Speckbacher et al., 2020b).

1.6 *Trichoderma atroviride*: a photomorphogenic and injury response fungal model

Light is one of the most ubiquitous and universal environmental clues for all organisms on the planet. It is a major source of information (Schmoll et al., 2010). Therefore, not surprisingly, organisms had generated signaling pathways to perceive different light wavelengths and producing different responses to them (Corrochano, 2019; Schmoll et al., 2010).

In *T. atroviride,* light responses have been studied since 1957 when Gutter first described its light-induced conidiation phenotype (Gressel and Galun, 1967; Gutter, 1957). In complete darkness, the fungus grows as a flat, septate, hyaline and white mycelia. After 30 seconds of blue light exposure, only the narrow region of the mycelium produced just before illumination generates conidia. A few hours later after light exposure, abundant branching of aerial hyphae and increased number of septa can be observed, producing new aerial hyphae that leads to conidiophore development, generating a ring of conidia in the exposed tissue (Figure 1.3) (Casas-Flores et al., 2004; Galun and Gressel, 1966; Schmoll et al., 2010). After a light pulse, several biochemical changes occur in the fungi: changes in membrane potential, ATP levels, transient biphasic oscillations in cAMP levels, activation of adenylyl cyclase, and phosphorylation proteins that finally drive sporulation (Casas-Flores et al., 2006). In constant light, conidiation occurs continuously, and in light/dark cycles, several rings of conidia can be seen (Figure 1.3).

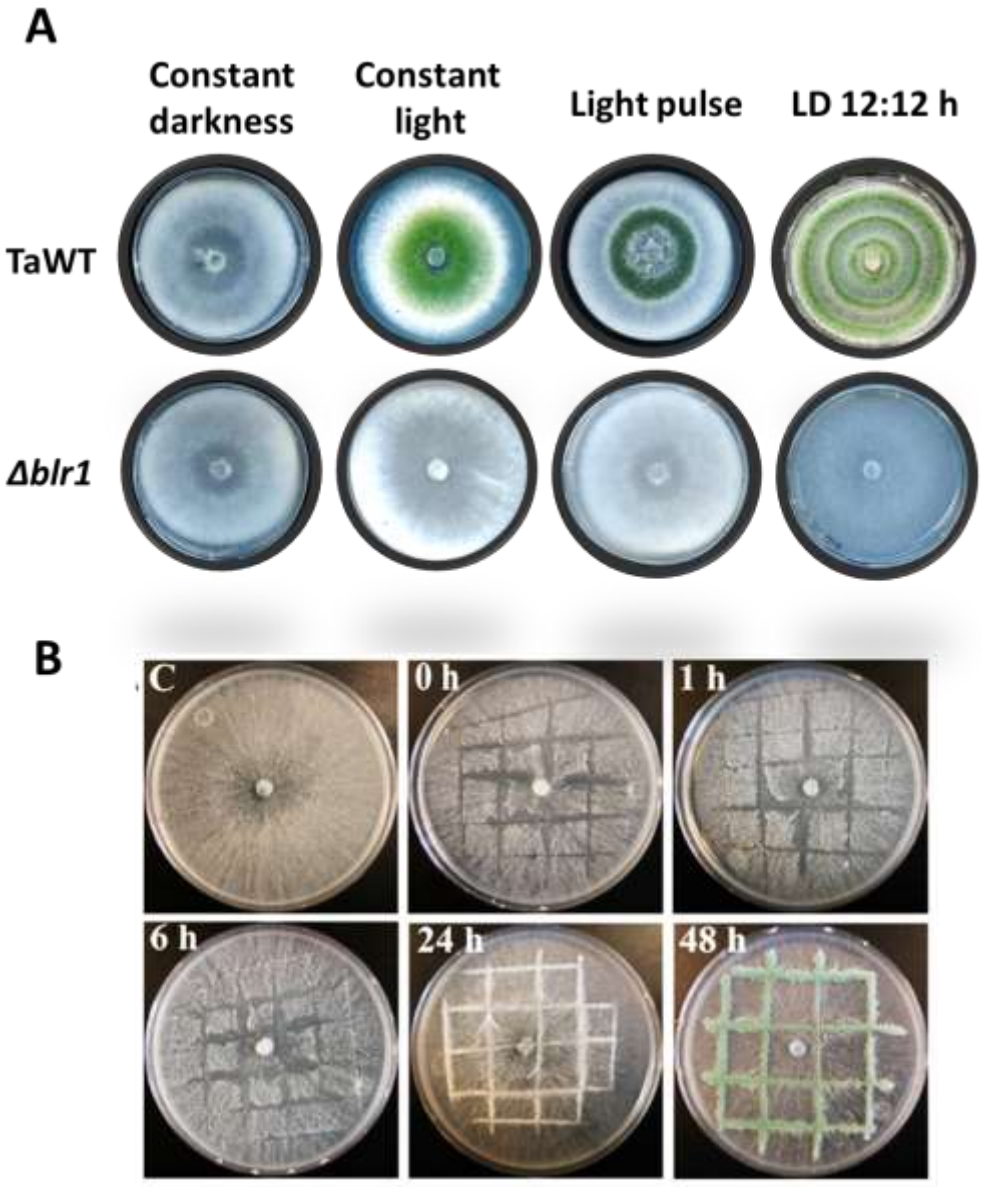

Figure 1.3 Photo and damage-induced conidiation *in T. atroviride*. A) Conidiation in *T. atroviride* IMI 206040 is induced in the presence of light. The wildtype (TaWT) fungus grows as a white mycelium in constant darkness. After 3 days of cultivation under constant light conditions, green conidia are uniformly formed. If the colony receives a short light pulse after 32h of growth in the dark, 48h later, a ring of conidia is formed only in the younger tissue formed previous the light pulse. When *Trichoderma* grows under 12h.:12 h. LD (Light: Dark) cycles, several rings of green conidia are formed in response to the 12 h. light meanwhile white mycelium rings are formed during its grow in 12 h. darkness. **B)** Damage-induced conidiation of *Trichoderma* at different times after injury. Damage was first performed in a dark-growing colony. After 24 h., aerial hyphae are formed (white tissue in the edged of the damage) and 48 h. later, conidia was observed only in the damaged area (Modified from Hernández-Oñate et al., 2012)

Different approaches have been used to elucidate the photoreceptor responsible for light responses in *T. atroviride.* Horwitz in 1985 attempted to find the photoreceptor through mutagenesis, screening mutants unable to sporulate in light, but no photoreceptor was identified (Schmoll et al., 2010). However, the answer came with the analysis of the light responses in *T. atroviride.* It was demonstrated that the expression of the photolyase gene *phr*-1 is light regulated, because no *phr-1* mRNA is detected in the dark, but after a light pulse, *phr-1* undergoes a fast transcriptional activation (Berrocal-Tito et al., 2000). The promoter of *phr-1* showed the presence of putative WCC-binding boxes, suggesting that blue-light responses in *T. atroviride* could be regulated by homologues to WCs from *N. crassa* (Berrocal-Tito et al., 2000; Casas-Flores et al., 2004). Indeed, based on the WCs sequences, Casas-Flores et al. (2004) isolated two genes homolog to *wc-1* and *wc-2* from *N. crassa* and named them as blue light regulator 1 and 2 (*blr1* and *blr2*). BLR proteins have almost the same domain composition as the WCs, except for the absence in BLR-1 of a polyglutamine region present in C-terminal of WC-1(Casas-Flores et al., 2004). BLR1 and BLR2 proteins have 1020 and 484 aa., respectively, and these proteins display a 53% and 52% identity with WC-1 and WC-2, respectively. Both BLR possess a GATA zinc finger DNA-binding domain and BLR1 has 3 PAS domains, and only 1 is present in BLR2. In BLR1, one of the PAS domains belongs to the LOV (Light Oxygen Voltage) domain that binds a flavin chromophore that participates in light sensing (Casas-Flores & Herrera-Estrella, 2013). Absence of BLRs proteins impaired conidiation in response to light and carbon deprivation and no expression of *phr-1* was detected after light exposure (Casas-Flores et al., 2004; Cervantes-Badillo et al., 2013).

Interestingly, there is another kind of stimulus that induces conidiation independently of light in *T. atroviride*. If the mycelium of dark-growing colony of *T. atroviride* is cut with a

scalpel, the borders of the damaged zone starts a differentiation process producing conidia. This conidiation triggered by mechanical injury was first described by Casas-Flores et al. (2004), but only in 2012, microscopic and molecular description of this process were obtained. After causing physical injury to the mycelia, the first step is the formation of aerial hyphae, and 48 h after, conidia is observed in the damaged area. Microscopically, the damaged hyphae start to regenerate, establishing newly developed hyphae 1 h. after the injury. Phialides (basal cell with elongated and flask shaped that extend out the mycelium serving as basal cell for conidia formation, (Minter et al., 1983)) are formed after 24 h, and conidiophores are observed at 48 h (Hernández-Oñate et al., 2012) **(Figure 1.3B)**. Immediately after the injury, an oxidative response is activated through the generation of ROS by NADPH oxidases (Nox). Also, activation of calcium signaling pathways and altered lipid metabolism, specifically, oxylipins, are some of the responses triggered by mechanical damage (Hernández-Oñate et al., 2012). Release of extracellular ATP (eATP) and Ca^{2+} by the damaged cells is also necessary to trigger the injury response, activating MAPK pathways. Two MAPK, previously mentioned in the text, have been described as relevant in this process: Tmk1 and Tmk3. A *tmk1* mutant is incapable of forming aerial mycelium after injury and a *tmk3* mutant, despite being able to form aerial hyphae, cannot form conidia after injury (Medina-Castellanos et al., 2014). Interestingly, the MAPK pathway does not only participate in injury-induced conidiation but it is also involved in photo-induced conidiation and expression of early response genes to light, showing an interesting interplay between light perception and stress signaling via Tmk proteins (Esquivel-Naranjo et al., 2016). A transient light-induction of phosphorylation of Tmk3 that depends on BLR1/BLR2 has been observed and a possible interaction between BLRs and Tmk3, as part of a transcriptional complex has been suggested (Esquivel-Naranjo et al., 2016). These findings show the

importance of MAPK signaling pathway and light perception through BRL1/BLR2 in conidiation, suggesting intricate crosstalk between light perception, stress signaling, and development in *T. atroviride.*

1.7 Circadian rhythms in *Trichoderma*

To date, only a "circadian rhythm" of conidiation in the specie *T. pleuroticola* has been described. Nevertheless, no description of a circadian oscillator controlling the mentioned rhythm has been reported (Steyaert et al., 2010b). Moreover, these phenotypic studies cannot conclude that the observed pattern of conidiation is strictly circadian and not just a mere rhythm driven by environmental cycles. However, the presence of FRQ and a WCC in several members of the genus *Trichoderma* suggests the potential of functional circadian clock in this group (Montenegro-Montero et al., 2015). In *T. atroviride* the extensive studies on *blr1* and *blr2* and the presence of a *frq* homolog that is light-induced in a BLR1 dependent manner, suggest a possible role in this hypothetical – yet not validated - circadian clock in *T. atroviride.*

1.8 Research proposal

The scientific literature discussed above clearly shows that the outcome between biotic interactions depends on, or can be modulated by, the circadian regulation of at least one of the interacting partners. Additionally, there is evidence pointing out to the presence of a circadian clock in *T. atroviride* that has not been analyzed. Herein, we propose to study the relevance of circadian regulation in the interaction between fungi, such as the mycoparasite interaction between *T. atroviride* and *B. cinerea*, to determine whether the clock of one of them (or both) modulates the outcome of the interaction.

1.9 Hypothesis

"The intensity of the mycoparasitic interaction between *Botrytis cinerea* and *Trichoderma atroviride* is subjected to daily variations as a result of circadian regulation by at least one of the interacting partners".

General aim

To characterize the effect of circadian regulation in the dynamics of the interaction between *B. cinerea* and *T. atroviride.*

Specific aims

1.- To characterize, at the molecular level, a circadian oscillator in *T. atroviride.*

2.- To determine the effect of the circadian clock of *B. cinerea* and *T. atroviride* in their interaction.

CHAPTER 2

Circadian oscillations in *Trichoderma atroviride* and the role of core clock components in secondary metabolism, development, and mycoparasitism against the phytopathogen *Botrytis cinerea.*

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2.1 Abstract

Accumulative evidence highlights the importance of circadian clocks for individuals' fitness, while recent studies underline their role in the outcome of organismal interactions, such as in the dynamics of *Arabidopsis thaliana* and the phytopathogen *Botrytis cinerea.* However, so far, the relevance of circadian clocks on fungal-fungal interactions has been seldom addressed. Here, we sought to characterize the presence of a functional clock in the biocontrolling fungus *Trichoderma atroviride*, to understand its importance in the mycoparasitic interaction against the abovementioned phytopathogen.

By utilizing luciferase reporters to monitor the *T. atroviride* core-clock, we confirmed the existence of circadian oscillations that can be modulated by environmental cues such as light and temperature. Confrontation assays between WT and clock mutant strains of *T. atroviride* and *B. cinerea*, in constant light or darkness, revealed an inhibitory effect of light in *Trichoderma's* mycoparasitic capabilities. Interestingly, when confrontation assays were performed under light/dark cycles, *Trichoderma's* overgrowth capacity was enhanced when inoculations were at dawn compared to dusk. Deletion of core-clock negative elements had dissimilar effects on this phenotype as the Botrytis's (BcFRQ1), but not Trichoderma's one (TaFRQ), was key in such daily differential behavior, suggesting that the Botrytis' clock has a greater influence in this interaction. Additionally, *T. atroviride* clock components modulate development and secondary metabolism in this fungus, affecting the production of several molecules, including volatile compounds, such as 6-PP. Finally, we detected the rhythmic production of several *T. atroviride* VOCs, which depend on a functional circadian clock. Thus, this study not only provides evidence on how clock components impact diverse aspects of *T. atroviride* lifestyle, but also on how circadian gears and variables modulate fungal interactions and dynamics.

2.2 Introduction

Trichoderma species are cosmopolitan soil fungi found in several substrates including decaying wood, fungal fruiting bodies, in association with plants (Carreras-Villaseñor et al., 2012; Kubicek et al., 2019; Vos et al., 2015), or stablishing beneficial interactions with these and other microorganisms (Druzhinina et al., 2006; Guzmán-Guzmán et al., 2019; Harman et al., 2004; Sood et al., 2020). *Trichoderma* species are well-known mycoparasites as they can degrade and grow over other fungi, a distinctive feature that has been exploited in agriculture as biocontrol agents of different phytopathogens (Naglot et al., 2015; Rahman et al., 2009), including *Botrytis cinerea*.

The mycoparasitic behavior in *Trichoderma* starts with the secretion of low levels of cell wall degrading enzymes (CWDE) (Druzhinina et al., 2011; Seidl et al., 2009; Vos et al., 2015) that deconstruct the fungal prey's hyphae. Release of small molecules derived from the prey can function as molecular signals in *Trichoderma*, allowing its recognition (Zeilinger et al., 2005), re-directing hyphal growth towards it, coiling around the host mycelium, followed by strangulation. The synergistic effect of enhanced CWDE and toxic secondary metabolites production allow *Trichoderma's* hyphae to penetrate the prey's lumen, achieving its killing (Bélanger et al., 1995; Druzhinina et al., 2011; Sharma et al., 2011; Steyaert et al., 2003).

Trichoderma species present developmental responses to different stresses, in which conidiation induced by light and mechanical damage have been extensively studied (Casas-Flores & Herrera-Estrella, 2013). Mechanical damage of mycelia in *T. atroviride* triggers a developmental injury response, leading to conidia production in the injured area through the generation of ROS and activation of MAPK signaling pathways (Hernández-Oñate et al.,

2012; Medina-Castellanos et al., 2014). Additionally, light is also perceived as a stress signal, triggering conidiation: a light pulse given to a dark grown *T. atroviride* generates several biochemical changes driving the formation of a green ring of conidia at the edge of the colony (Casas-Flores et al., 2004, 2006; Galun and Gressel 1966; Schmoll et al., 2010). In *T. atroviride*, two genes called blue light regulator 1 and 2 (*blr1* and *blr2*) were identified as responsible for light-induced conidiation, because their respective mutant strains fail to form conidia in response to light. The BLR proteins are homologs of the transcription factors WC-1 and WC-2, which in *Neurospora crassa* conform the WCC (White Collar Complex), responsible of light and circadian regulation in this fungus (Casas-Flores et al., 2004; Froehlich et al., 2002; Montenegro-Montero et al., 2015). Importantly, light can be directly perceived thanks to the presence of a Light-Oxygen-Voltage (LOV) domain in WC-1/BLR1 (Corrochano, 2019). Furthermore, BLRs have also been described as critical for the effect of light on growth, oxidative stress responses and light-controlled gene expression (Cervantes-Badillo et al., 2013; Esquivel-Naranjo et al., 2016; Friedl et al., 2008a; García-Esquivel et al., 2016). Extensive studies on BLR1 and BLR2 and the presence of a *frq* homolog (see below) that is induced in a BLR1/light-dependent fashion (García-Esquivel et al., 2016; Steyaert et al., 2010a), suggests the potential of a functional clock, and of underlying circadian regulation, in this fungus. However, to date, neither circadian oscillations of *tafrq*/TaFRQ levels, nor circadian phenotypes have been reported in *T. atroviride* whereas, diverse studies have failed at detecting overt or molecular circadian rhythms in several fungi across fungal clades (Cascant-Lopez et al., 2019; Hevia et al., 2016; Larrondo and Canessa 2019).

Circadian rhythms have emerged as an adaptation to Earth's rotation around its axis, allowing organisms to keep track of time, anticipating and adapting to periodic and

predictable environmental changes through the temporal coordination of gene expression, metabolic pathways, physiological responses and even behaviors (Bell-Pedersen et al., 2005; Hurley et al., 2016). Recent findings have shown that also interactions between organisms may be subjected to circadian regulation (Hevia et al., 2016; Larrondo and Canessa 2019). Thus, several reports have highlighted the importance of the plant clock in plant-pathogen interactions, whereas there has been little information from the pathogen perspective. Yet, it has been reported that the interaction between the phytopathogenic fungus *B. cinerea* and *Arabidopsis thaliana* largely depends on the fungal functional circadian clock, with virulence reaching higher levels at nighttime (Hevia et al., 2015; Ingle et al., 2015).

The ascomycete *N. crassa* is the leading model in fungal chronobiology due to its robust circadian phenotype of daily spore production at dawn, and a wealth of molecular resources (Baker et al., 2012). The central oscillator comprises a negative transcriptional-translational feedback loop (TTFL), a shared central architecture across phyla (Dunlap, 1999; Dunlap & Loros, 2017; Loros, 2020). In Neurospora, the WCC activates the expression of the TTFL negative element: *frequency* (*frq*): soon after produced, the FRQ protein recruits kinases and negatively regulates its own expression by phosphorylating and inactivating the WCC. FRQ itself is progressively phosphorylated until reaching its maximum phosphorylation state at night, losing its affinity for WCC, allowing a new cycle to start. Levels of *frq* mRNA and FRQ protein oscillate during this process with a period close to 22.5 h (Larrondo et al., 2015; Montenegro-Montero et al., 2015). Albeit *wc-1*, *wc-2*, and *frq* homologs are present in several fungi, the description of overt clock phenotypes, as the molecular characterization of circadian rhythms in fungal species besides *N. crassa*, has been scarce (Montenegro-Montero et al., 2015; Salichos and Rokas 2010). Indeed, despite different studies addressing the latter, there are limited examples of characterized

circadian oscillators in other fungi, or studies exploring circadian phenomena and clock components. Among these is *B. cinerea*, where rhythms in FRQ levels were characterized, along with FRQ's key role in conferring time of the day difference on virulence potential. Remarkably, the evidence also revealed that in Botrytis FRQ displayed extra-circadian phenotypes, as it regulates developmental decisions under conditions where clock function is abrogated, such as constant light (Hevia et al., 2015).

In order to evaluate the presence of a functional circadian clock in *T. atroviride*, we sought to assess daily oscillations of TaFRQ expression under free-running conditions. In addition, we also assessed canonical clock properties, and the ability of the clock to be entrained by environmental stimuli. By generating clock mutant strains, we evaluated the impact of core clock components in *T. atroviride* secondary metabolism, developmental programs, and in its mycoparasite abilities against different *B. cinerea* strains including those deficient in circadian regulation. In the aggregate, our results reveal the presence of a functional circadian clock in *T. atroviride*, a role of core-clock components in the modulation of mycoparasitic interactions and extra circadian roles for TaFRQ.

2.3 Materials and Methods

2.3.1 Fungal strains and culture conditions

T. *atroviride* IMI 206040 strain initially isolated from a plum tree of the Czar variety, in an orchard in Urshult near Växjö in southern Sweden (EFSA 2015), was used as wild type strain (TaWT). The Δ*blr1* strain was described by Casas-Flores et al., 2004 and was kindly provided by Dr. Alfredo Herrera-Estrella. Strains were maintained in Potato Dextrose Agar (PDA) in constant light at 25°C. *B. cinerea* B05.10 Pers. Fr. [*Botryotinia fuckeliana* (de Bary) Whetzel] was initially isolated from *Vitis vinifera* (Germany) and was provided by the Tudzinsky laboratory (WWU, Germany). *B. cinerea* clock mutant strains Δ*bcwcl1* and Δ*bcfrq1* used in this study were described and obtained by Canessa et al., 2013 and Hevia et al., 2015. *B. cinerea* strains were cultivated in PDA at 20°C in LD 12:12-h cycles. All fungal strains were grown in Percival incubators, equipped with cold white-light fluorescent tubes (light intensity up to 100 μM/m2/s; wavelength 400–720 nm).

2.3.2 Generation of Δ*tafrq* and OE::*tafrq* mutant strains

The *tafrq* ORF was replaced by the hygromycin resistance cassette (*hph*), as described (Canessa et al., 2013) (Supplementary Fig. 1 & Supplementary Table V). To overexpress *tafrq,* we placed *tafrq* under control of the actin promoter with *hph* as a selection marker, and the construct was integrated by homologous recombination into the intergenic region of *blu17* (Supplementary Table VI) (Balcázar-López et al., 2016).

2.3.3 Generation of luciferase reporter strains

The TaFRQ^{LUC} strains were designed and generated as previously reported by Larrondo et al., (2012). The *tafrq* gene was fused, by homologous recombination at its endogenous locus, to a *N. crassa* codon-optimized luciferase gene (*luc*), and a hygromycin resistance cassette (*hph*) was used as a selection marker (Supplementary Fig. 2 & Supplementary Table VII). The transcriptional reporter strain, termed $_{nc}$*c-box-luc*, was designed as reported by Larrondo et al., 2015. We cloned a 505 bp sequence containing the *c-box* sequence from the *N. crassa frq* promoter to control *luc* expression, and the construct was integrated into the intergenic region of *blu17*, which has been previously demonstrated not to affect mycoparasite behavior (Supplementary Table VIII) (Balcázar-López et al., 2016). To develop transcriptional reporters of hypothetical clock-controlled genes (*ccg*) in *T. atroviride,* we used a promoter region of different genes as follows: 768 bp from TRIATDRAF_77441, homologous to *ccg-9* (trehalose synthase in *N. crassa)*, 1159 bp from TRIATDRAF_297741, homologous to glyceraldehyde 3-phosphate dehydrogenase of *N. crassa*, 485 bp from TRIATDRAF_298583 homologous to superoxide dismutase of *N. crassa* and 1000 bp from TRIATDRAFT_291013 homologous to *con-10*. The promoters were used to control *luc* expression and the construction was integrated into the intergenic region of *blu17* (Supplementary Table VIII) (Balcázar-López et al., 2016)

2.3.4 Transformation of *T. atroviride*

PEG-mediated protoplast transformation was performed as described by Casas-Flores et al., 2004 and Herrera-Estrella et al., 1990. All transformants were subjected to 3-5 rounds of single spore isolation, and gene replacement and integration events were verified using Southern Blot using the DIG Easy Hyb Hybridization solution (Roche) following manufacturer's instructions. The *hph* gene was used as a probe (Supplementary Fig. 1 & 2).

2.3.5 Assessment of luminescence *in vivo*

To evaluate rhythmic levels of bioluminescence produced by the different luciferase reporter strains, spores from 7-day old colonies were resuspended in sterile water, filtrated using sterile glass wool, and kept for 48 h at 4°C. Then, 30 µL of spore suspension was inoculated in a 96-well plate containing distinct culture media (Supplementary Table I). Luciferin was added to each media to a 2.5 mM final concentration. The reporter strains received LD 12:12 entrainment at 20°, 22°, 25°, and 28° C. Luminescence traces were captured using a PIXIS 1024B CCD camera (Princeton Instruments) under constant darkness (DD) conditions at the corresponding temperature. For LL reset, strains were grown 72 h. in LL at 25 °C and transferred to DD for bioluminescence measure. For the 4°C pulse, strains were incubated for 48 h in LL (constant light) at 25°C and then transferred to 4°C for 6 h in complete darkness prior measurements. For the light pulse experiment, strains were incubated for 48 h in LL, transferred to DD for 24 h and received a 20 min light pulse at 25°C, and transferred back to DD to measure light traces. Bioluminescence was acquired using the WinView software (version 2.5.23.0) and subsequently analyzed using ImageJ

employing a custom-made macro. As indicated therein, in different graphs we present raw or normalized data (align to mean). Circadian parameters, such as period and phase, were calculated using fast Fourier transform nonlinear least squares (FFT-NLLS), which fits a sine wave to data, estimating period, phase and amplitude using the BioDare online platform (Zielinski et al., 2014).

2.3.6 Protein extracts and immunoblots.

To detect TaFRQLUC oscillations at protein levels, TaFRQLUC reporter strains were subjected to a time course experiment in GYEC+peas culture media, using a light pulse as cue. Samples were taken every 4 h. for 56 h. Proteins were extracted as described by Kilani et al., 2020. Protein concentration was determined by the Bradford method, using the BioRad Protein Assay (Bio-Rad Laboratories, Inc., CA, USA). For immunoblot analysis, total protein extracts (50 μg of protein per lane) were separated in 4-15% Mini-PROTEAN TGX Precast gels (Bio-Rad #456-1086) and transferred to PVDF membranes (Bio-Rad) using the Trans-Blot Turbo RTA Transfer Kit (Bio-Rad #170-4272).The membranes were blocked overnight in phosphate-buffered saline solution (PBS-T: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4 and 0,1% Tween-20) with 5% non-fat dried milk at 4ºC. To detect FRQLUC protein a Luciferase antibody C-12 (sc-74548, Santa Cruz Biotechnology Inc., TX, USA) was used at a 1:250 dilution and as secondary antibody Goat Anti-Mouse IgG(H+L)-HRP Conjugate at a 1:5000 dilution.

2.3.7 Confrontation assay between *B. cinerea* and *T. atroviride*

To evaluate the role of circadian regulation on *B. cinerea* and *T. atroviride* interactions, confrontations assays we conducted as a modified version of Bell 1980. Overgrowth was calculated as the percentage of the *B. cinerea* colony that was covered by Trichoderma. TaWT and clock mutant strains of both fungi were grown in PDA at 20°C for *B. cinerea* and PDA and GYEC at 25°C for *T. atroviride*, during three days either in constant light (LL), darkness (DD) (Fig. 3A), or in opposite LD cycles (LD AM and LD PM) (Fig. 4A). Mycelial plugs of each strain were inoculated in opposite sides of 90 mm x 15 mm Petri dishes, 1 cm from the edge and kept at 20°C in LL, DD, and LD conditions (as indicated in each case). Digital images were acquired after 7 days of interaction. The analysis of the images was performed using the ImageJ software employing an external calibration scale.

2.3.8 Conidiation response assays

For light-induced conidiation, we cultivated *Trichoderma* strains in PDA at 25°C in LL for seven days and collected conidia at 3 and 7 days of growth and quantified. For light-pulse conidiation assay, strains were grown in PDA at 25°C in DD for 36 h. A white-light pulse of 1 and 60 min was given and maintained in DD for an additional 48 h and conidia were quantified. For injury-induced conidiation, strains were grown in PDA at 25°C in DD for 32-36 h and damaged with a scalpel in DD using a safety red light and incubated for an additional 48 h in the dark. Subsequently, conidia were collected in sterile water and quantified by direct counting in a Neubauer chamber. For statistical analysis, ANOVA was used, and significance was determined using Tukey's test with a threshold of $P < 0.05$.

2.3.9 Secondary metabolic fingerprinting

The effect of core clock components and light in *Trichoderma* secondary metabolites was determined by growing the strains in LL and DD for 48 h in PDA at 25°C. A group of dark-growing strains received a 4 h light pulse. For circadian metabolic profiles, TaWT and Δ*tafrq* strains were grown in GYEC with 0.5% peas during three days in LD 12:12 h cycle, and transferred to DD. Samples were taken every 4 h during 48 h after transition to DD.

The metabolic profile of the diffusible compounds was carried out using direct liquid injection mass spectrometry (DLI-MS). Mycelium of the strains grown in PDA in LL, DD, and LP conditions, was harvested and extracted with 1ml of a mix of high-performance liquid chromatography (HPLC)-grade ethyl acetate, methanol and dicholoromethane 3:2:1 with 1% formic acid. The samples were sonicated for 20 min at room temperature at a frequency of 40 kHz and centrifugated at 15000 rpm for 20 min. The supernatant was transferred to a clean Eppendorf tube, and the solvent was evaporated at room temperature. Extracts were weighed and resuspended in methanol to 0.05 mg/mL. A 1/10 dilution was injected in the DLI-MS in positive ESI mode, using an LCQ Fleet ion trap mass spectrometer. The spectra were acquired in the positive mode in an m/z range of 500 to 2000. For VOCs analysis, the Low Temperature Plasma Mass Spectrometry (LTP-MS) device was used as described by Martínez-Jarquín et al., 2016 and Martínez-Jarquín & Winkler, 2013**.** The Petri dish was placed under the plasma**.** The spectra were acquired in the positive mode in an m/z range of 50 to 500. In both analyses (DLI and LTP MS), six biological replicates were used for each condition.

2.3.10 Data analysis

The mass spectra raw data were converted to mzML open format with ProteoWizard and analyzed in the R statistical programing language using the MALDIquant package to import and create a matrix with all the spectrums aligned and corrected. For LL, DD, and LP culture conditions, data was filter using interquartile range (IQR) and scale using autoscaling (mean-centered and divided by standard deviation of each variable). Significative differences in mean abundance among metabolites in different samples were assessed with Two-way ANOVA with a threshold of $P < 0.05$. A heatmap correlation matrix was built to visualize metabolic variations in all analyzed strains and experimental conditions with metabolites identified as statistically significant by ANOVA. The Euclidean distance and Ward algorithm were used for clustering in the MetaboAnalyst platform (Pang et al. 2020). Fold change and T-test was calculated in pairs, using the mean value of raw data for each comparison. A 2-fold change cutoff was stablished for diffusible and 1.5-fold change for volatile compounds.

For circadian analysis and VOCs analysis, a Bayesian analysis was made using all ten micro-scans taken as individual scans to perform ion identity related to the sample or noise (ambient noise and for GYEC + 0.5% peas medium). The Bayesian analysis shows the percentage of the identity of the ion, related to the sample. Ions with 0% of identity with samples were eliminated, and ions intensities with less than 100% of identity to the sample were used. Adjustment of intensities was made on the spectra matrix made with means of the micro-scans. Determination of period and rhythmicity was carried out in the BioDare platform (Zielinski et al., 2014), using FFT NLLS and BD2eJTK with $p<0.05$.

2.4 Results

2.4.1 *T. atroviride* bears a functional circadian clock.

We developed TaFRQ^{LUC}, a translational fusion reporter between luciferase and TaFRQ (Supplementary Fig. 2.2), to assess oscillations of this putative core-clock negative element and quantify bioluminescence levels in different culture media under free-running conditions (DD). Monitoring LUC activity after three-days of constant light (LL) or LD 12:12 h entrainment cycles at 25°C, failed to reveal oscillations in different media (Supplementary Fig. 2.3 & Supplementary Table I) with only one exception: GYEC, where weak but distinguishable oscillations were observed (Fig. 2.1A). Since *T. atroviride* interacts with plants, we decided to modify the latter media by adding plant-derived material, (GYEC + peas) observing that the quality of the oscillations improved ostensibly (Fig. 2.1B, Supplementary Fig. 4). Period estimation of the oscillations by FFT NLS using the BioDare Platform (Zielinski et al., 2014) revealed an average period of 26.5 ± 0.36 h. While rhythms can be observed in DD after three days of LD entrainment, transfer from the same amount of days in LL to DD also leads to oscillations, albeit less robust ones (Fig. 2.1C). As shown in Fig. 2.1D, after 48 h. in DD a short 20 min. light pulse was able to reset the clock, elevating TaFRQ^{LUC} levels and generating sustained oscillations for at least 4 days. (Fig. 2.1D). Likewise a low temperature pulse of 4 °C for 6 h also served as a cue to reset the clock, shifting its phase almost 12 h, similar to what can be seen in Neurospora (Larrondo et al., 2012) (Fig. 2.1E). After both pulse protocols, period also remained in the 26-h range (26.30 h. and 26.49 h., respectively), even exhibiting better oscillations after the perturbation, as previously reported for clock reporters under some conditions (Shi et al., 2007). These findings indicate that circadian oscillations of TaFRQ can be entrained by LD cycles or

restarted by light and temperature pulses, as expected from a *bona fide* circadian clock. The analyses of these perturbations (Supplementary Fig. 2.5) reveal a mean phase delay of 4.29 h. between LD and light pulse entrainment and 10.85 h. between LL reset and 4°C pulse, further confirming that this oscillations behave as one would expect from a circadian oscillator (Heintzen & Liu, 2007). Finally, to further expand these results, using a western blot with anti-LUC antibodies, we confirmed TaFRQ^{LUC} oscillations in constant darkness over a 56 hrs. time course, ratifying the circadian variations of TaFRQ (Supplementary Fig. 2.6).

Importantly, we also evaluated if the observed oscillations could maintain period in a range of physiological temperatures, a key circadian property known as temperature compensation. TaFRQ^{LUC} rhythms were evaluated after three days in 12:12 LD entrainment regimes at 20, 22, 25, and 28 °C and then released in DD at the corresponding temperatures. While low amplitude oscillations were observed at 20 °C (Fig. 2.1F, Supplementary Fig.2. 7), at 22 °C changes in TaFRQ^{LUC} levels exhibited higher amplitude than at 25 ºC, indicating that of the tested temperatures, the Trichoderma's clock runs better at 22 °C (Fig. 2.1G, Supplementary Fig. 2.7) while, on the other hand, oscillations at 28 °C were lost (Fig. 2.1H, Supplementary Fig. 2.7). To quantify the degree of temperature compensation, we calculated the Q_{10} between 20 and 25 ºC (Supp. Fig. 2.8**)**, obtaining a Q_{10} of 1.05 ± 0.07, which is in the range for temperature compensation described in other systems (Anderson et al. 1985; Kusakina et al. 2014; Mattern et al. 1982). Thus, the results show that the *T. atroviride* circadian clock can compensate in a temperature range between 20 and 25 °C, further supporting that the observed rhythms are generated by a *bona fide* circadian clock.

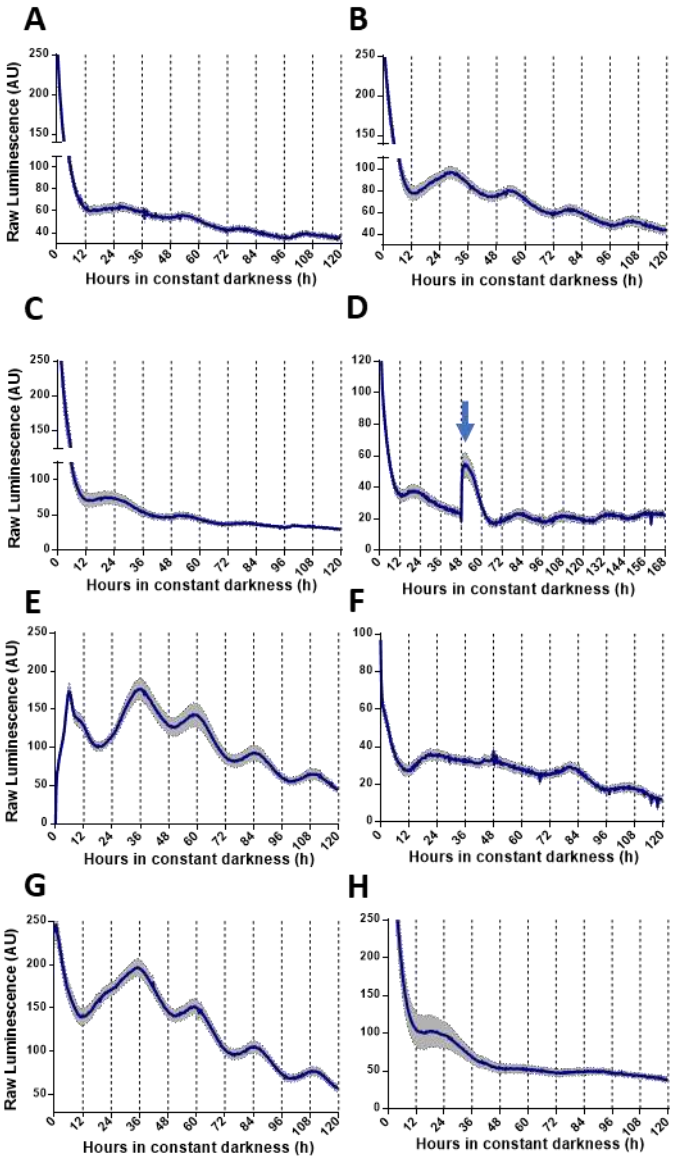

Fig. 2.1. Circadian oscillations of TaFRQ^{LUC} are reset by light and temperature, and are temperature compensated. a, b. TaFRQ^{LUC} levels were quantified under constant darkness in strains previously entrained for 3 days under LD (12:12 h.) cycles in GYEC (**a**) or GYEC+peas (**b**) culture media. **c.** Culture in LL for 72 h. at 25°C and transition to DD can generate oscillations. **d.** The effect of a light pulse (blue arrow) was evaluated in DD on cultures that were also entrained in LD cycles as previously mentioned. **e.** A 4 °C temperature treatment efficiently resets rhythms, as shown in cultures grown for 48 h in LL, cold pulsed in the dark for 6 h, and then monitored in DD. **f, g, h.** TaFRQ^{LUC} expression was monitored at 20 (**f**) 22 (**g**) and 28°C (**h**) to assess rhythmicity at different temperatures. GYEC + peas media was used in all cases except in **a**, and bioluminescence was recorded at 25° C except indicated otherwise. In all cases mean values of raw data is presented from two biological and 8 technical replicates each (SEM represented with grey area).

2.4.2 Functional conservation of a c-*box* sequence from *N. crassa* in *T. atroviride.*

In *N. crassa, frq* transcriptional activation under constant darkness requires WCC recognition of the *clock box* (*c-box*) in its promoter, a *cis*-element which is necessary and sufficient to generate and maintain *frq* daily rhythms (Froehlich et al. 2003). To evaluate the conservation of the circadian system of *T. atroviride,* we transformed this fungus with *luc* under the control of the Neurospora *c-box* ($_{Nc}$*c-box-luc*). Notably, this reporter revealed clear rhythms of *luc* patterns under free running conditions (DD), which also responded to light and temperature cues. The reporter traces were analyzed using BioDare (Zielinski et al., 2014), revealing an average 25.67 ± 1.01 h. period for two independent $_{Nc}$*c-box-luc* strains, in agreement with the TaFRQ^{LUC} data. This provides additional proof of a functional circadian clock in *T. atroviride* and demonstrates that a pivotal *Neurospora cis*-element ($_{Nc}$*c-box*) can be recognized in *T. atroviride* (Fig. 2.2, Supplementary Fig. 2.9A). Albeit the measured period with this reporter and TaFRQ^{LUC} is a slighter longer in the latter, this also recapitulates what has been seen when comparing both types of reporters in Neurospora, most likely due to discrete changes in FRQ structure/dynamics (Larrondo et al. 2012). Notably, in response to a light-pulse a clear and acute augment in *luc* expression was seen, similar to what is observed for light-responsive elements in Neurospora, arguing that this $_{Nc}$*c-box* could be integrating both light and clock information in Trichoderma, a hypothesis that will be challenged in future experiments. In an attempt to attest additional (and putative circadian) transcriptional reporters, we subjected *luc* expression under the control of four *T. atroviride* promoters of interest. These were selected from homologs to *N. crassa* genes encoding trehalose synthase, glyceraldehyde 3-phosphate dehydrogenase, superoxide dismutase and CON-10, which have been reported as rhythmic in Neurospora and other

organisms (Lakin-Thomas et al., 2011; Lillo, 1993; Shinohara et al., 2002; Yoshida et al., 2008). Although we observed high luminescence levels, no oscillations were seen nor recognized by BioDare in DD, at least under the tested culture conditions (Supplementary Fig. 2.9B & Supplementary Table IV**).** Despite the disappointing lack of oscillation of these putative clock-controlled genes (*ccg*s) reporters, such result reinforces the idea that the rhythms yielded by the $_{Nc}$*c-box-luc* reporter strains are due to the circadian nature of the *cis*-element, rather than oscillations driven by external factors. As the DNA binding domains of WC1 and BLR1 share a 97.2% aa identity, we hypothesize that the BRL1/BLR2 complex can recognize the $_{Nc}$*c-box* element driving circadian oscillations and that a similar *c-box* sequence could be found in the *tafrq* promoter. Furthermore, as both fungi belong to the *Sordariomycetes* class, a conserved circadian regulation mechanism is not unexpected in this group and, in fact, two putative light response elements in *tafrq's* promoter with a putative GATA box consensus sequence at proximal and distal locations (-355 to -319 and -1180 to -1171 from the TSS, respectively) have been postulated (Cervantes-Badillo et al. 2013), of which one of them could be a putative $_{Ta}$*cbox* sequence.

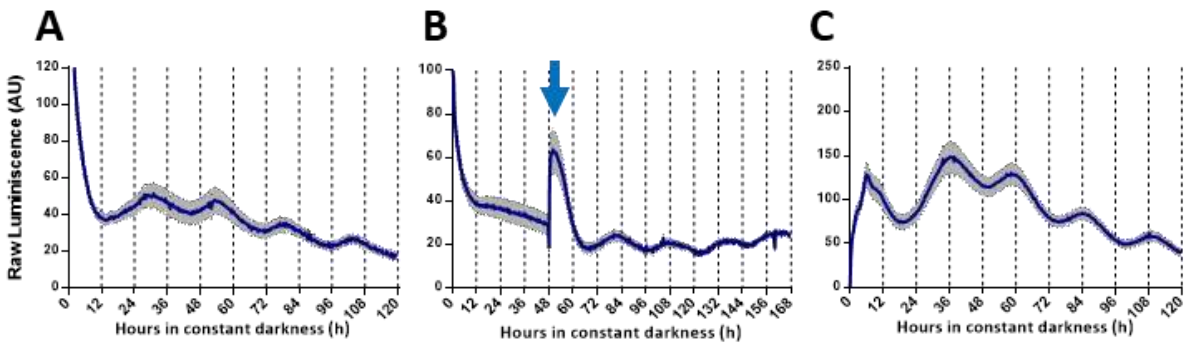

Fig. 2.2. The *c-box* of the *N. crassa frq* promoter exhibits circadian rhythmic expression in *T. atroviride*. a, b. $_{Nc}$*c-box-luc* reporter strains were entrained for 3 days under LD (12:12 h.) cycles at 25°C and then transferred to DD where they were monitored unperturbed (**a**), or examined after a discrete light pulse (blue arrow), which produces a perceivable phase shift (**b**). **c.** Strains were grown for 48 in LL and then transferred to DD, where a 4 °C temperature treatment for 6 h. was applied and then bioluminescence was monitored under constant darkness and 25°C. GYEC + peas culture media was used in all cases. Raw data is presented with mean values of 2 biological replicates with 3 technical replicates (SEM is represented with grey area).

2.4.3 TaFRQ is required for conidiation after light exposure and mechanical injury.

While in Neurospora FRQ appears to mainly serve clock functions, in *B. cinerea* it was reported that BcFRQ1 exhibits extra-circadian roles, impacting developmental phenotypes. Indeed, enhanced microconidiation and sclerotia formation could be observed for Δ*bcfrq1* even in conditions where a fungal circadian clock does not run, such as constant light (Hevia et al., 2015). This distinctive phenotype, which contrast the copious production of macroconidia of WT Botrytis in LL and LD, prompt us to ask whether *tafrq* might be also serving a role in fungal development in *T. atroviride*. Therefore, we constructed *tafrq* deletion (Δ*tafrq)* and *tafrq* overexpressing strains (OE::*tafrq*), and evaluated light induced conidiation during 7 days in LL. After 72 h. of cultivation in LL, conidia in the Δ*tafrq* strain exhibits a dark-green color in comparison to the TaWT and the OE::*tafrq* strains (Supplementary Fig. 2.10A), which displayed the characteristic yellowish/ green coloration. Importantly, at 3 days post inoculation (dpi), the Δ*tafrq* strain had produced significantly fewer conidia, albeit this difference was not significant by day 7 where all strains exhibited similar amounts of conidia, indicating that conidiation in Δ*tafrq* is delayed, but not compromised. In addition, conidia formation in response to a discrete light pulse was significantly decreased in Δ*tafrq*, regardless of the extension of the light pulse, whereas no significative differences were observed between TaWT and OE::*tafrq* in both experiments (Supplementary Fig. 2.10B).

We also evaluated whether the injury-response mechanism that drives the formation of conidia in *T. atroviride* upon physical damage (Hernández-Oñate et al., 2012) was affected in the mutant strains. We injured in constant darkness WT and the mentioned clock mutant strains, including Δ*blr1*, employing a sterile scalpel and quantified conidia formation 48 h.

afterwards. Interestingly, Δ*blr1* showed a 51% average reduction of conidia production in comparison with the TaWT strain, which had not been previously reported. In contrast, while the OE::*tafrq* showed no significant differences compared to TaWT, Δ*tafrq* exhibited almost a 100-fold reduction in injure-triggered conidiation (Supplementary Fig. 2.10A&C). These results indicate that TaFRQ has an underlying role in promoting conidiation in response to an acute stress stimulus, providing evidence of an additional role for TaFRQ in *T. atroviride* besides its expected functions as negative element of the clock.

2.4.4 Light and core clock components regulate the mycoparasitic interaction between *T. atroviride* and *B. cinerea*.

In order to evaluate the role of *T. atroviride* core clock components in its mycoparasitic capabilities, we performed confrontations assays between this biocontroller and *B. cinerea*, utilizing the corresponding clock mutant strains (for *wc* and *frq*) (Canessa et al., 2013; Hevia et al., 2015). Experiments were first conducted under LL and DD conditions, evaluating the mycoparasitic behavior of *T. atroviride* as its ability to overgrow *B. cinerea*, as shown in Fig. 2.3A.

In the TaWT-B05.10 interaction, TaWT displayed a higher overgrowth in DD than in LL over *B. cinerea* (50% versus 20% of overgrowth, respectively). Increased overgrowth in DD (90%,) versus LL was also seen for *Δblr1* against B05.10, being the most aggressive of the tested *Trichoderma* strains, while Δ*tafrq* showed similar behavior as TaWT in LL and DD against B05.10 (Fig. 2.3B&C, Supplementary Table II). *Trichoderma* strains interaction against *B. cinerea* Δ*bcfrq1*, was similar to what was observed for B05.10 in LL and DD, except for the Δ*tafrq*-Δ*bcfrq1* interaction in LL, where no overgrowth was observed. Finally,

under LL, Botrytis Δ*bcwcl1* was easily overgrown by TaWT but not by the *Δblr1* or *Δtafrq* Trichoderma mutants. However, in the absence of light, Δ*bcwcl1* was easily overgrown circa an 80% by all *Trichoderma* strains (Fig. 2.3B&C, Supplementary Table II). In the aggregate, these results show that constant light inhibits the overgrow capacity of *T. atroviride* irrespective of the absence/presence of *blr1* or alternatively, that mycoparasitism is enhanced in darkness. Core clock components also modulated the outcome of these mycoparasitic interactions, suggesting an inhibitory role of BLR1 as its absence enhances overgrowth in DD against all Botrytis strains. Meanwhile, *tafrq* does not seem to have a relevant role in the outcome of the interaction when challenged with B05.10, but has a weaker mycoparasitic effect when interacting with Δ*bcwcl1* and Δ*bcfrq1 Botrytis* strains in LL. Similarly, the absence of *B. cinerea bcfrq1* does not affect the fungus when challenged with TaWT, but the absence of *bcwcl1* increases the susceptibility of *B. cinerea* to *T. atroviride*, suggesting a diminished capacity to respond to this biotic stress in this mutant.

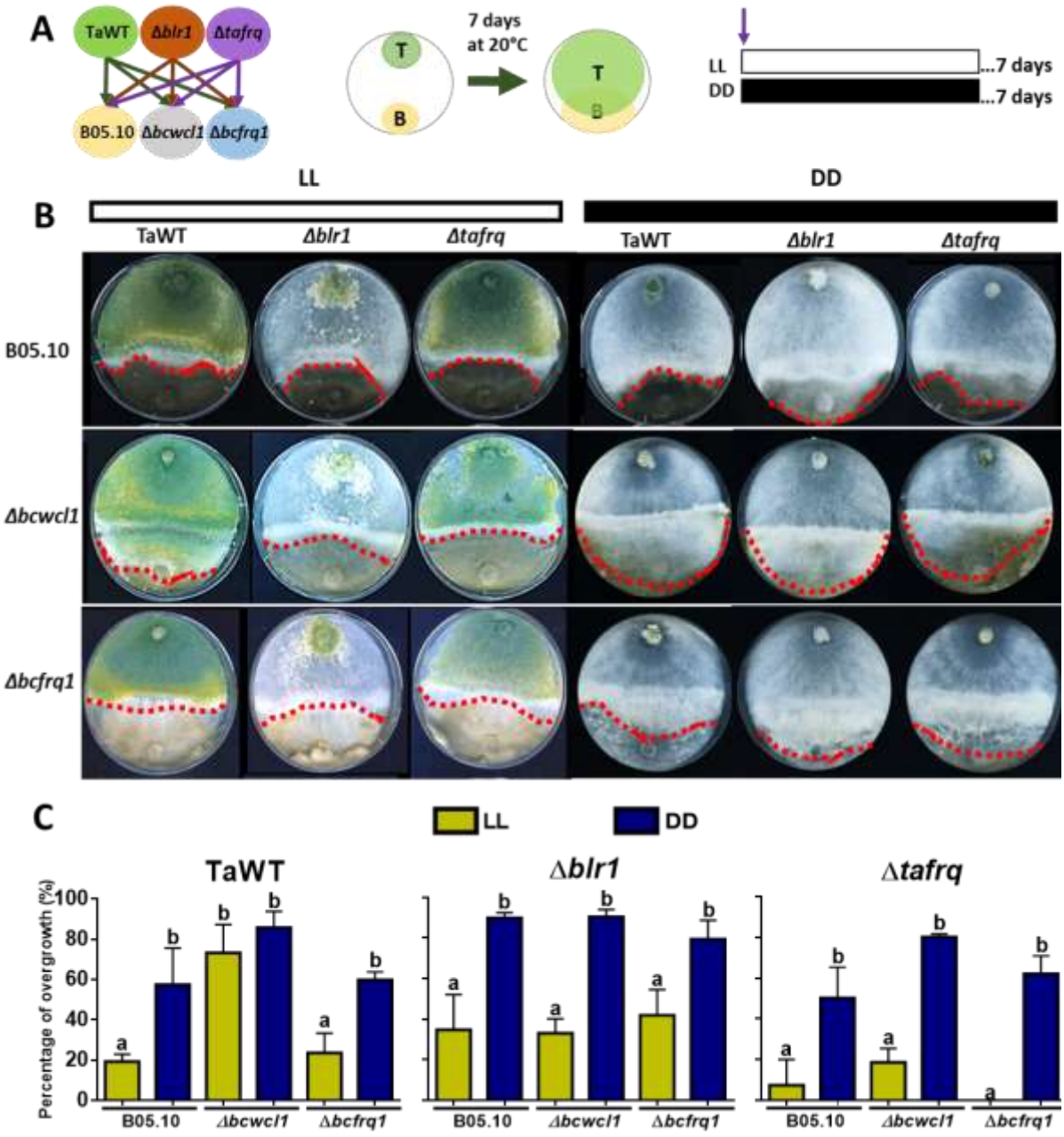

Fig. 2.3. Core clock components, light and darkness modulate the outcome of the *T. atroviride* and *B. cinerea* interaction. a. Schematic representation of the experimental design (left) and confrontation assay (right). **b.** After 7 days of cultivation, *T. atroviride* overgrowth was evaluated in LL and DD culture conditions. Red dotted lines indicate the edge of *Trichoderma* over *B. cinerea*. A representative image of three biological replicates is shown. **c.** Percentage of *B. cinerea* colony area covered by *Trichoderma* (percentage of overgrowth). Error bars represent the means ± SD of 3 biological replicates. Different letters indicate significant differences based on Tukey's test ($p < 0.05$).

2.4.5 Differential mycoparasitic behavior of *T. atroviride* against *B. cinerea* in light/dark cycles.

To fully understand the role of core clock components in the mycoparasite interaction between *T. atroviride* against *B. cinerea*, under a circadian paradigm, we performed out-of-phase confrontations assays in LD 12:12 h. cycles. Confrontations assays were inoculated at "dawn" when lights are turned on in the LD (AM) cycle and at "dusk" before lights are turned off in the DL (PM) regime (Dark/Light cycle) (Fig. 2.4A). Confrontations were kept in their respective photoperiod cycles for 7 days, after which Trichoderma's strains overgrowth on Botrytis strains was evaluated.

In TaWT-B05.10 interactions under LD cycles, Trichoderma's overgrowth was enhanced at dawn but not at dusk, as overgrowth was greater for plates inoculated at dawn ("AM" protocol), rather than at dusk ("PM" protocol) (Fig. 2.4B&C, Supplementary Table II**).** The Δ*tafrq* – B05.10 interactions behaved similarly as TaWT-B05.10, suggesting that TaFRQ does not cause the differential mycoparasitic behavior seen in the light: dark cycles (enhanced capacity under the AM inoculation protocol). In contrast, Δ*blr1* was capable of overgrowing WT Botrytis to the same extent in both AM and PM inoculation protocols, suggesting that indeed, BRL1 has a role in the differential outcome of interactions performed in LD cycles (Fig. 2.4B&C, Supplementary Table II**)**.

Centering the analysis on Botrytis, Δ*bcfrq1* was overgrown by all *Trichoderma* strains to the same extent in both cycles (AM or PM), revealing the disappearance of the circadian phenotypes observed for B05.10. Thus, *frq*-mutants in Trichoderma and Botrytis display major differences regarding their behavior, in which the absence of TaFRQ does not alter the time-of-the-day difference in mycoparasitic interactions (against B05.10 and Δ*bcwcl1* strains), whereas the absence of BcFRQ1 does, suggesting a

major role for the latter in the outcome of the circadian interactions. Regarding Δ*bcwcl1*, it mimics the differences seen for B05.10, but being more aggressively overgrown by *Trichoderma* in the AM protocol. In the aggregate, these results show that both, core clock components (WC and FRQ) and the time of the day in which the interaction is staged, affects the outcome of the fungal-fungal interactions, where the Botrytis (BcFRQ), but not the Trichoderma (TaFRQ) negative element appears as critical.

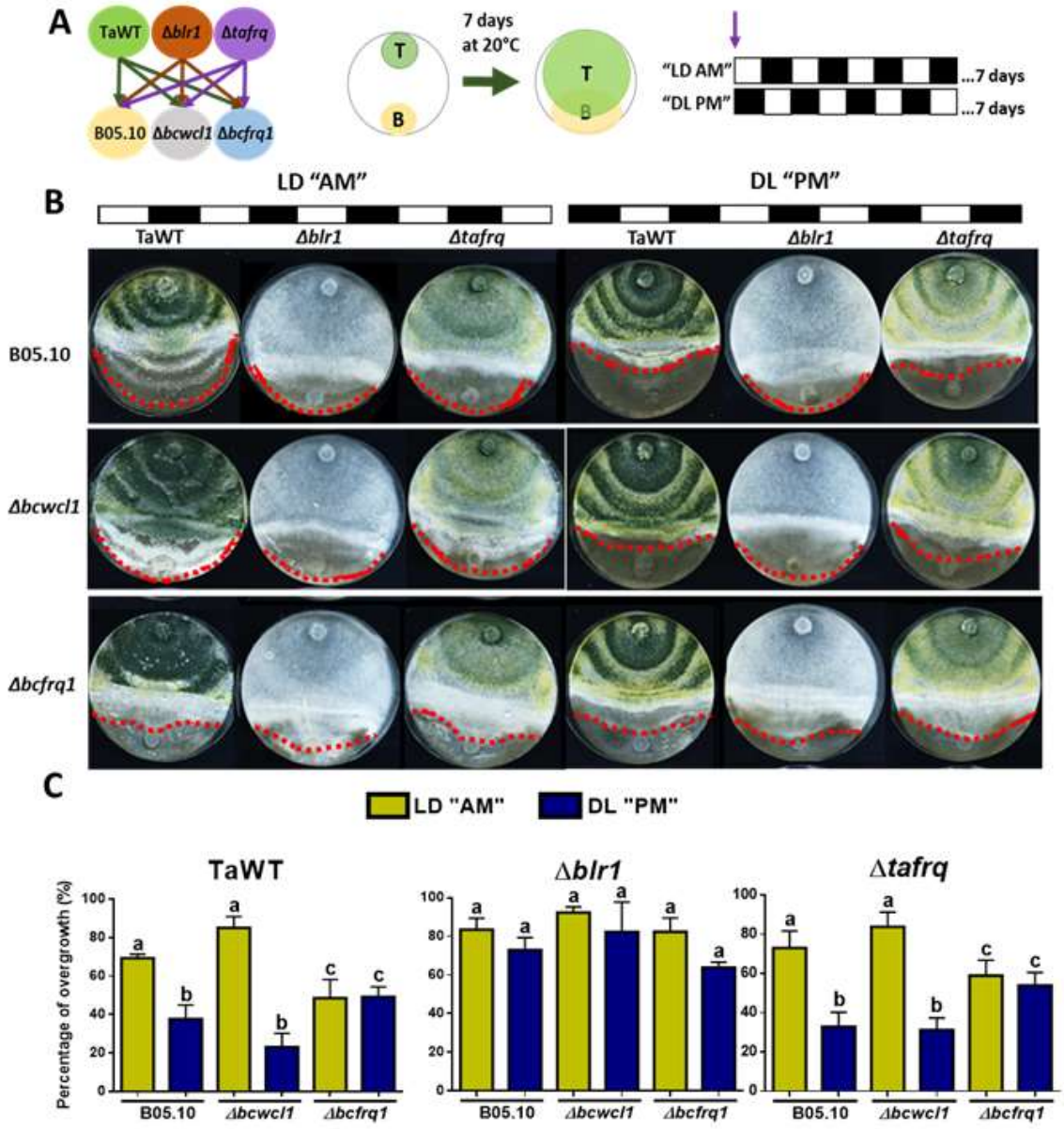

Fig. 2.4. Core clock components of *B. cinerea* and *Trichoderma* have a role in the differential mycoparasitic interaction observed under LD cycles. a. Schematic representation of the experimental design (left) and confrontation assays (right) in which fungal strains were inoculated in PDA at the beginning (dawn) of a light: dark (LD or AM) cycle or at the beginning (dusk) of a dark: light (DL or PM) regime. In both cases, cultures were kept at 20°C during 7 days under the corresponding light regimes. **b.** After 7 days of cultivation, *T. atroviride* overgrowth was evaluated. Red dotted lines indicate the edge of *Trichoderma* over *B. cinerea*. A representative image of three biological replicates is shown. **c.** Percentage of *B. cinerea's* colony area covered by *Trichoderma* (percentage of overgrowth). Error bars represent the means ± SD of 3 biological replicates. Different letters indicate significant differences based on Tukey's test ($p < 0.05$).

Secondary metabolism is affected in clock mutant strains of *T. atroviride*

Trichoderma species are prolific producers of secondary metabolites (SM) that have been implicated in the effectiveness of mycoparasitism against pathogens (Reino et al., 2008). To identify if core clock components could affect SM production, we carried out a metabolic fingerprinting to obtain an overview of global SM produced by TaWT and core-clock components mutant strains under LL, DD and after a 4 h. light pulse.

Extracts of diffusible compounds were analyzed by direct injection to MS. The production of 230 compounds were found to be statistically different among strains and conditions. The overview of diffusible compounds produced by the TaWT, Δ*blr1*, *Δtafrq* and OE::*tafrq* in DD, LL and LP (Fig. 2.5A) indicates that production of diffusible compounds is particularly enhanced in *Δtafrq* in all conditions compared to TaWT, Δ*blr1* and OE::*tafrq*. In TaWT, 103 and 15 molecules showed a a 2-fold induction in LL and LP compared to DD, respectively, which suggests that light induces production of diffusible compounds in *T. atroviride.* Comparison of diffusible compound production between TaWT and Δ*blr1*, showed that in the mutant strain 129 molecules were at least 2-fold reduced in LL, meanwhile a large number were induced after a LP and a few others in DD (Table I). Among the diffusible compounds, the molecule 1679.08 m/z had an interesting behavior between strains and light conditions. In TaWT, this molecule had the greatest fold induction in LL and in LP compared to DD. On the contrary, in Δ*blr1*, its production was reduced in LL, but it was induced in DD compared to TaWT (Fig. 2.5B). This suggests that BLR1 is necessary for light-induction of several diffusible compounds in TaWT, including 1679.08 m/z, as their production is severely dampened in the Δ*blr1* mutant in LL. However, it seems that a subset

of diffusible are either repressed in DD by BLR1 and/or additional photoreceptors are involved in light-induction of diffusible after a LP in Δ*blr1*.

Table I. Induced and repressed diffusible and volatile compounds in each mutant strain compared to TaWT in DD, LL and LP.

	Difussibles*						VOCS**					
	DD		LL		LP		DD		LL		LP	
	I	R	I	R	I	R	I	R	I	R	I	R
Δ*blr1*/TaWT	26	3	-	129	66	-	-	5	17	-	-	-
Δ*tafrq*/TaWT	67	-	137	2	218		23	13	16	1	10	11
OE::*tafrq*/TaWT	34	-	38	-	128	-	-	1	-	-	10	-

I=Induced, R=Repressed
*Diffusible compounds with at least 2.0-fold change
**Volatile compounds with at least 1.5-fold change

As mentioned, diffusible production is enhanced in *Δtafrq,* with several molecules with at least a 2-fold induction compared to TaWT. The production of some molecules like 381.77 m/z, downregulated in TaWT and Δ*blr1,* but enhanced in *Δtafrq* (Fig. 2.5A & B, Table I). This suggests that TaFRQ plays an extra-circadian role, repressing production of diffusible compounds under these conditions. On the contrary, TaFRQ could also have a positive role in the light-mediated biosynthesis of molecule 1679.08 m/z, which is one of the few downregulated compounds in *Δtafrq* in LL. Despite our findings, we did not observe a correlation between enhanced production of diffusible compounds with enhanced overgrowth of Trichoderma' strains, as *Δtafrq* has a weak overgrowth behavior (Fig. 2.3). Finally, production of the detected compounds in OE::*tafrq* is also upregulated in the 3 experimental conditions compared to TaWT, but less than in *Δtafrq* (Table I). This shows that the absence of a functional clock due to overproduction of TaFRQ (even in LL where the fungal clock is not functional) causes an upregulation of SM, reinforcing the idea of extra-circadian roles for TaFRQ in secondary metabolism.

Taken together, these analyses show that light induced the production a group of diffusible compounds and that BLR1 and TaFRQ have different/opposite roles in the regulation of such secondary metabolites.

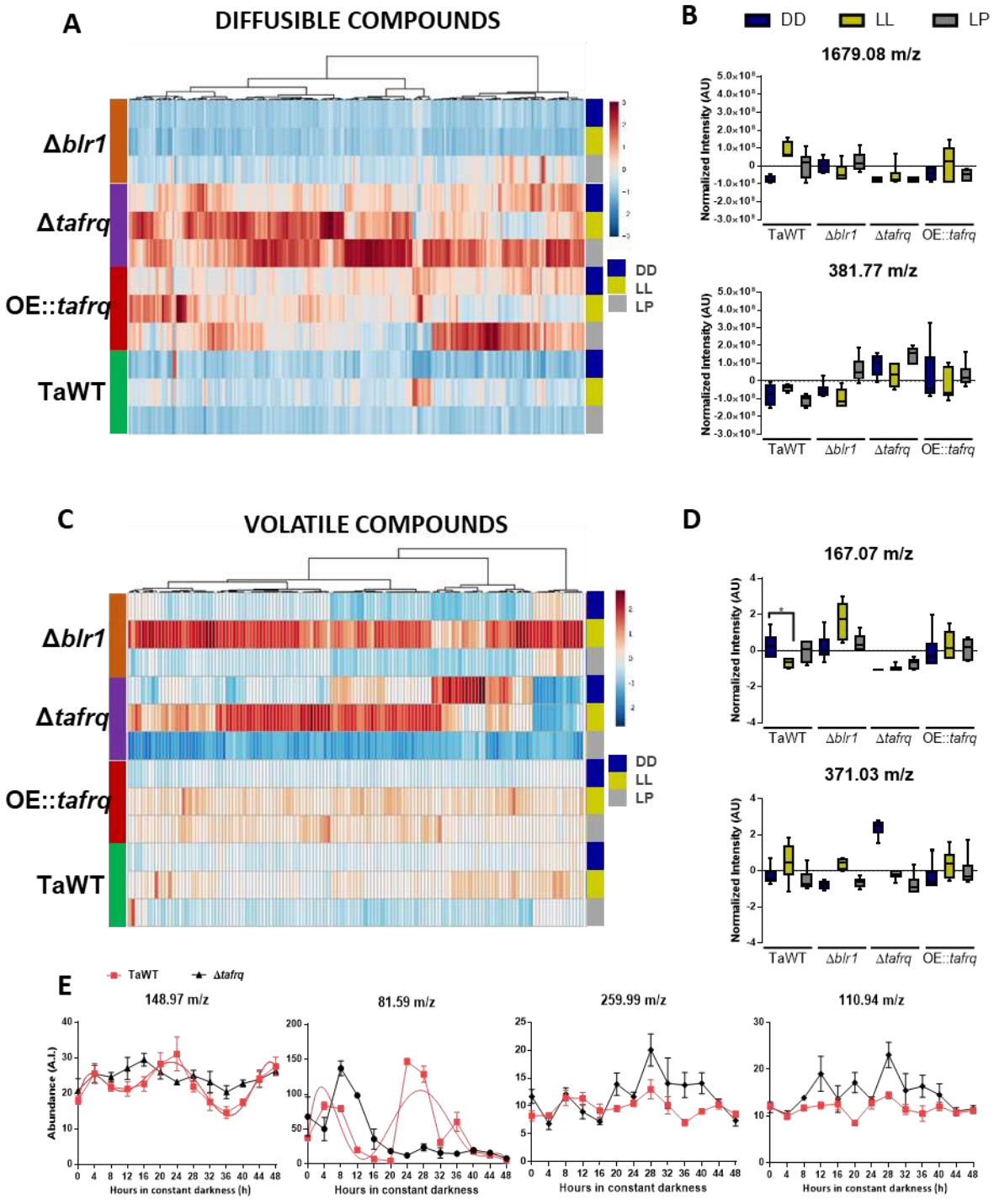
A
DIFFUSIBLE COMPOUNDS
Δblr1
Δtafrq
OE::tafrq
TaWT
DD
LL
LP
B
DD
LL
LP
1679.08 m/z
Normalized Intensity (AU)
TaWT
Δblr1
Δtafrq
OE::tafrq
381.77 m/z
C
VOLATILE COMPOUNDS
D
167.07 m/z
371.03 m/z
E
TaWT
Δtafrq
148.97 m/z
81.59 m/z
259.99 m/z
110.94 m/z
Abundance (A.I.)
Hours in constant darkness (h)
Hours in constant darkness

Fig. 2.5. Secondary metabolism is regulated by light conditions and *T. atroviride* core clock components. Global overview of secondary metabolites produced by TaWT and clock mutant strains grown in LL, DD and after a light pulse (LP). **a.** Diffusible compounds are enhanced in Δ*tafrq*. **b.** Normalized intensity values for two particular diffusible compounds is shown: 1679.08 m/z is light induced in TaWT and light repressed in *Δblr1* and *Δtafrq*; 381.77 m/z showed enhanced production in *Δtafrq* and OE::*tafrq* in all culture conditions. **c.** Volatile compounds are induced by light in Δ*blr1* and Δ*tafrq*. **d.** Normalized intensity values for two VOCs: 6-PP (167.07 m/z) and 371.03 m/z. **e.** Circadian levels of ions 148.97 m/z and 81.59 m/z in TaWT, but arrhythmic in *Δtafrq*. Compounds 255.99 m/z and 110.94 m/z do not oscillate in neither strains.

As diffusible production does not clearly correlate with the biocontrol abilities of Trichoderma's strains, we also analyzed the volatile organic compounds (VOCs) profile, by measuring their production *in vivo* with a Low-Temperature Plasma (LTP) ionization MS (LTP-MS) under the same conditions. The analysis revealed that the production of 131 compounds, in a 50-500 m/z range, were statistically different across all strains and conditions. A global view of VOCs shows an enhanced production in LL in *Δblr1* and *Δtafrq,* but a group of VOCs are particularly induced in *Δblr1* and reduced in *Δtafrq*. On the contrary, in DD, a large group of volatile compounds are upregulated in *Δtafrq* and downregulated in *Δblr1* (Fig. 2.5C).

As volatile compounds showed higher dispersion and narrow range of variation, we analyzed significative molecules with 1.5-fold change between TaWT and mutant strains. Comparison between Δ*blr1* and TaWT, detected light-induced as well as dark-repressed compounds (Table I) in Δ*blr1*. In Δ*tafrq,* several compounds were induced in DD, LL and LP compared to TaWT, but also some repressed compounds were observed in the 3 conditions in Δ*tafrq* (Table I). In *Δtafrq,* the compound 371.03 m/z had the highest production in DD compared to TaWT (Fig. 2.5D). No significative differences were found between OE::*tafrq* and TaWT, except for enhanced production of few compounds after a LP in OE::*tafrq* (Table I).

In TaWT, few VOCs showed differences between light conditions, being one of them the *Trichodermas's* well-known antifungal volatile compound 6-PP (molecule 167.07 m/z). This compound showed a 3-fold induction in DD compared to LL, suggesting an inhibitory effect of light in 6-PP production (Fig. 2.5D). In Δ*blr1,* 6-PP production showed a 2.7-fold induction in LL, but no differences were observed in DD and LP compared to TaWT. A

possible explanation is that BLR-1 could act as a repressor of the production of 6-PP in LL (which is consistent with the light repression of this molecule observed in TaWT and the augmented production in LL in Δ*blr1*) and that other photoreceptors could be involved in the activation of 6-PP production in LL in Δ*blr1* (Fig. 2.5D). Interestingly, a dramatic reduction of 6-PP production was observed in *Δtafrq* in DD, LL and LP (61, 10 and 3 negative fold change, respectively) (Fig. 2.5D). These results clearly show that the profile of volatile compounds is also broadly altered in *Δtafrq*. The differences found in VOCs, and particularly 6-PP production, in light conditions and strains correlates to the different biocontrol capacities of each strain in LL and DD.

Finally, we performed a circadian metabolic profile measuring VOCs every 4h. during 48 h. in DD conditions for both TaWT and Δ*tafrq* strains in GYEC+peas plates. Periodicity and rhythmicity of VOCs levels was calculated using BioDare (with FFT NLS and BD2eLTK cycle algorithms with a p-value < 0.05), where 17 compounds exhibited a circadian behavior with a calculated period between 24 and 26 h. in TaWT (Supplementary Table III). In Δ*tafrq*, 4 out of the 17 compounds defined as rhythmic in TaWT (63.83 m/z, 90.83 m/z, 388.87 m/z and 389.77 m/z) also showed oscillatory behavior in some of their replicas (see Supp. Table III). However, after visual inspection of their dynamics in Δ*tafrq* it was obvious that they failed to exhibit true circadian behavior (Supp. Fig. 2.11 & Supp. Table III), so we decided to include them in the final list of rhythmic compounds in TaWT. The circadian abundance of 2 ions (148.97 and 81.59 m/z) and 2 arrhythmic ions (259.99 and 110.94 m/z) are depicted in Fig. 2.5E. *In toto*, these results suggest that the production of some VOCs in *T. atroviride* is under circadian control.

2.5 Discussion

While putative core-clock components encoding genes can be found in fungi distributed in several orders (Montenegro-Montero et al. 2015; Salichos and Rokas 2010), the phenotypic description of circadian phenomena in them has been less common and the molecular characterization of those rhythms has been even scarcer. One of the few examples (besides *N. crassa*) has been the characterization of the core-clock in *B. cinerea* and its effect on its virulence (Hevia et al., 2015). In our efforts to further understand the role of circadian clocks in a fungus-fungus interaction, herein we provide evidence of a functional clock in the mycoparasitic fungus *T. atroviride*. TaFRQ^{LUC} reporter strains revealed circadian oscillations with a free-running period of ~26 h., which could be entrained by LD cycles and reset by light and temperature pulses, consistent to what has been described in Neurospora and other organisms (Liu et al., 1998). These oscillations showed temperature compensation between 20°-25° C where, notably, the best rhythms in terms of amplitude were observed at 22°C, whereas they were lost at 28°C. While such narrow range of temperatures that allow clear visualization of these molecular rhythms may be puzzling, it might as well be associated with the environment in which *Trichoderma* is usually found -soil- in which temperature shifts are overall buffered (Rodriguez-Romero et al., 2010). Interestingly, we found evidence of strong functional conservation of clock regulatory motifs between Neurospora and Trichoderma, as the *c-box* sequence from the former fungus is recognized in the latter. The high conservation of WC-1 and BLR1 DNA binding domains (Casas-Flores et al. 2004; Cervantes-Badillo et al. 2013; Froehlich et al. 2002), indicate that they are expected to recognize a similar motif considering general rules of eukaryotic transcription factor sequence specificity (Weirauch et al., 2014), and allow predicting the presence of a *c-box*-

like element in the *tafrq* promoter. Regrettably, we did not observe oscillations of the additional luciferase transcriptional reporters of potential *T. atroviride ccg*s, which could be due to *i*) the lack of circadian transcription of the assayed genes, *ii*) the selected promoters failed to capture elements necessary for their circadian control or, *iii)* the assay conditions were not appropriate to visualize their rhythmic expression (see below). Further analysis of the transcript levels of those genes will be performed under different culture conditions in order to confirm whether their expression is eventually circadian.

One of the striking findings in our study was the strong influence of culture media composition in allowing the visualization of molecular circadian oscillations. Indeed, rhythms in TaFRQ^{LUC}, or $_{Nc}$*c-box-luc* levels, were only observed in GYEC media, which were further improved when plant-derived tissue was included. While the impact of nutrient availability in certain aspects of circadian rhythms has been observed from mammals to *N. crassa* (Bae et al., 2019; Oosterman et al., 2015), our results indicate an even bigger dependence of culture conditions in allowing the correct visualization of the Trichoderma clock. Thus, although the quality and characteristics of *frq* and clock-controlled genes can vary, depending on media characteristics in Neurospora (Díaz & Larrondo, 2020; Dunlap & Loros, 2017; Hurley et al., 2014; Larrondo et al., 2015; Olivares-Yañez et al., 2016) we were surprised to observe such a dramatic effect in Trichoderma: that is, an almost "conditional" media-dependent rhythmicity. Albeit currently we ignore the reasons for the latter, we believe that this may serve as a cautionary tale for other attempts aiming to characterize circadian rhythms in different fungi. Having had limited our analysis to a small set of media and higher temperatures we would have been prone to conclude that there were no rhythms in Trichoderma TaFRQ levels. Yet, hitting the right conditions allowed us confirming their existence and describing the idea of a functional clock in this organism.

In *T. atroviride* light stimulates growth and conidiation, and these processes have shown to be dependent on the carbon source. Moreover, it has been reported that BLR1 has critical role in carbon-source selectively of these processes (Friedl et al. 2008a; Friedl et al. 2008b). Additionally, it has been described the importance of organic nitrogen source in culture media composition for mycoparasitic behavior in fungi (Danielson & Davey, 1973; Nolan & Nolan, 1972). Also, C:N ratios are a relevant environmental factor influencing conidiation in Trichoderma (Steyaert et al., 2010c) and that the addition of plant tissue improves the biocontrol activity of *Trichoderma* species (El-Nagdi et al., 2019). These antecedents prompt us to hypothesize that nutrients found in GYEC media (high amounts of organic nitrogen derived from casamino acids and yeast extract, absent in the other media used, plus peas) to some extent, may mimic the composition of *Trichoderma's* natural niche, in which different nitrogen sources derived from its preys are available, as well association with plants provide different sugars (Druzhinina et al. 2011; Steyaert et al. 2010c). These findings reinforce the idea of the importance of defined nutrient/temperature conditions to monitor the clockworks, and could help future fungal circadian studies in visualizing otherwise weak oscillations, such as in *Pyronema confluens* and *Ophiocordyceps kimflemingiae* (de Bekker et al., 2017; Traeger & Nowrousian, 2015). This could also help monitoring *frq* molecular oscillations in fungi where previous attempts have failed, like in *Verticillium dahliae, Aureobasidium pullulans*, and *Magnaporthe oryzae* (Cascant-Lopez et al., 2019; Deng et al., 2015; Franco et al., 2017).

While *frq* in Neurospora does not have a described role outside the pacemaker (Aronson et al. 1994), in *B. cinerea* it exhibits extra-circadian roles associated with developmental programs (Hevia et al., 2015). In *T. atroviride* we observed that the core clock components play a role in development, secondary metabolism and mycoparasitism. TaFRQ

is critical for conidia formation after a light pulse and mechanical damage, showing a dramatic reduction of conidiation in the later. These findings, along with those found in *B. cinerea*, opens new questions regarding the role in developmental decisions that FRQ has acquired or lost across the fungal kingdom and moreover, raises questions of the extra-circadian role of negative elements of circadian clocks in other organisms. In fungi, little is known about FRQ besides Neurospora and Botrytis, and a recent study of *frq* in the phytopathogen *V. dahlia* found no major phenotypic differences in the deletion strain regarding sporulation and microsclerotia formation in DD or LD (Cascant-Lopez et al., 2019). In contrast, we found altered developmental responses and enhanced production of both diffusible and volatile compounds in Δ*tafrq*, suggesting a general repressor regulatory role of TaFRQ in secondary metabolism. Interestingly, the antifungal compound 6-PP, a key feature of Trichoderma species, was dramatically reduced in the Δ*tafrq* strain even in LL, a condition where clock function is expected to be abrogated, suggesting a potential extra-circadian activator role of TaFRQ in 6-PP production.

Secondary metabolites have been related to developmental programs in fungi and could have a role in response to damage in *T. atroviride* (Atriztán-Hernández et al., 2019; Schmoll et al., 2010; Tisch & Schmoll, 2010). In fact, reports show that after injury and fungivory, a group of VOCs, including 6-PP, increase in TaWT but not in the MAPK mutant *Δtmk3* (Atriztán-Hernández et al., 2019), which shows a dampened conidiation after injury as was seen for Δ*tafrq*. This suggests that reduced 6-PP production in Δ*tafrq* could be behind its dramatic reduction on injury-induced conidiation. Additionally, oxylipin production, previously suggested as involved in signaling after damage, could be dampened in Δ*tafrq* (Medina-Castellanos et al., 2014). On the contrary, Δ*blr1* showed repressed production of several compounds (diffusible and VOCs), suggesting a role of light-perception in the

activation of secondary metabolism. This is consistent to the light-induced production of several compounds in TaWT. Interestingly, Δ*blr1* produced higher amounts of 6-PP in DD and even in LL, whereas in TaWT 6-PP production is inhibited by light. This suggests a repressor role of light and BLR1 in the production of this molecule (and also some diffusible compounds as seen for 1679.08 m/z) and that other photoreceptors could activate its production in the absence of BLR1 (García-Esquivel et al., 2016). Similarly, light-repression effect on 6-PP production was observed in *T. atroviride* P1 and IMI 206040 strains, when 6-PP production was analyzed under LD and DD conditions (Speckbacher et al., 2020a) and when grown in different light-wavelengths (blue, green, yellow and red) (Moreno-ruiz et al., 2020). Light regulation of secondary metabolites has been reported in fungi (Tisch & Schmoll, 2010) as well the involvement of WC-1 homologue in *C. zeae-maydis* as a repressor of cercosporin production in DD (Kim et al., 2011). The involvement of TaFRQ and BLR1 in the production of secondary metabolites in *T. atroviride* (or FRQ of any other fungus) has not been reported to date.

In addition to the circadian oscillations of $TaFRQ^{LUC}$, we observed rhythmic levels of volatile secondary metabolites in *T. atroviride* under constant conditions (DD), that was absent in *Δtafrq*, suggesting a *bona fide* circadian control of these volatile compounds. The identity of the circadian compounds (and some of those found in the light/dark experiments) is unknown and would be of great interest to dive into their chemical identification and their specific role in *T. atroviride's* lifestyle. This reinforces the idea of the importance of the environmental perception (including time) in *Trichoderma*'s biology, in particular, in the production of secondary metabolites that is a key feature of this fungus, and that is involved in several processes such as development and mycoparasitism. On the contrary, while studies in *Neurospora* have systematically shown the influence of the clock on the transcriptome,

and the proteome (Hurley et al., 2014, 2018; C. Sancar et al., 2015), it has not been reported so far, how daily rhythms impact the production of different compounds. Temporal compartmentalization of metabolism at large has been described in *Neurospora*, with catabolic and anabolic reactions tending to occur at dawn and dusk, respectively (Hurley et al. 2016). Our work contributes presenting proof of concept that fungal circadian control can also impact the production of secondary metabolites. These studies, and future comprehensive analyses of circadian metabolic profiles in classic models such as *Neurospora*, and other fungi, will help bridging the gap on how clock regulation impacts the daily life of a fungus.

An interesting role of core clock components and light-dark conditions in the outcome of the mycoparasite interaction between *T. atroviride* and *B. cinerea* was found. The *Δblr1* strain showed the strongest mycoparasitic behavior, meanwhile *Δtafrq* did not show major differences to TaWT in most interactions, except a slightly weaker mycoparasitic behavior against *Δbcwcl1* and *Δbcfrq1* in LL. The antagonistic capacity of *Trichoderma* species is correlated with their ability to produce 6-PP (Reino et al., 2008). The *Δblr1* strain produces higher amounts of this molecule whereas, in *Δtafrq* its production is severely dampened, which is consistent with their different mycoparasitic abilities. The antagonist capacity of *Trichoderma* was reduced in LL, which in turn was enhanced in darkness and this could be due to differential metabolites production found in LL and DD. Similar inhibitory effects of light in mycoparasitism in *T. atroviride* have been reported against *Fusarium oxysporum* (Moreno-ruiz et al. 2020; Speckbacher et al. 2020b), along with differential VOCs production upon illumination during the confrontation assay. High amounts of 2-heptanone is produced during confrontation against *F. oxysporym* in a light-dependent manner that negatively correlates with *Trichoderma* antagonism, which has led to

propose that 2-heptanone production was a result of a stress response elicited by *F. oxysporum* in the light.

Finally, mycoparasite interactions under a circadian paradigm, showed enhanced overgrowth of TaWT and Δ*tafrq* over B05.10 and Δ*bcwcl1* when interactions started at dawn under LD cycles, in contrast to a reduced overgrowth at dusk. We have previously observed differential interactions in the context of *B. cinerea* and *A. thaliana*, where fungal virulence was enhanced at dusk (Hevia et al., 2015). The differential effect on the Trichoderma-Botrytis dynamic was lost when Δ*blr1* was used against all Botrytis strains, a similar phenomenon was observed by Hevia et al., 2015, when *A. thaliana* was infected with Δ*bcwcl1*, which did not show a circadian behavior. Disruption of BLR1 abrogates both light and circadian responses, so the effect observed for Δ*blr1* could be also due to a light-driven phenomena (besides a circadian effect) because the absence of the blue-light perception annuls the differential effect observed in TaWT. Yet, it is noteworthy that Δ*tafrq* yields results comparable to what is seen in TaWT, suggesting that the time-of-the-day effect we observe does not appear to depend strictly on the Trichoderma clock.

On the other hand, under LD cycles the loss of BcWCL1 does not considerably affect the dynamics between both fungi, whereas for Δ*bcfrq1* the differential outcome of the daily interaction is completely absent. Lack of BcFRQ1 implies that time perception in *Botrytis* is abrogated and therefore the circadian outcome of the interaction is lost an effect that, nevertheless, is not seen when Trichoderma losses TaFRQ. This suggests that the *Botrytis* clock is critical in the outcome of the interaction with tested organisms – both plants and fungi – suggesting a complex mechanism in which light and time perception interact giving rise to the observed phenotypes. Even though we lack a potential mechanism underlying this phenomenon and integrating the role of the different clock/light-perception components, this

it is the first description of an interplay of core and light regulation in a mycoparasite interaction. If we consider that in nature organisms live under light/dark transitions, this differential attack-resistance behavior depending on the time (or the light/darks cycles) at which the assays are established could have an impact on biocontrol strategies in agriculture, optimizing the application of *Trichoderma* in crops.

Acknowledgments

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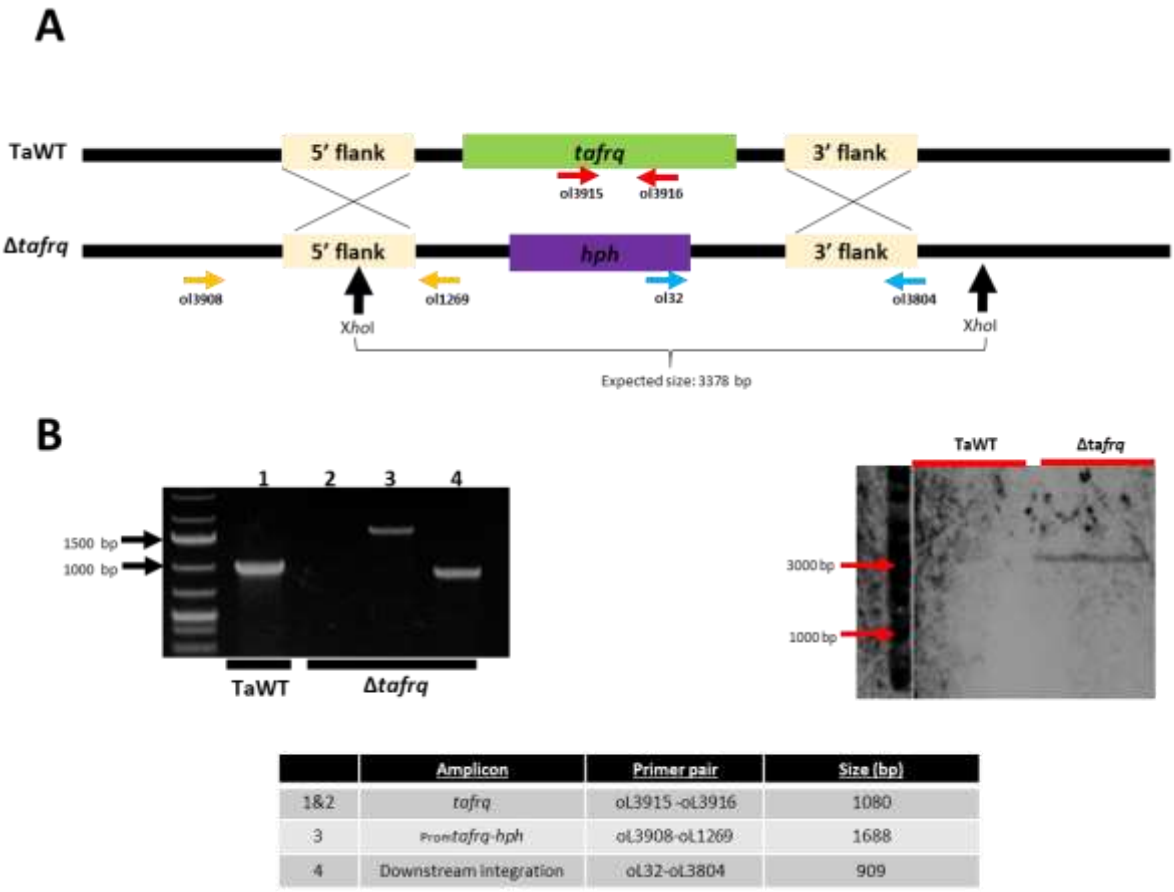
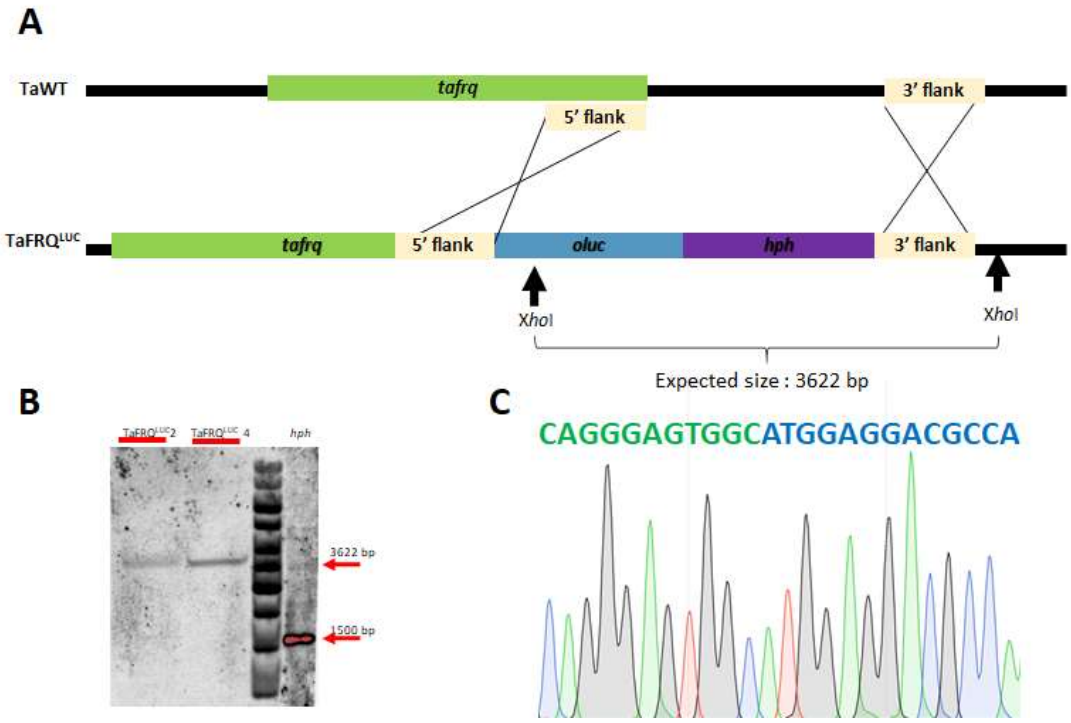
2.6 Supplementary information

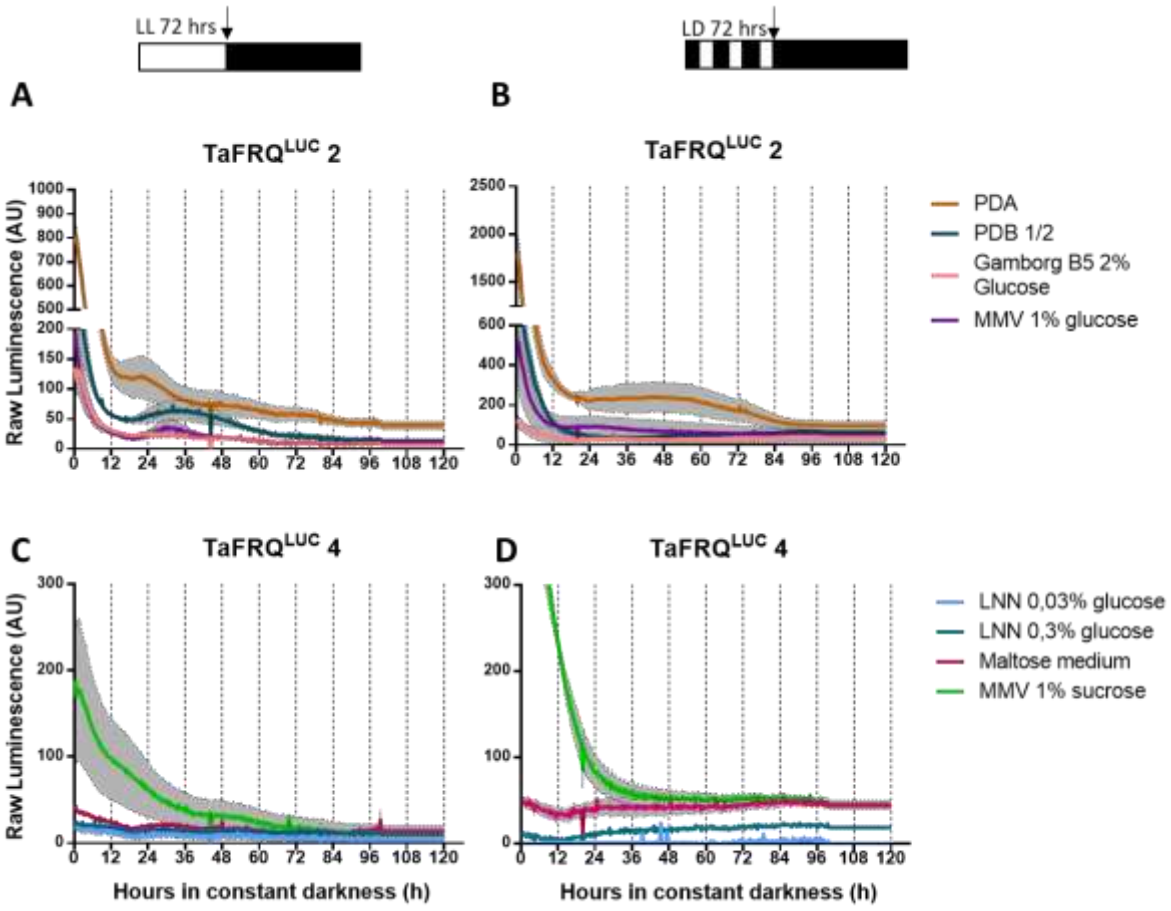


	Amplicon	Primer pair	Size (bp)
1&2	*tafrq*	oL3915 -oL3916	1080
3	Prom*tafrq-hph*	oL3908-oL1269	1688
4	Downstream integration	oL32-oL3804	909

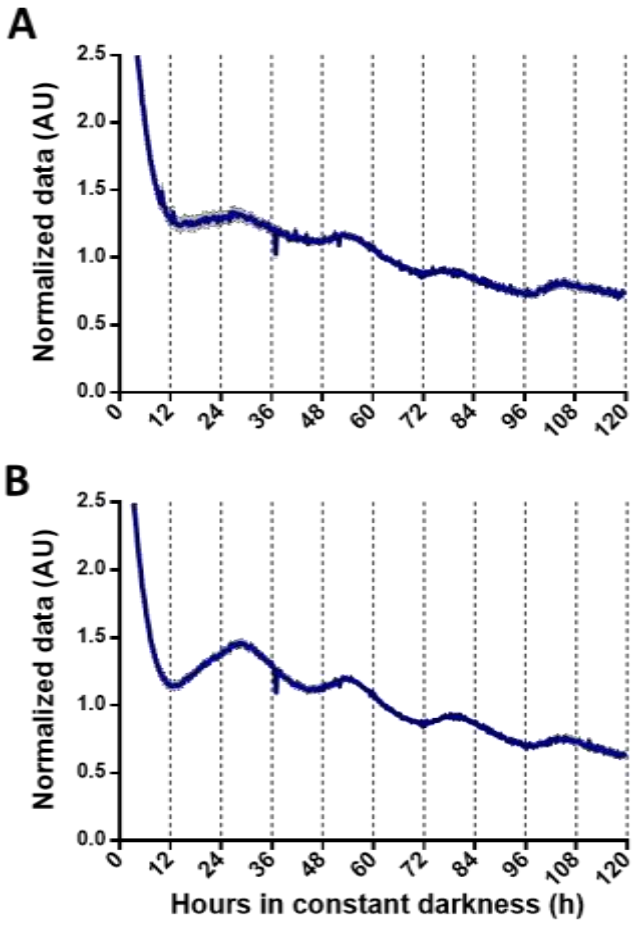
Supp. Fig. 2.1. Generation of Δ*tafrq* strain in *T. atroviride*. a. Representation of the *tafrq* locus and the replacement with the *hph* cassette by homologous recombination. Arrows (horizontal) show primers used for diagnostic PCRs, indicating their relative position and orientation. Black arrows show expected *XhoI* restriction sites used for southern blot. **b.** Diagnostic PCR showing integration (left) and southern blot analysis (right) of both correct and single copy integration of the replacement cassette. The *hph* amplicon was used as a probe. The table at the bottom shows expected PCR amplicon size (lanes indicated in the first column).



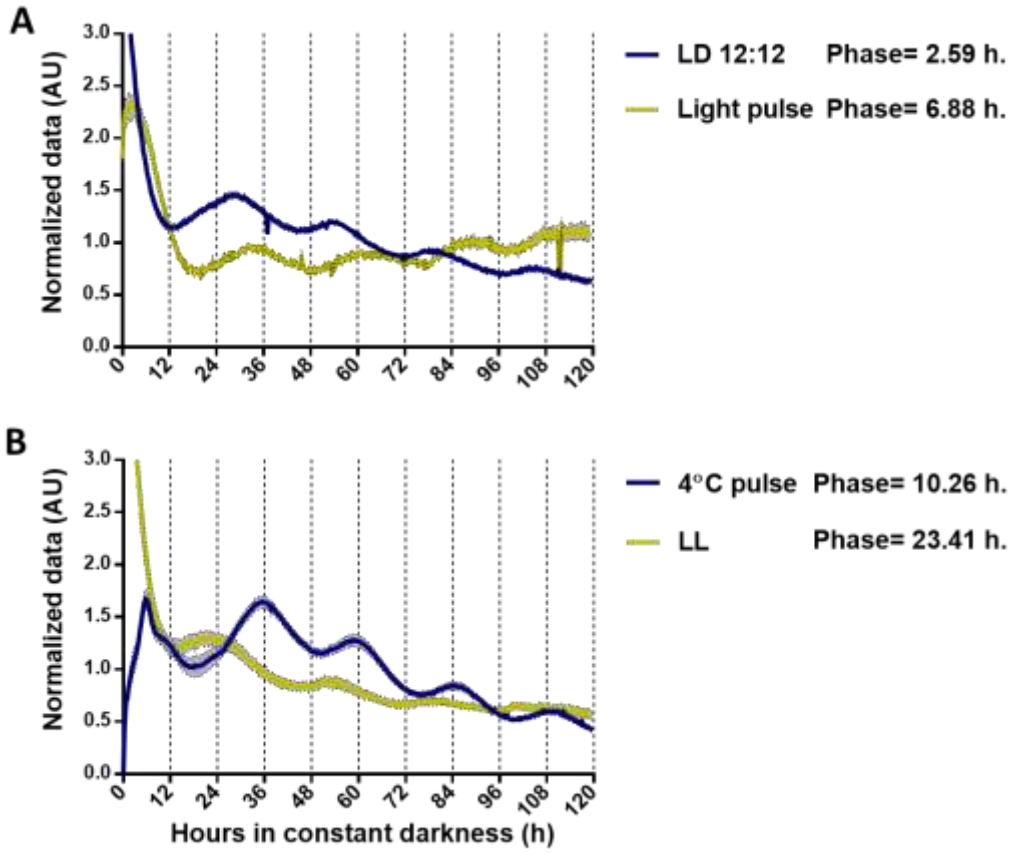
Supp. Fig. 2.2. Generation of TaFRQ^{LUC} strains. a. Representation of the homologous recombination strategy used for the integration of *luc* yielding TaFRQ^{LUC} at the endogenous locus. Black arrows show expected *XhoI* restriction sites used for southern blot. **b.** Southern blot analysis showing both correct and single copy integration of the replacement cassette using *hph* as probe. **c.** Sequence chromatogram showing the correct fusion of *tafrq* (in green, without stop codon) and *luc* (in blue).



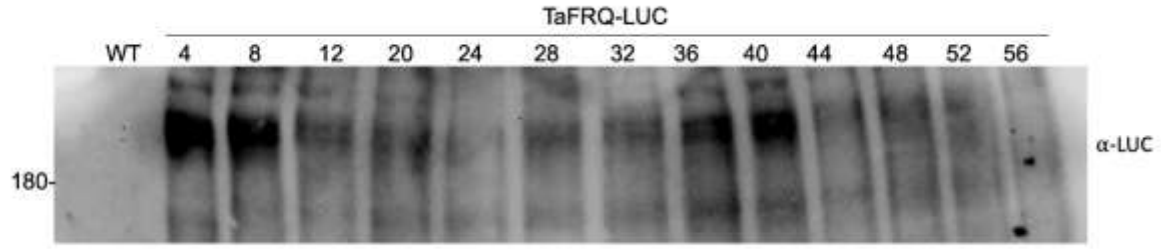
Supp. Fig. 2.3. Evaluation of TaFRQ^{LUC} oscillations in different culture media and entrainment protocols. TaFRQ^{LUC} reporter strains (2 and 4) received 2 treatments: each strain was cultivated first under LL culture conditions for 3 days to reset the clock (**a** & **c**) or entrained during 3 days under LD conditions (**b** & **d**), both at 25 °C. The schematic representation at the top of each panel denote the lights-on period and the lights-off period (white and black boxes, respectively). LUC acquisition of the TaFRQ^{LUC} reporter strains started at the beginning of the free-running conditions (DD, indicated with a vertical black arrow) monitored during 5 days at 25 °C. Eight different culture media were tested for each strain (indicated with different colors), but no oscillations were determined. Raw data is presented (SD is represented with grey area).



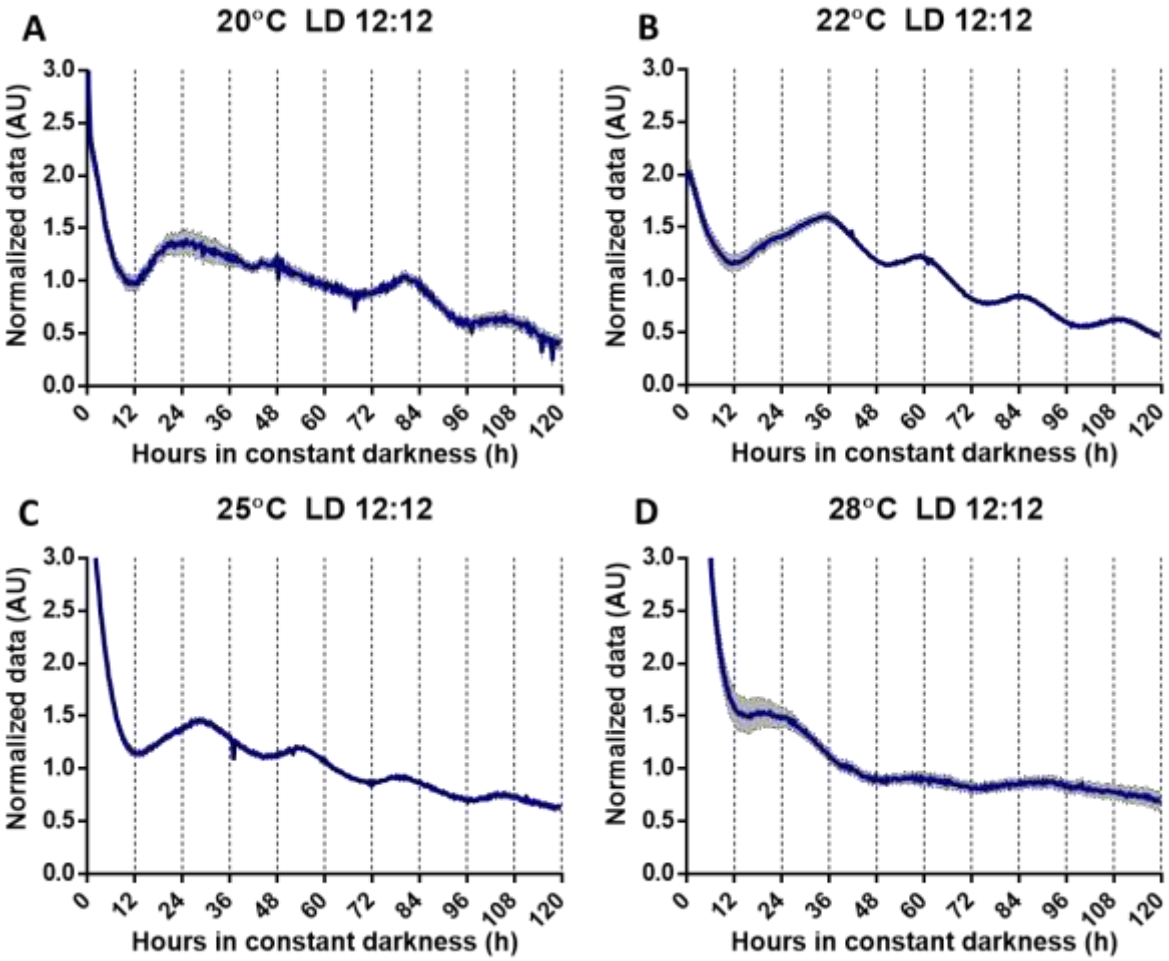
Supp. Fig. 2.4. Normalized data of TaFRQ^{LUC} oscillations in GYEC. TaFRQ^{LUC} reporter strains (TaFRQ^{LUC} 2 and 4) were entrained during 3 days under LD (12:12 h.) cycles at 25 °C. **a.** GYEC culture media. **b.** GYEC supplemented with 0.5% peas (w/v). In all experiments, LUC activity was monitored under DD at 25 °C for 5 days. Data was normalized to mean. Average of 2 biological replicates and 8 technical replicates is presented (SEM is represented with grey area).



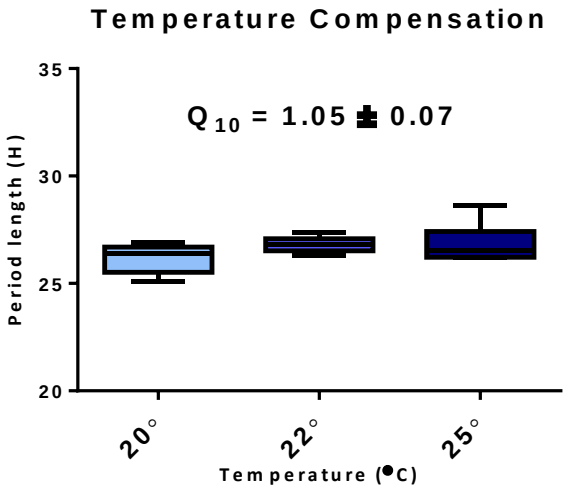
Supp. Fig. 2.5. Phase shift of TaFRQ^{LUC} oscillations after different light entrainment and reset conditions. **a**. TaFRQ^{LUC} reporter strains (TaFRQ^{LUC} 2 and 4) received LD 12:12 h. 25 °C entrainment (blue) and a 20 min. light pulse (yellow). A 4.29 h. phase shift is observed after a LP. **b.** TaFRQ^{LUC} received a 4 °C temperature pulse (blue) and 3 days at LL (yellow). A 10.85 phase shift is observed after the 4 °C temperature pulse compared to LL reset. Data was normalized to mean. Average of 2 biological replicates and 6-8 technical replicates is presented (SEM is represented with grey area). Phase shifts were calculated using the BioDare platform using first peak and circadian unit.



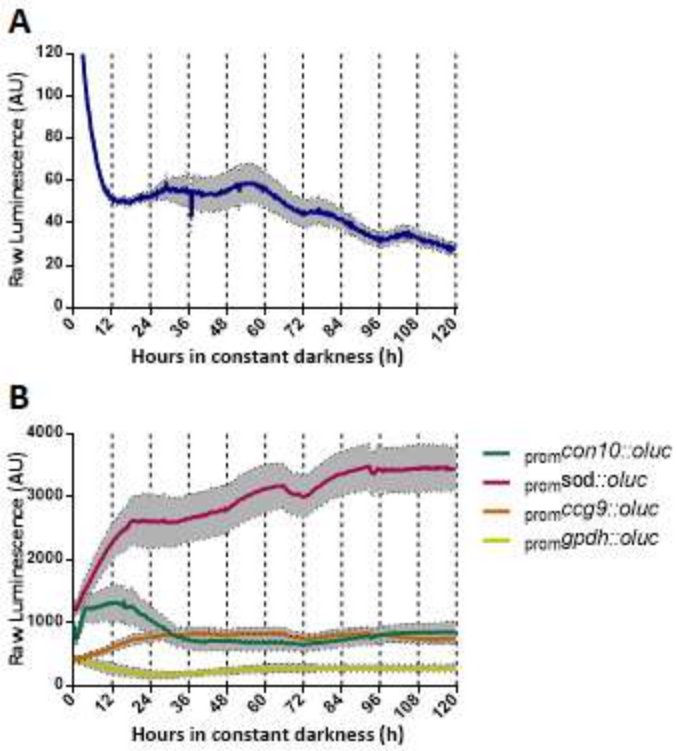
Supp. Fig. 2.6. TaFRQ^{LUC} protein oscillation under constant conditions. Oscillations of TaFRQ^{LUC} protein levels in DD during a 56 h. time course. Reporter strains were inoculated in GYEC+peas and received a light pulse as input to reset the clock and drive oscillations. Samples were taken every 4 hrs in DD at 22°C.



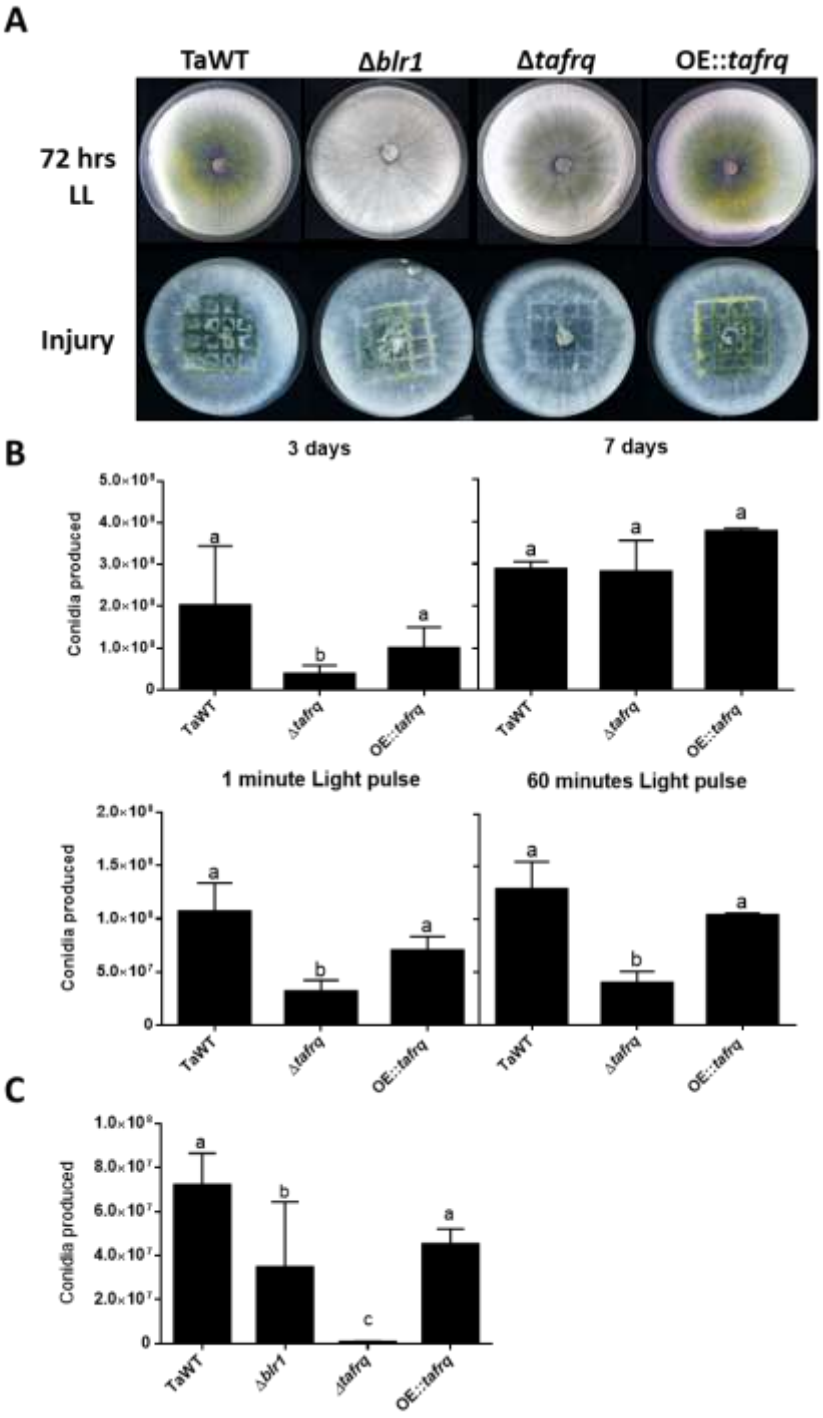
Supp. Fig. 2.7. TaFRQ^{LUC} oscillations observed at different temperatures demonstrate temperature compensation in *T. atroviride*. (a-d) TaFRQ^{LUC} reporter strains were entrained during 3 days under LD 12:12 h. cycles at 20, 22, 25 and 28 °C, respectively, and transferred to DD to the indicated temperatures. TaFRQ^{LUC} strains display low amplitude oscillations at 20 °C, robust oscillations at 22 and 25 °C, and no oscillations at 28 °C. In all experiments, LUC activity was monitored under DD at the given temperature, for 5 days. GYEC+peas culture media was used in all experiments. Data was normalized to mean. Average of 2 biological replicates and 8 technical replicates is presented (SEM is represented with grey area).



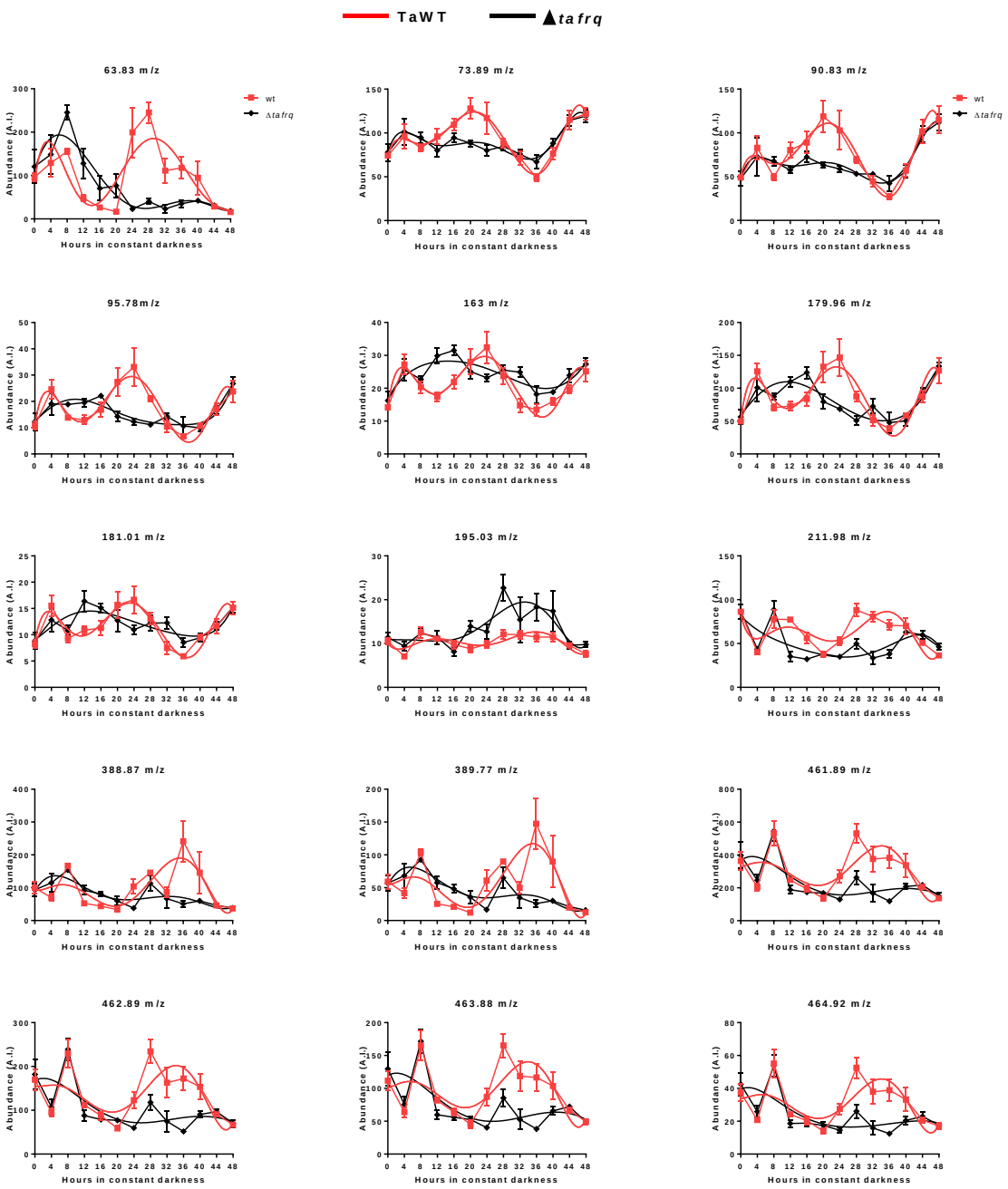
Supp. Fig. 2.8. Oscillations of TaFRQ^{LUC} compensate between 20 and 25°C. Average period of the 2 TaFRQ^{LUC} strains for each experimental condition (n=3). The mean Q_{10} value obtain for the 20-25 °C is presented.



Supp. Fig. 2.9. Evaluation of transcriptional luciferase reporters in *T. atroviride.* a. Oscillations of $_{Nc}$*c-box-luc* in *T. atroviride* in GYEC culture media after 3 days at LD 12:12 h. cycle entrainment. Raw data and mean values of 2 biological replicates with 3 technical replicates each is presented. **b.** Selected promoters from *T. atroviride* do no drive oscillations of *luc* under DD. Each transcriptional reporter strain was entrained during 3 days under LD (12:12 h.) conditions at 25° C in GYEC+peas culture media. Thereafter, LUC activity was monitored under DD at 25° C for 5 days (SEM is represented with grey area).



Supp. Fig. 2.10. Light and injury-induced conidiation is altered in clock mutant strains of *T. atroviride.* TaWT, Δ*blr1*, Δ*tafrq* and OE::*tafrq* strains were grown in PDA at 25 °C. **a.** Sporulation was observed at 72 h. in LL. **b.** Conidia produced at 3 and 7 days of growth and after a 1 and 60 min light pulse. **c.** Conidia produced after mechanical damage. Errors bars represent the mean ± SD of 3 biological for B and 4 for C replicates for each experiment. Different letters indicate significant differences based on Tukey's test ($p < 0.05$).



Supp. Fig. 2.11. Circadian production of volatile organic compounds (VOCs) in *T. atroviride*. VOCs were measured using LTP-MS. TaWT and Δ*tafrq* strains were inoculated in GYEC+peas media, entrained during 3 days in LD cycles and then transferred to DD. Samples were taken every 4 h. for 48 h. (N=6). Period and rhythmicity were calculated in BioDare platform (see Supplementary Table III) as described in the main text. A trend line is depicted in red.

Supp. Table I. Culture media used for *in vivo* luminescence assessment.

Culture media	Composition
PDA	Potato Dextrose Agar (Difco). 39 grs per liter.
PDB	Potato Dextrose Broth (Difco). 12 grs per liter.
Gamborg B5	2% glucose, 0.31625% Gamborg B5 (Duchefa Biochemie), 1.5% agar.
MMV 1% Glucose	1% Vogel's salts, 1% glucose, 1.5% agar.
LNN 0.03% Glucose	1% Vogel's salts, 0.03% glucose, 0.05% arginine, 50ug biotin,1.5% agar.
LNN 0.3% Glucose	1% Vogel's salts, 0.3% glucose, 0.05% arginine, 50ug biotin,1.5% agar.
Maltose	1% Vogel's salts, 0.5% maltose, 1.5% agar.
MMV 1% Sucrose	1% Vogel's salts, 1% sucrose, 1.5% agar.
GYEC	0.5% glucose, 0.3% yeast extract (Difco), 0.5% casaminoacids (Difco), 1.5% agar.
GYEC+peas	0.5% glucose, 0.3% yeast extract (Difco), 0.5% casaminoacids (Difco), 0.5% peas, 1.5% agar.

Supp. Table II. Mean overgrowth area of *T. atroviride* over *B. cinerea* in all confrontation assays performed.

	Condition	B05.10	Δ*bcwcl1*	Δ*bcfrq1*
TaWT	LL	19.29 ± 3.58	73.24± 14.03	23.55± 9.85
	DD	57.46 ±18.06	85.75± 8.06	59.76± 4.07
	LD AM	69.35± 2.06	85.09± 5.83	48.54± 9.69
	DL PM	37.65± 7.32	23.02± 7.29	49.21± 5.13
Δ*blr1*	LL	34.68± 17.38	33.04± 7.06	41.91± 12.52
	DD	89.86± 2.49	90.41± 3.57	79.35± 9.29
	LD AM	83.46± 5.95	92.25± 3.07	82.36± 7.20
	DL PM	72.86± 6.42	82.26± 15.48	63.85± 2.89
Δ*tafrq*	LL	7.32± 12.68	18.54± 6.93	0.00
	DD	50.27± 15.21	80.30± 1.48	62.25± 8.77
	LD AM	73.07± 8.63	83.83± 7.49	58.94± 7.88
	DL PM	32.76± 7.41	31.18± 6.19	53.88± 6.62

Supp. Table III. Circadian metabolites of *Trichoderma atroviride.* Period was estimated using FFT NLSS and rhythmicity was evaluated using BD2eJTK in BioDare platform.

TaWT						
Group Label	N	Period	Period Std	GOF	ERR	Rhythmic
63.83*	4	24.60	0.10	0.76	0.91	**TRUE**
73.89	5	25.14	0.46	0.64	0.69	**TRUE**
81.59	4	24.44	0.11	0.74	0.87	**TRUE**
90.83*	6	25.12	0.28	0.63	0.83	**TRUE**
95.78	5	24.34	0.50	0.54	0.58	**TRUE**
148.97	5	24.72	0.59	0.68	0.78	**TRUE**
163	4	24.53	0.32	0.63	0.8	**TRUE**
179.96	4	24.62	0.41	0.68	0.78	**TRUE**
181.01	4	24.60	0.38	0.74	0.77	**TRUE**
195.03	4	23.48	0.46	0.63	0.8	**TRUE**
211.98	4	24.07	0.74	0.7	0.79	**TRUE**
388.87*	3	24.00	NA	NA	NA	**TRUE**
389.77*	4	24.00	NA	NA	NA	**TRUE**
461.89	4	24.16	0.22	0.48	0.63	**TRUE**
462.89	4	24.21	0.19	0.62	0.76	**TRUE**
463.88	4	24.08	0.28	0.49	0.64	**TRUE**
464.92	4	24.31	0.30	0.45	0.66	**TRUE**
Δ*tafrq*						
Group Label	N	Period	Period Std	GOF	ERR	Rhythmic
63.83*	3	24.40	0.15	0.55	0.95	**TRUE**
73.89*	1	23.13	0	0.45	0.63	FALSE
81.59*	2	24.28	0.06	0.54	0.93	FALSE
90.83*	3	24.70	1.83	0.81	0.95	**TRUE**
95.78	0	NA	NA	NA	NA	FALSE
148.97	0	NA	NA	NA	NA	FALSE
163	0	NA	NA	NA	NA	FALSE
179.96	0	NA	NA	NA	NA	FALSE
181.01*	1	24.37	0	0.55	0.99	FALSE
195.03	0	NA	NA	NA	NA	FALSE
388.87*	4	24.00	NA	NA	NA	**TRUE**
389.77*	4	24.00	NA	NA	NA	**TRUE**
211.98	0	NA	NA	NA	NA	FALSE
461.89	0	NA	NA	NA	NA	FALSE
462.89	0	NA	NA	NA	NA	FALSE
463.88	0	NA	NA	NA	NA	FALSE
464.92	0	NA	NA	NA	NA	FALSE

*For those molecules in Δ*tafrq* that showed a period and/or rhythmicity according to BD2eJTK, we compared the graphics of those molecules in the mutant and TaWT strain.

Supp. Table IV. Period analysis of transcriptional reporters in *T. atroviride* using BioDare platform.

Data	Period	P.Std	Phase	Ph.Std	Amp.	Amp.Std
***Nc*c-box-luc**	25.67	1.01	3.53	1.96	4.86e+0	1.92e+0
ccg-9*$_{prom}$*-luc	NaN	NaN	NaN	NaN	NaN	NaN
sod*$_{prom}$*-luc	NaN	NaN	NaN	NaN	NaN	NaN
con-10*$_{prom}$*-luc	NaN	NaN	NaN	NaN	NaN	NaN
gpdh*$_{prom}$*-luc	NaN	NaN	NaN	NaN	NaN	NaN

Supp. Table V. List of primer used for *tafrq* replacement cassette and diagnostics PCR. (Fw: direct orientation; Rv: reverse orientation).

Target DNA	Primer Name	Orientation	Sequence 5' → 3'	Size (bp)
5' flank upstream *tafrq* gene	oL3867	Fw	AGCGGATAACAATTTCACACAGGAAACAGCACTTCCACCAGCCCACTCCT	600
	oL3884	Rv	AAAAATGCTCCTTCAATATCATCTTCTGTCAAATGCCTACCTAGGCTTGC	
3' flank downstream *tafrq* gene	oL3807	Fw	GACCGGGATCCACTTAACGTTACTGAAATCAATTAGGGATTCCCTCCCCCC	527
	oL3808	Rv	GTAACGCCAGGGTTTTCCCAGTCACGACGGAAATGCGTCGAGTGCCTGT	
hph	oL768	Fw	GACAGAAGATGATATTGAAGGAGC	1435
	oL769	Rv	GATTTCAGTAACGTTAAGTGGAT	
Construct amplification	oL83	Fw	GGCAGTGAGCGCAACGCAAT	2562
	oL84	Rv	ATTCAGGCTGCGCAACTGTT	
Upstream integration	oL1269	Fw	GCACCAAGCAGCAGATGATA	1680
	oL3908	Rv	AAACAAAAAAAAAAACAGGGCCAGTATGGTACAGTGTATACGCGCATACAG	
Downstream integration	oL32	Fw	ATGGCTGTGTAGAAGTACTC	909
	oL3804	Rv	GTAACGCCAGGGTTTTCCCAGTCACGACGAAAGTGAGACGAGATAAAGGG	
tafrq ORF	oL3915	Fw	GAAAATTTGCAGGTCGGCATGCCCGTCGGAACCAGGCTAGCCAAACGGCA	1080
	oL3916	Rv	ATAACAAATACGTCGTAGGGG	

Supp. Table VI. List of primer used for OE::*tafrq* insertion cassette. (Fw: direct orientation; Rv: reverse orientation).

Target DNA	Primer Name	Orientation	Sequence 5' → 3'	Size (bp)
5' flank intergenic region *blu17* gene	oL4189	Fw	GCGGATAACAATTTCACACAGGAAACAGCTAGCGCCTCGTTGCTGAAAT	1500
	oL3810	Rv	GACCGGGATCCACTTAACGTTACTGAAATCTATTCGCTACAACGGACAGT	
3' flank intergenic region *blu17* gene	oL3813	Fw	GAGGTGTTTCTTAAGTAGTT	1500
	oL4188	Rv	GTAACGCCAGGGTTTTCCCAGTCACGACGAGGACCTATTCTGGAGAGATA	
Complete *tafrq* ORF	oL3857	Fw	GAAAATTTGCAGGTCGGCATGCCCGTCGGAACCAGGCTAGCCAAACGGCA	3053
	oL3855	Rv	ATAACAAATACGTCGTAGGGG	

V5His6	oL526 oL1586	Fw Rv	GGATCTAGAGGGCCCTTCGAA TCAATGGTGATGGTGATGAT	93
Actin terminator	oL3856 oL3853	Fw Rv	CGTACCGGTCATCATCACCATCACCATTGAAAGCTACGCGAGGTCAAAGA CAGTCCTGTAAACTACTTAAGAAACACCTCGAATTCCATCTAGCGTTAAAGATACT	335
Actin promoter	oL3848 oL3854	Fw Rv	AAAAATGCTCCTTCAATATCATCTTCTGTCAAAGCGGCCGCTTAGTCGTCAGGAGA GGGAGGC TTTCGGAGGATTGCCCTCTGTCGGCTGCATTGTGACTGATTATAGGATGA	1210
hph	oL768 oL769	Fw Rv	GACAGAAGATGATATTGAAGGAGC GATTTCAGTAACGTTAAGTGGAT	1435
Construct amplification	oL83 oL84	Fw Rv	GGCAGTGAGCGCAACGCAAT ATTCAGGCTGCGCAACTGTT	9126

Supp. Table VII. List of primer used for luciferase translational reporter (TaFRQ^{LUC}) insertion cassette.
(Fw: direct orientation; Rv: reverse orientation).

Target DNA	Primer Name	Orientation	Sequence 5' → 3'	Size (bp)
5' flank *tafrq* w/o stop codon	Frq-Luc 5 flank oL3800	Fw Rv	GCGGATAACAATTTCACACAGGAAACAGCTACTACAGTGGTGCTCCGT GCCCTTCTTGATGTTCTTGGCGTCCTCCATGCCACTCCCTGCGGAGCTGGT	1027
3' flank downstream *tafrq* gene	oL3803 oL3804	Fw Rv	GACCGGGATCCACTTAACGTTACTGAAATCGCCAGTTTAAGGCTATACTG GTAACGCCAGGGTTTTCCCAGTCACGACGAAAGTGAGACGAGATAAAGGG	498
hph	oL768 oL769	Fw Rv	GACAGAAGATGATATTGAAGGAGC GATTTCAGTAACGTTAAGTGGAT	1435
luc	oL24 oL25	Fw Rv	ATGGAGGACGCCAAGAACAT TCAGAGCTTGGACTTGCCGC	1741
tafrq 3'UTR	oL3801 oL3802	Fw Rv	GCCAAGAAGGGCGGCAAGTCCAAGCTCTGATTGTACGGGACATTTCATATT AAAAATGCTCCTTCAATATCATCTTCTGTCATAACAAAAGCTCATTAAAC	299
Construct amplification	oL83 oL84	Fw Rv	GGCAGTGAGCGCAACGCAAT ATTCAGGCTGCGCAACTGTT	5000

Supp. Table VIII. List of primer used for luciferase transcriptional reporter insertion cassette. (Fw: direct orientation; Rv: reverse orientation).

Target DNA	Primer Name	Orientation	Sequence 5' → 3'	Size (bp)
5' flank intergenic region *blu17* gene	oL4189 oL3810	Fw Rv	GCGGATAACAATTTCACACAGGAAACAGCTAGCGCCTCGTTGCTGAAAT GACCGGGATCCACTTAACGTTACTGAAATCTATTCGCTACAACGGACAGT	1500
3' flank intergenic region *blu17* gene	oL3813 oL4188	Fw Rv	GAGGTGTTTCTTAAGTAGTT GTAACGCCAGGGTTTTCCCAGTCACGACGAGGACCTATTCTGGAGAGATA	1500
Putative *sod* promoter	oL4898 oL4899	Fw Rv	TAACCTGCACACTGTCCGTTGTAGCGAAtGCGGCACCCAGCCTCGGCTTA GCCCTTCTTGATGTTCTTGGCGTCCTCCATTGTGGCGGTTGGATTTTTAC	485
Putative *con10* promoter	oL4915 oL4916	Fw Rv	TAACCTGCACACTGTCCGTTGTAGCGAATGGATGAACATTGATAGTGCAC GCCCTTCTTGATGTTCTTGGCGTCCTCCATGGTGAAATATGCTTGGTTTG	1000
Putative *ccg9* promoter	oL4896 oL4897	Fw Rv	TAACCTGCACACTGTCCGTTGTAGCGAAtGAGGAAGCGTACATGTAAATT GCCCTTCTTGATGTTCTTGGCGTCCTCCATTTTGGAAATCAAGGACTCAA	768
Putative *gpdh* promoter	oL4894 oL4895	Fw Rv	TAACCTGCACACTGTCCGTTGTAGCGAATGGTTGGTGCCGGGTCCCAGCC GCCCTTCTTGATGTTCTTGGCGTCCTCCATGATGGCGGTTTTGTGAACTG	1159
c-box from *N. crassa*	oL3920 oL3921	Fw Rv	AAAAATGCTCCTTCAATATCATCTTCTGTCCGGAATTATACGATTTAGGT GCCCTTCTTGATGTTCTTGGCGTCCTCCATATTCTAGTGGAAAGGGGAGG	505
luc	oL24 oL25	Fw Rv	ATGGAGGACGCCAAGAACAT TCAGAGCTTGGACTTGCCGC	1741
hph	oL768 oL769	Fw Rv	GACAGAAGATGATATTGAAGGAGC GATTTCAGTAACGTTAAGTGGAT	1435

CHAPTER 3

SUPPLEMENTARY INFORMATION.

3.1 Materials and Methods

3.1.1. Assessment of luminescence *in vivo*

To evaluate rhythmic levels of bioluminescence produced by the transcriptional reporters, spores from 7 days old colonies were resuspended in sterile water, filtrated using sterile glass wool, and kept for 48 h. at 4°C. Then, 30 µL of spore suspensions were inoculated in a 96-well plate containing GYEC+peas culture media (Supp. Table I). The reporter strains received LD 12:12 entrainment at 25° C. The luminescence was capture using a Pixis 1024B CCD camera (Princeton Instruments) under DD conditions at 25° C. Bioluminescence was acquired using the WinView program and subsequently analyzed using ImageJ.

3.1.2 Stress response assay

For stress-response assay, we cultivated *Trichoderma* strains in PDA at 25°C in LL adding different stressor agents to culture media. To evaluate osmotic stress tolerance, we added sorbitol, NaCl and KCl at 50 and 100 mM. To evaluate oxidative stress, hydrogen peroxide (40 and 80 mM) and menadione (500 and 750 µM) were added. To evaluate cell wall stress, we employed Congo red (0.2, 0.4 and 0.8 mM). Growth evaluation was performed after 3 days of culture in the mention conditions, using Image J. For statistical analysis, ANOVA was used, and significance was determined using Tukey's test with a threshold of $P < 0.05$.

3.1.3 Laser Desorption Low-Temperature Plasma Mass Spectrometry Imaging (MSI)

For mass spectrometry imaging (MSI) experiments, we used laser desorption low-temperature plasma (LD-LTP) ionization (Rosas-Román et al., 2020). Laser desorption (LD) was performed with a 405 nm continuous wave (CW) ultraviolet (UV) diode laser, and for low-temperature plasma (LTP) post-ionization we employed a 3D-printed LTP probe. The system was mounted on the Open LabBot platform Kuturabi SA de CV, Mexico, http://www.kuturabi.com), as described earlier by Rosas-Roman et al., 2020. The parameters of the LTP probe were as follows: 0.1 L helium (technical grade) flow, 3 kV voltage and 12 kHz frequency. Activated charcoal powder (Merck) was added to the surface of the Petri dishes containing the biological samples. Activated charcoal was used for two reasons; its ability to absorb odours, decreasing the dispersion of volatile molecules and darkening the surface of the sample allowing the laser to impact and heat the sample. The laser was used at a power of 304 MW.

3.2 Results

3.2.1 Different input signal can drive oscillations in *T. atroviride*

In the first attempts to find circadian oscillations of the reporter strains, we tested 9 different culture media conditions using different entrainment. In Figure 3.1, a 3 days

temperature entrainment of 12 h. at 20° C and 12 h. ° C was given followed by 2 days at 20° C. In Figure 3.1 A, we present the data of the GYEC media. The first 3 days, luminescence is high during the 12h. at 28° C and fall at 20° C. These luminescence fluctuations are due to fluctuation in luciferase activity derived of temperature cycling. Once temperature is set at 20° C, 2 mild oscillations can be seen, suggesting that temperature cycling can entrain the clock.

We decided to perform the 3-day temperature entrainment prior luminescence recording and transferred the plates at 20° C for 5 days to the CCD camera. In figure 3.2B, 4 weak oscillations can be seen in GYEC culture media, that are absent in the other media. These results showed that in GYEC media, temperature can entrain the clock, resulting in weak oscillations.

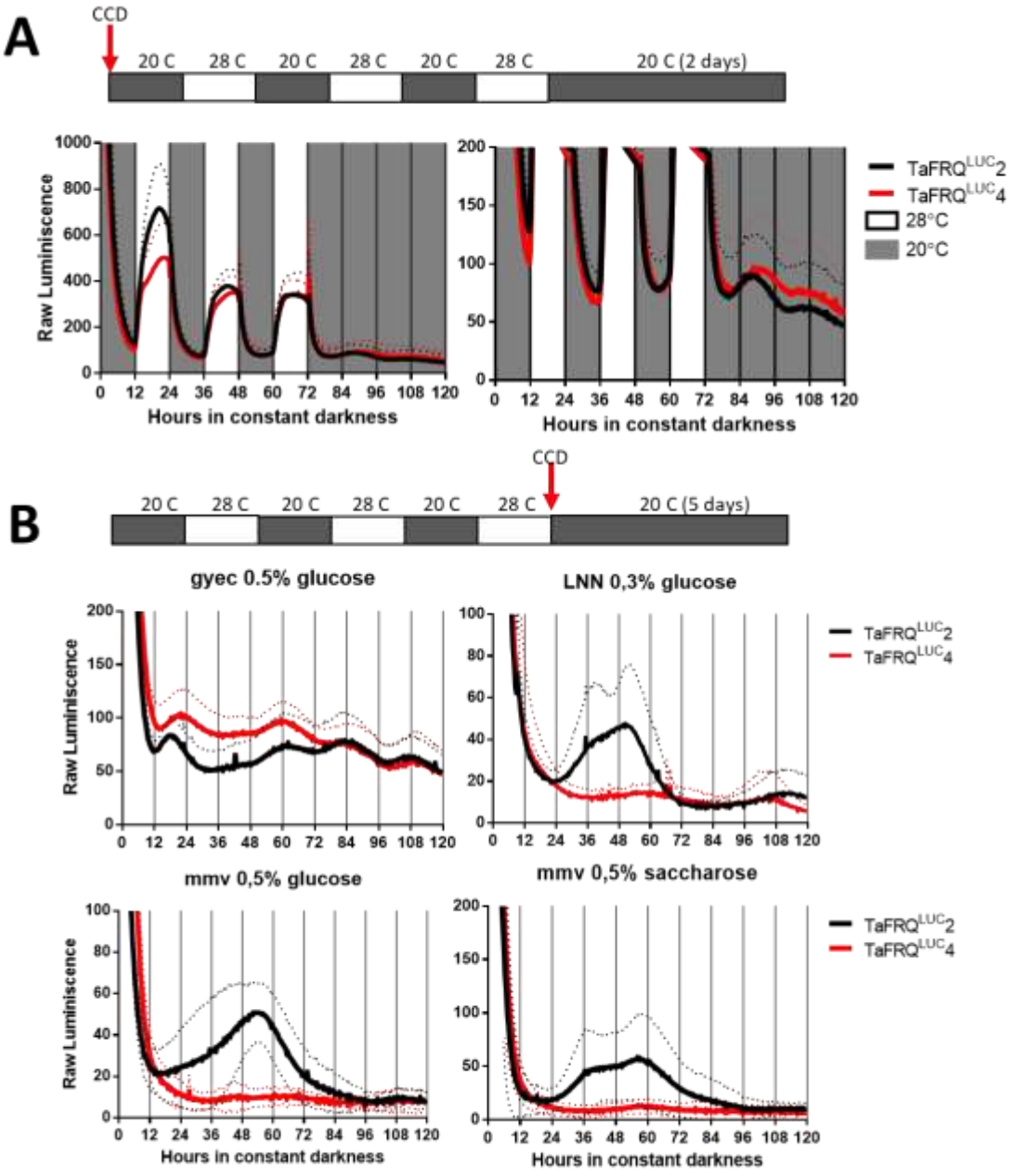


Figure 3.1. Oscillations of FRQ^{LUC} can be seen after temperature entrainment only in GYEC.

Two independent translational reporter strains, TaFRQ^{LUC} 2 and TaFRQ^{LUC} 4, were grown in 8 different culture media in 96 wells plate **A)** The plates were inoculated and then transferred to the CCD camera to capture luminescence. Temperature cycled for 3 days for 12 h. at 20° C and 12 h. at 28°C and then kept at 20° C for 2 more days. Only the results of GYEC culture media are shown. **B)** The plates were inoculated and kept in temperature cycling of 12 h. at 20° C and 12 h. at 28°C for 3 days in the incubator. Later, the plates were transferred to the camera and luminescence was recorded for 5 days at 20 °C.

3.2.2 BLR1 is necessary for osmotic and cell wall stress resistance.

To evaluate if *T. atroviride* clock components have a role in oxidative, osmotic and cell wall stress responses, TaWT, Δ*tafrq* and Δ*blr1* strains were grown in the presence of hydrogen peroxide, menadione, NaCl, KCl, Sorbitol, or Congo red under LL conditions during three days. As shown in Figure 3.2, the Δ*blr1* strain is more resistant to osmotic stress when compared to TaWT and Δ*tafrq* in Sorbitol, NaCl and KCl. The OE::*tafrq* strain has higher osmotic tolerance than the Δ*tafrq* and TaWT strains in KCl and Sorbitol 100 mM. When the strains are subjected to hydrogen peroxide, growth is seriously hampered, but the Δ*tafrq* has a slight resistance at 40 mM compared to the rest of the strains, whereas at 80 mM, growth is equally affected in all strains. In the presence of 500 µM of menadione, growth reduction in Δ*blr1* and OE::*tafrq* is greater than in TaWT and Δ*tafrq.* At high concentrations of menadione, all strains were equally affected. Concerning cell wall stress, Δ*blr1* is more susceptible than the rest of the strains (Figure 3.2).

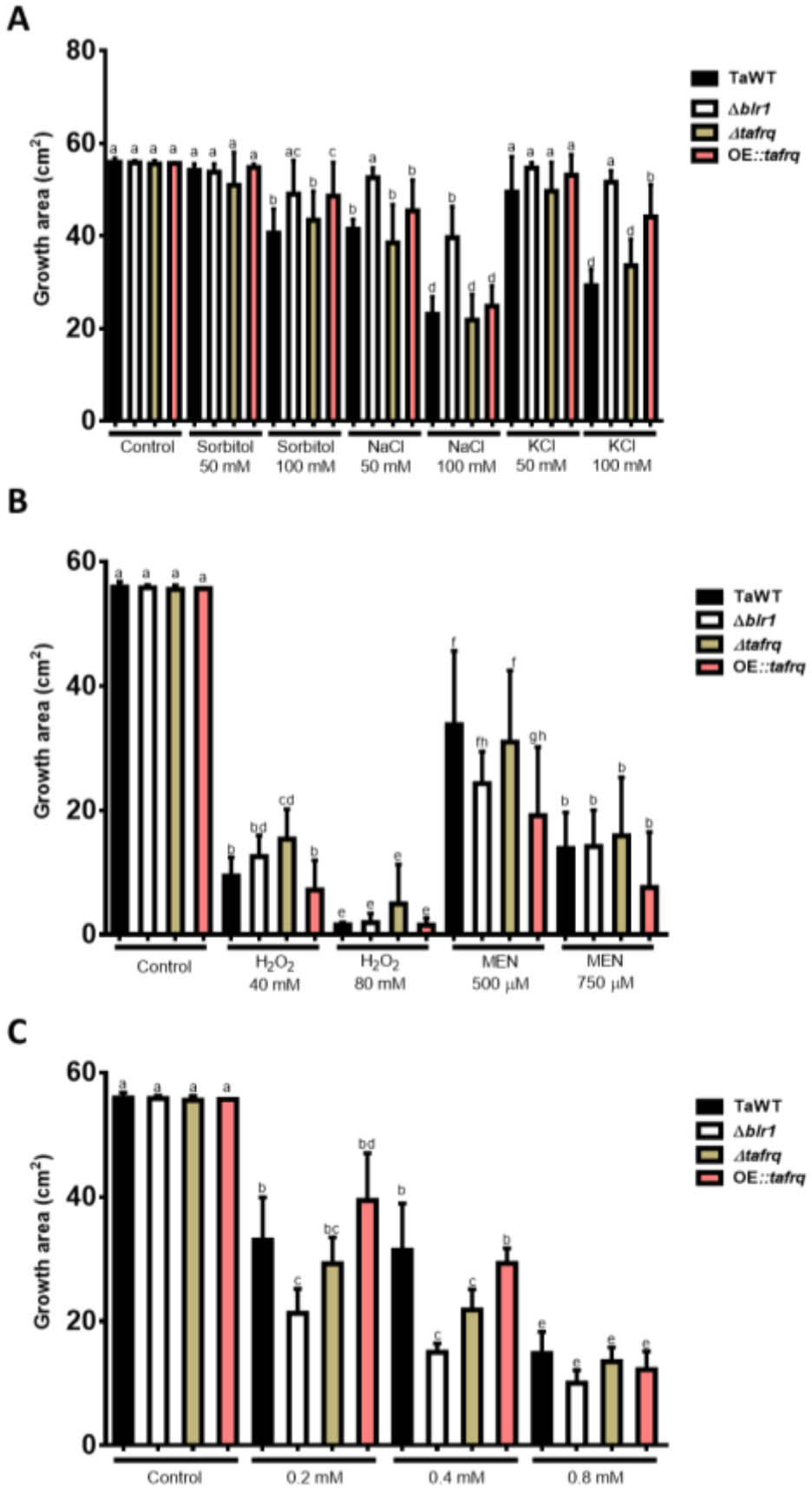

A
Growth area (cm²)
80
60
40
20
0
TaWT
Δblr1
Δtafrq
OE::tafrq
Control
Sorbitol 50 mM
Sorbitol 100 mM
NaCl 50 mM
NaCl 100 mM
KCl 50 mM
KCl 100 mM
B
Growth area (cm²)
60
40
20
0
TaWT
Δblr1
Δtafrq
OE::tafrq
Control
H2O2 40 mM
H2O2 80 mM
MEN 500 µM
MEN 750 µM
C
Growth area (cm²)
60
40
20
0
TaWT
Δblr1
Δtafrq
OE::tafrq
Control
0.2 mM
0.4 mM
0.8 mM

Figure 3.2 Stress response of *T.atroviride* WT and clock mutant strains.

Strains where grown in PDA at 25 °C in constant light, adding different stress agents. Growth area is presented. **A)** Osmotic stress (Sorbitol, NaCl and KCl), **B)** Oxidative stress (hydrogen peroxide and menadione (MEN)) **C)** Congo red. Errors bars represent the means ± SD of 4 biological replicas for each experiment. Different letter indicates significant differences based on Tukey's test ($p < 0.05$).

3.2.3 Spatial localization of 6-PP in a confrontation assay between *Botrytis cinerea vs. Trichoderma atroviride* performed in LD cycles.

To help understand the role of volatile compounds in the mycoparasitic behavior of *T. atroviride*, we performed a mass spectrometry imaging (MSI) (Rosas-Roman et al., 2020) on confrontation assays between *T. atroviride* and *Botrytis cinerea.* This technique allows to analyze the spatial distribution of molecules in a given sample *in vivo*.

We performed confrontations between TaWT and *B. cinerea* B05.10 and Δ*blr1*-B05.10 and kept the assays in LD cycles 12:12 h. and took images at day 2 of interaction. We were particularly interested in analyzing and tracking the behavior of 6-PP and its differences between TaWT and Δ*blr1* strains. The imaging analysis of each confrontation took approximately 90 minutes. Each imaging file contains between 9,500 to 12,000 spectra corresponding to the number of pixels that generate one image.

The results show that production of 6-PP by TaWT strain matches the LD cycles. The plates were incubated in LD 12:12 h. cycles, starting with 12 h. of darkness. A ring of 6-PP production is observed and co-localized with the first ring of aerial mycelia formed by the 12 h light received in the TaWT strain (bottom left Figure 3.3). As we observed in Figure 2.5, 6-PP production is repressed in LL in TaWT, so the phenomenon we observe here could suggest that the production of the molecules is a result of the activated developmental program that is induced by light. Meanwhile, the tissue grown during the 12 h. dark (the ring at the begging of colony growth and the tissue following the aerial mycelium), produces lower amounts of 6-PP. On the other hand, the production of 6-PP by the strain Δ*blr1* does

not show a ring pattern, showing a continuous production of the molecule in the fungus (Figure 3.3). No molecules were observed in *B. cinerea* colony.

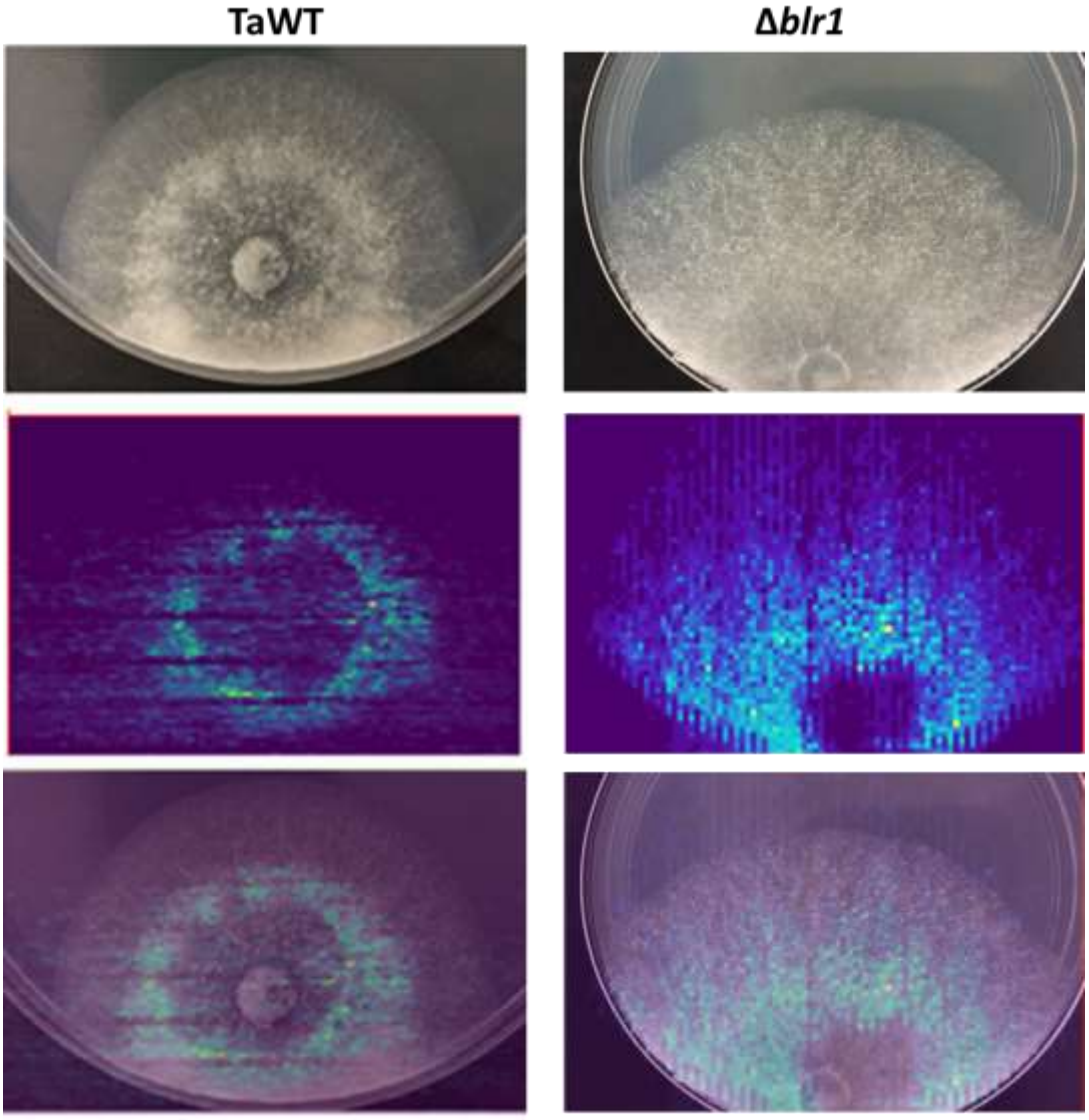


Figure 3.3. Spatial distribution of 6-PP on *T. atroviride* WT and *Δblr1* strains grown in LD cycles. MSI of a confrontation assay between TaWT and Δ*blr1* against B05.10. Upper image shows the *Trichoderma* colony after 2 days of growth in LD cycles. The incubation of the plates started with 12 h. dark followed by 12 h. light. In the middle, image represents the localization and intensities of the 6-PP molecule in the mycelium of each strain. Bottom images are the overlay between up and middle images. No molecules were detected in B05.10, so no images are shown.

CHAPTER 4

General Discussion.

4.1 Circadian oscillations in *T. atroviride*

The first attempts to find a circadian behavior in *T. atroviride* were unsuccessful, and (Steyaert et al. 2010a), suggested that probably the appropriate culture conditions to visualize circadian behavior in *T. atroviride* were yet to be determined. Moreover, in *T. atroviride* rhythmic conidiation under LD cyles have been described (Steyaert, 2007), however this is not observed under constant conditions. In the model fungus *N. crassa*, a strong circadian conidiation phenotype has transformed it in one of the main models in fungal chronobiology. However, the "WT" strain that is used, actually corresponds to a mutant strain called *band* (*bd*), which has a point mutation in the *ras-1* gene which enhances circadian conidiation, facilitating its visualization (Larrondo & Canessa, 2019). In *B. cinerea*, like in *T. atroviride*, no circadian conidiation is observed, but a light dependent conidiation has been described (Canessa et al., 2013). The B05.10 strain of Botrytis produces macroconidia in LD meanwhile it produces sexual reproductive structures in DD, corresponding to microconidia and sclerotia. In contrast, another popular Botrytis strain, T4, produces conidia in both LD and DD ("always conidia phenotype"). Circadian analysis in *Botrytis* were conducted on the B05.10 strain (Hevia et al., 2015) due to its genetic stability and high rates of homologous recombination (Hevia et al., 2016). These antecedents show the importance of selecting a well-characterized strain to conduct circadian phenotypic and molecular analysis (Larrondo & Canessa, 2019). In this study, we used the strain *T. atroviride* IMI 206040, which its light

responses have been well-characterized, in which conidiation occurs in the presence of light and no conidiation is observed in the darkness, in opposition to the P1 strain (also commonly used), where conidiation is independent of the light (Speckbacher et al., 2020b).

Through the use of luciferase-based reporters TaFRQ^{LUC} and the transcriptional reporter, $_{nc}$*cbox::oluc,* we showed for the first time circadian oscillations in the mycoparasitic fungus *T. atroviride,* rhythms that are strongly affected by culture media composition. As mentioned above, we firstly evaluated oscillations of our reporters in different culture media, but we only observed oscillations in GYEC (Figure 2.1 A), and the addition of plant-derived tissue (peas) improved oscillations (Figure 2.1 B).

In past years, the importance of nutrients in circadian regulation has been highlighted in mammals, in which nutrient metabolism is under circadian control, and organisms must manage diurnal nutrient fluctuations (Bae et al., 2019). Also, even though there is a light entrained central oscillator, peripheral oscillators can be entrained by several factors, of which feeding is the most important, setting the phase of oscillations (Oosterman et al., 2015). However, in fungi, little is known about how nutrients can influence the circadian clock at a molecular level. In the model fungi *N. crassa*, robust oscillations of luciferase reporters (both FRQ^{LUC} and $_{frq}$*cbox::luc*) can be observed in different carbon sources from simple sugars to complex mixtures of cellulose-containing media such as straw, silver grass, avicel, etc. (Diaz & Larrondo, 2020). However, it has been emphasized that culture media and growth conditions must be taken in to account when assessing circadian rhythms in this organism: oscillations of luciferase transcriptional reporters of *ccgs* vary depending on culture media used, meaning that circadian expression might be altered depending on nutritional conditions (ex.: a shift from ammonium to nitrate) (Dunlap & Loros, 2017; Hurley et al., 2014). Likewise, visualization of *frq*/FRQ rhythms can be affected by media

conditions, particularly in some mutants. Thus, for example, while analyses of *frq/FRQ* oscillations in the Neurospora Δ*fwd-1* mutant, utilizing liquid cultures failed to show rhythms (He et al., 2003), studies utilizing *luc* and solid media confirmed that the clock is functional in that mutant (Larrondo et al., 2015). Likewise, media conditions also permitted to reclassify another Neurospora gene product (RCO-1), as not an essential clock component (Olivares-Yañez et al., 2016), although early studies under different media conditions had proposed it was part of the clock. On the other hand, several elements have been identified as relevant linking the circadian clock and nutritional conditions. The global transcription repressor CSP-1 has a role in maintaining nutrient compensation in high glucose concentration, repressing *wc-1* expression (Sancar et al., 2012). In the *prd-1* mutant, a lengthened period was observed, which was restored when oscillations were observed under limiting nutritional carbon conditions (Emerson et al., 2015). Additionally, it has been suggested that the TOR pathway and nutritional sensing of nitrogen and carbon source, play a role in maintaining rhythmicity in *N. crassa* (Ratnayake et al., 2018).

Even though there is no information on the interplay between circadian clock and nutrient in *T. atroviride*, evidence points out a link between carbon source, conidiation, growth, and light. In *T. atroviride*, lights generate changes in plasma membrane potential, biphasic oscillations in cAMP levels that finally drives conidiation. Analysis of the impact of several carbon sources on conidiation upon illumination found 45 of 95 different carbon source that enables conidiation in the dark in the TaWT strain. However, only 11 supported darkness sporulation in *Δblr1* and *Δblr2* mutants, suggesting that BLR proteins are involved in carbon source-dependent conidiation (Friedl et al., 2008a). Casas-Flores et al., 2006, suggested that BLR proteins may also play a role in transducing signals from the lack of glucose, besides its role in photoreception. Hypothetically, BLR could be acting as redox-

oxygen sensors of the different redox potential generated by a variety of carbon source inducing different levels of conidiation (Friedl et al., 2008a). Likewise, photostimulation of *T. atroviride's* growth varies upon different carbon sources, with higher growth rates on mono and disaccharide (Friedl et al., 2008b).

Interestingly, photostimulation of growth occurs on carbohydrates and polyols that are metabolically related to cellulose and hemicellulose and it has been seen that BLR1 is responsible for carbon source selectivity in this process (Friedl., et al., 2008b). Based on these, it has been proposed that the type of carbon source for which photostimulation of growth occurs, somehow reflects the composition of the aerial niche of *T. atroviride.* In other words, for example, the presence of simple sugars as carbon source (derived from decaying plant debris in the soil litter layer) and light could be signals for the fungus to enhance growth rate to reach conidiation faster and disperse its spores in the aerial environment (Friedl., et al., 2008b).

Taking these antecedents into account, we hypothesized that GYEC culture media could be reflecting components of *T. atroviride's* natural niche. The organic nitrogen from yeast extract and casamino acids present in GYEC could mimic the composition of its natural niche, in which different nitrogen sources derive from its hosts are available. The addition of plant-derived material could be resembling it association with plants that provide different carbon sources (Druzhinina et al., 2011). Yeast extract and casamino acids have been studied as necessary in culture media composition for mycoparasite fungi, been in some cases determinant for spore germination and parasitic behavior against a prey (Barnett, 1963). Also, casamino acids enhance fungal growth, particularly in *Trichoderma* species, instead of a synthetic mix of amino acids (Nolan & Nolan 1972, Danielson & Davey 1973) and addition of plant tissue improve biocontrol activity of Trichoderma species (El-Nagdi et al., 2019).

These findings suggest that culture composition could be playing a role by synchronizing fungal cells by resembling its niche, allowing rhythms visualization or could be resetting the clock in *T. atroviride*. Even though there is not enough evidence to prove one or the other, it is interesting to find such a strong correlation between nutrient and visualization of circadian oscillations in *T. atroviride*. Finally, the previous shows the importance of nutrient as circadian clock input, which is logical considering that nutrient availability in their respective ecological niche can vary through time (Dunlap & Loros, 2017).

4.2 Light and temperature effects on circadian oscillations in *T. atroviride*

Further analysis of TaFRQ^{LUC} oscillations (Figure 2.1 D-E) demonstrates that a light pulse and temperature can reset rhythms. Firstly, we observed oscillation of luciferase reporters using a three days LD entrainment, but we could also reset the clock with 72 h. in LL (Figure 2.1C) or a 20 min light pulse. In *N. crassa*, a short light pulse has proven to induce mRNA *frq* and FRQ levels rapidly, and this is associated with light-induced clock resetting (Liu et al., 1998). In TaFRQ^{LUC} reporter strains, luminescence increases considerably after a 20 min light pulse indicating the induction of TaFRQ (which is also consistent with the described increase of *tafrq* levels after a light pulse (García-Esquivel et al., 2016)), triggering sustained oscillations.

Temperature, on the other hand, can mimic the effects of light/dark, in which temperature steps (up or down) can lead to phase change in *N. crassa*. Temperature cycles of 12:12 h. can entrain the clock in *N. crassa* (Pregueiro et al., 2005). We observed that in *T. atroviride* temperature cycles can also entrain the clock in *T. atroviride* generating mild

oscillations compared to the others stimulus used (Figure 3.1). Additionally, in *N. crassa* a 6 h. 4°C pulse (and subsequent transfer to 25°C) generates a phase advance in the first peak, but the rest of the oscillations maintain phase and period (Francis & Sargent, 1979). In *T. atroviride*, the same is observed when reporter strains are subjected to a 4°C pulse. It seems that the clock "stops" when exposed to cold temperatures and resume oscillations once established at higher temperatures. Interestingly, resetting the clock with a 4°C pulse, generated more robust oscillations than the LD and temperature 12:12 h. entrainment.

Another important property of a circadian rhythm is temperature compensation, this means that the period of the oscillator is constant at different temperatures. Mathematically, the Q_{10} value must be in a range of $0.85 < Q_{10} < 1.15$ (Kurosawa et al., 2017).To test whether *T. atroviride's* clock could compensate, we evaluated oscillations between 20 and 28 °C. Oscillations are lost in 28 and maintain between 20-25 °C, with robust oscillations at 22° C. For 20º and 25º, an average Q_{10} of 1,05 ± 0.07 was found. These results show that the clock can compensate in a range between 20 and 25 °C and is sensitive to higher temperatures. Therefore, the temperature is critical for *T. atroviride's* clock visualization, considering that regular culture temperature used in laboratories for Trichoderma is 25°C or above, therefore higher temperatures may not be reflecting optimum physiological temperatures. Temperature compensation in *N. crassa* occurs between 20°-30°C; meanwhile, we found that in our experimental conditions, *T. atroviride* can compensate in a 5°C range. The narrow range of temperature compensation found is consistent with the soil environment in which *Trichoderma* is usually found, in which temperature shifts are buffered (Rodriguez-Romero et al., 2010).

4.3 Circadian transcriptional regulation in *T. atroviride*

In the promoter of *frq* from *N. crassa,* two *cis*-acting elements are recognized by WCC and are involved in circadian and light regulation: the *clock-box* or *c-box*, responsible for *frq* rhythmic expression and a proximal light regulatory element (pLRE), necessary for acute light responses, entrainment and resetting (Froehlich et al., 2002, 2003). The WCs are GATA- zinc finger transcription factors that binds a consensus GATA sequence. In *the c-box* sequence, two GATG sequence repeats are found and are recognized by WCC (Froehlich et al., 2003). Meanwhile, LREs found in early light response genes (ELRE) possess a core consensus GATCB.

In *T. atroviride*, similar sequences to LREs are found in light-responsive genes. In the promoter of the BLR1-dependent light-induced photolyase gene *phr1*, several CGATB boxes are found, and two repeats of inverted CGATB motifs play a role in light induction. Alignment of *phr-1* promoters from *T. atroviride, N. crassa,* and other Sordariomycetes, showed high conservation of core LRE sequences, that varies in their polarity and organization. Two conserved LRE motifs are found in *tafrq* promoter, one between -1180 to -1171 and -335 to -319, which is consistent with its BLR1-light regulation (Cervantes-Badillo et al., 2013). Such strong conservation of GATA sequences in light responses genes between *T. atroviride* and *N. crassa* and the fact that DBD of WC1 and BLR1 share a 97% identity, may explain the fact that *c-box* from *N. crassa* is functional in *T. atroviride,* driving sustained oscillations and the ability to be entrained or reset by light (Figure 2.2). In the future, a putative *c-box* could be described in *T. atroviride* using promoter alignment and functional analysis with transcriptional reporters.

We also developed luciferase transcriptional reporters of putative clock-controlled genes in *T. atroviride* (Supp. Figure 2.8). We search for the promoters of genes in *T. atroviride* homologous to the circadian express genes *ccg-9, gpdh, sod* and *con-10* from *N. crassa,* and fused to *oluc*. Unfortunately, even though luminescence was elevated in the reporter strains, no oscillations were observed. This could be due to 1.- There is not circadian transcription of those genes 2.- Promoter selection was not complete. Further analysis of the transcript levels of those genes could be done in order to confirm if its expression is not circadian.

4.4 Clock core components of *T. atroviride* impacts on its mycoparasite lifestyle

Mycoparasitism is a complex process that involves the secretion of CWDE, secondary metabolites, and ROS during the interaction (Druzhinina et al., 2011). We demonstrated that light and circadian clock components of both *B. cinerea* and *T. atroviride* influence the outcome of mycoparasite interaction, and that light inhibits *T. atroviride* overgrowth over its prey. Such influence of light on mycoparasite interactions have been described and can affect both, the mycoparasite and the prey (Barnett, 1963). Recently, Speckbacher et al., (2020b) found that mycoparasitism of *T.atroviride* P1 and IMI206040 strains is enhanced in darkness, but repressed in LD cycles. Indeed, we found that overgrowth of *Trichoderma* strains over *Botrytis* is higher in darkness, and the absence of BLR1 in *T. atroviride* enhances the ability to overgrow *Botrytis* compared to TaWT and Δ*tafrq* strains in DD, suggesting that blue-light sensor somehow repress mycoparasitism. However, the light also repressed *Δblr1* ability to overgrow *Botrytis*, suggesting that other photoreceptors might be additionally involved in the interaction (García-Esquivel et al., 2016) or that light causes

additional stress that the strain cannot overcome. Interestingly, the absence of BcWCL1 in *Botrytis* makes it more susceptible to the attack on both LL and DD conditions than the B05.10 strain, implying that light perception is crucial from *Botrytis* to resist *Trichoderma's* attack. This is consistent with BcWCL1 role in managing excessive light, oxidative stress and reaching full virulence in *B. cinerea* (Canessa et al., 2013). Regarding the role of FRQ, it seems that *tafrq* plays a mild role in overgrowth because Δ*tafrq* can still overgrow *Botrytis*, but in some conditions is not as strong as TaWT.

Interestingly, when the interaction is established under LD cycles ("AM" or "PM"), differential behavior is observed dependent on if both fungi were inoculated at "dawn" or "dusk." TaWT and Δ*tafrq* strains can overgrow B05.10 more at "dawn" than at dusk, behavior that its absence in the Δ*blr1*-B05.10 interaction (Figure 2.4), suggesting that from *Trichoderma's* side, light perception (BLR1) is playing a role in the interaction. However, when *Trichoderma* establishes interaction with Δ*bcfrq1* strain, the strain is overgrown to the same extent in both LD cycles by all Trichoderma strains, suggesting that from *Botrytis* side, BcFRQ1 is playing a role in the interaction. Meanwhile, when TaWT and Δ*tafrq* interacts with Δ*bcwcl1*, the overgrowth is greater at "dawn" than at "dusk" as seen for B05.10, but the effect its more dramatic in the mutant, which suggest that BcWCL1 does not have a role in the circadian mycoparasite interaction. In *Botrytis*, BcFRQ1 is responsible for time perception during the interaction and has a more critical role in the interaction than TaFRQ in *Trichoderma*. Even though we lack a potential mechanism underlying this phenomenon, it is the first description of an interplay of core clock components and light in a mycoparasite interaction. If we consider that in nature, organisms live under light/dark transitions, this differential attack-resistance behavior depending on the time that the interactions are established could have an impact on biocontrol strategies in agriculture,

optimizing the application of *Trichoderma* in crops. Likewise, light and clock components from both fungi could be further studied to develop more resistant or mycoparasite strains.

4.5 Involvement of *T. atroviride* circadian clock in development and secondary metabolism.

While in *N. crassa*, *frq* is essential to allow rhythmic conidiation under constant darkness or LD cycles, its absence does not appear to cause major phenotypic differences under constant lights. However, in *B. cinerea*, Δ*bcfrq1* strain presents a substantial alteration in its sexual/asexual reproduction and virulence. Δ*bcfrq1* grows producing sclerotia in LD cycles while the B05.10 strain only produces sclerotia in constant darkness. This phenotype can be reversed depending on the nitrogen source present in the culture media (Canessa et al., 2013; Hevia et al., 2015). These extra-circadian functions of BcFRQ1 are not well understood but raise questions about the role of *frq* in fungi besides its expected role as a negative element in the circadian clock. In this study, we found that *tafrq* has a role in conidiation and secondary metabolism. Conidiation production under constant light is delayed in the Δ*tafrq* and conidia production after a light pulse is reduced compared to the TaWT strain. Thus, these FRQ extra-circadian function could be part of the norm, and not the exception to it.

Interestingly, conidiation triggered by mechanical damage is severely affected. After the injury, all strains produced aerial mycelia, but clock mutant strains produced fewer conidia than the TaWT, with a dramatic reduction in Δ*tafrq*. The mechanism in which *tafrq* may be playing a role in conidiation is still unknown. However, the phenotype of Δ*tafrq* after the mechanical injury is similar to Δ*nox1* and Δ*noxR* strains in *T.*

atroviride, which are unable to produce ROS, essential signaling that triggers injury-induced conidiation (Hernández-Oñate et al., 2012). Also, cATP, calcium, and possible oxylipin production are related to injury-response in *T. atroviride,* because genes related to those processes are repressed in Δ*nox* (Hernández-Oñate et al., 2012; Medina-Castellanos et al., 2014). TaFRQ could be playing a role in some of these mechanisms yet to be determined. However, this evidence supports this new view of possible extra circadian roles of *frq* in fungi, related to development and conidiation.

In *Trichoderma,* secondary metabolites vary in a range of molecular weight, and it has been proposed that this could have different purposes in its lifestyle. For example, high molecular weight metabolites (diffusible) such peptaibols with antifungal activity, can exert its effect directly in the soil while *Trichoderma* establishes interactions with microbial hosts. Meanwhile, low molecular weight metabolites (VOCs) are released in the soil and could be expected to travel over distance, inhibiting microbial competitors, thus favoring *Trichoderma* dispersion in the environment (Reino et al., 2008). Production of secondary metabolites is strongly correlated with different stages in fungal development. Production of eight-carbon volatile compounds by *Trichoderma* species has been shown to stimulate conidiation and biosynthesis of peptaibols is associated with sporulation initiation (Steyaert et al., 2010a). The latter is influenced by light in a BLR-dependent manner because no peptaibols are found in Δ*blr* strains (Schmoll et al., 2010). These findings agree with other observations in fungi that have proposed a close relationship between developmental programs, secondary metabolism, and light responses (Schmoll et al., 2010; Tisch and Schmoll, 2010).

In our study, through metabolic fingerprinting, we observed significant differences in diffusible and volatile compounds patterns between TaWT and clock mutant strains, and

those differences were also light-dependent. In Δ*tafrq*, volatile compounds are up regulated in LL, but also presents low levels of some VOCS. The best example is the low production of antifungal compound 6-PP, while almost all diffusible compounds were upregulated in this strain compared to TaWT. This finding suggest that TaFRQ is critical for 6-PP production, which has not been reported before. Contrary, the Δ*blr1* showed to be a prolific producer of the volatile compounds 6-PP with the highest production of all strains (**Fig.2.5**).

Light also affected metabolites production in a differential manner: diffusible were upregulated in DD, LL and LP in Δ*tafrq* and OE::*tafrq*, whereas groups of VOCs were upregulated in LL and DD in Δ*blr1* and Δ*tafrq*. The 6-PP molecule was found to be higher in DD and LP and lower in LL in the TaWT. Interestingly, 6-PP production was higher in the blue-light blind strain Δ*blr1* in LL conditions compared to DD, suggesting 1) an additional light perception systems that have crosstalk with VOCs production or 2) BLR1 act as a repressor of 6-PP producing pathways.

Thus, the data speak about the impact of core clock components and light in secondary metabolism could have an impact on development, antagonist capacities, and lifestyle. Δ*tafrq* has an altered conidiation response to injury that could be related to an altered ROS production or diminished production of oxylipins, oxygenated secondary metabolites derived from fatty acids that have been proposed to be signal molecules generated after mechanical damaged (Hernández-Oñate et al., 2012). Further studies are needed to elucidate if Δ*tafrq's* secondary metabolism is affected in oxylipin production and its correlation with developmental programs.

On the other hand, dampened production of 6-PP in Δ*tafrq* could explain its less invasive behavior against *B. cinerea* in confrontations assays compared to TaWT, which is consistent with the overproduction of 6-PP by Δ*blr1* and its higher overgrowth capacity. This

is coherent to what it was observed in confrontations assays of *T. atrovitide* P1 and IMI20604 in LD and DD against phytopathogens, in which light showed an inhibitory effect on Trichoderma's biocontrol capacity (Speckbacher et al., 2020b), that correlates with a low 6-PP production in LD (Speckbacher et al., 2020a). Though we were unable to identify all compounds found in the metabolic fingerprinting, it could be expected that biosynthesis of other antifungal compounds was affected in the clock mutant strains in a light-dependent manner. For example, in *Aspergillus,* light negatively regulates aflatoxin and penicillin biosynthesis and mycotoxin production in *Alternaria alternata;* meanwhile, light enhances the production of other mycotoxins such ochratoxin 1 production in *A. ochraceus* and cercosporin biosynthesis in *Cercospora kikuchii* (Tisch and Schmoll, 2010). Analysis of VOCs production by 2 *T. atroviride* strains also found induction of 3-octanone, 2-methyl-butanol, and 3-methyl-1-butanol upon illumination and suggested that could be a response to stress induced by light (Speckbacher et al., 2020b). Accordingly, the production of VOCs has been observed to be induced in *Trichoderma* upon interaction with other fungi: 6-PP production is elicited in *T. harzianum* by the presence of *Rhizoctonia solani* (Reithner et al., 2007). Also, differential VOCs production was observed when *T. atroviride* confronts *R. solani* or *F. oxysporum* in a light-dependent manner, reinforcing the idea that secondary metabolites might be produced in response to stress, abiotic (light) or biotic (microbial) stimuli (Speckbacher et al., 2020b).

Intriguingly, under LD cycles, MSI analysis showed that 6-PP production in TaWT is localized in the aerial hyphae that is formed after light exposure and low production of the compound was observed in the dark-growing tissue, revealing a ring-shaped production of 6-PP (Figure 3.3). This is in contradiction to what was found during the VOCs measurement in LL **(**Figure 2.5) in which 6-PP production is higher in DD in TaWT. A plausible

explanation is that 6-PP production correlates with the developmental program induced by light in Trichoderma, in which hyphae branching and conidiophore formation are stimulated. Indeed, this ring pattern is only observed in the tissue that received the "first" 12 h. of light and not in the tissue that received the "second" 12 h. of light (edge of the colony). The later just received the light stimulus and has not yet started the developmental program, therefore reinforcing the idea that localization of 6-PP in the tissue that saw light is produced as a result of the light induced conidiation program. Meanwhile, in the Δ*blr1* strain the production of 6-PP is continuous and homogeneous in the mycelium, which correlates with the homogenous tissue developed by this strain. The production pattern of 6-PP is affected by the light/dark transitions and that pattern is absent in the Δ*blr1* strain. These could explain the enhanced overgrowth capacity of Δ*blr1* strain against *B. cinerea* than the TaWT in DD and LD cycles**.**

Finally, besides the circadian oscillations of TaFRQ^{LUC}, we found a circadian oscillation in the production of several volatile secondary metabolite in *T. atroviride* under constant conditions. We found that 17 out of 171 detected compounds, presented oscillations in the TaWT strain that were absent in the *Δtafrq*, suggesting that TaFRQ is necessary for the circadian oscillations of this volatile compound (Figure 2.5). Further analysis could be focused on identifying those compounds.

CONCLUSIONS

In this thesis, we have found evidence of circadian oscillations of TaFRQ in the mycoparasite fungus *Trichoderma atroviride.* Those oscillations were observed under specific nutritional conditions that could resemble the nutrients found in its niche. Also, using the luciferase reporters, we found that those oscillations are reset by light and temperature and are temperature compensated, as described for other functional circadian clocks in different organisms. Interestingly we found evidence that *N. crassa's c-box* sequence is also functional in *T. atroviride*, opening interesting questions regarding the shared origins and functional conservation of circadian clock regulation in these fungi.

Addressing the hypothesis, in this work we found that the intensity of the mycoparasite interaction between *T. atroviride* and *B. cinerea* varies according to the presence of a functional circadian clock and that depends on the time of day when the interaction begins, with an enhanced overgrowth activity from Trichoderma when the interactions starts at dawn. We found that lack of time perception in Botrytis (Δ*bcfrq1*) has a greater effect in the interaction than a clock-less Trichoderma strain (Δ*tafrq*). Despite the fact that the light and clock element BLR1 of *Trichoderma* represses its overall ability to overgrow *B. cinerea* and its absence disrupts the differential daily mycoparasite behavior, we cannot rule out a light effect rather than a clock effect of BLR1 in the interaction. On the other hand, BcFRQ1 and BcWCL1 from *B. cinerea* are necessary to cope *Trichoderma* attack suggesting a role in overcoming biotic stress such as mycoparasitism.

Finally, we also found that BRL1 and TaFRQ have a role in developmental programs induced by injury and production of secondary metabolite. The alterations observed in conidiation and enhance secondary metabolism in mutant strains opens new questions regarding the role of those elements, particularly TaFRQ outside the circadian clock and the impact in *Trichoderma's* lifestyle. Ultimately, to our knowledge this is the first description of circadian regulation in the genus *Trichoderma* or in biocontrol fungi and of circadian regulation of secondary metabolism in fungi.

REFERENCES

Anderson, R. W., Laval-Martin, D. L., & Edmunds, L. N. (1985). Cell cycle oscillators: Temperature compensation of the circadian rhythm of cell division in Euglena. *Experimental Cell Research*, *157*(1), 144–158. https://doi.org/https://doi.org/10.1016/0014-4827(85)90158-2

Aronson, B. D., Johnson, K. A., & Dunlap, J. C. (1994). Circadian clock locus frequency: protein encoded by a single open reading frame defines period length and temperature compensation. *Proceedings of the National Academy of Sciences*, *91*(16), 7683 LP – 7687. https://doi.org/10.1073/pnas.91.16.7683

Atanasova, L., Crom, S. Le, Gruber, S., Coulpier, F., Seidl-Seiboth, V., Kubicek, C. P., & Druzhinina, I. S. (2013). Comparative transcriptomics reveals different strategies of Trichoderma mycoparasitism. *BMC Genomics*, *14*(1). https://doi.org/10.1186/1471-2164-14-121

Atriztán-Hernández, K., Moreno-Pedraza, A., Winkler, R., Markow, T., & Herrera-Estrella, A. (2019). Trichoderma atroviride from predator to prey: Role of the mitogen-activated protein kinase tmk3 in fungal chemical defense against fungivory by drosophila melanogaster larvae. *Applied and Environmental Microbiology*, *85*(2), 1–15. https://doi.org/10.1128/AEM.01825-18

Austin, B. (1968). An Endogenous Rhythm of Spore Discharge in Sordaria fimicola. *Annals of Botany*, *32*(2), 262–278. https://doi.org/10.1093/oxfordjournals.aob.a084207

Bae, S.-A., Fang, M. Z., Rustgi, V., Zarbl, H., & Androulakis, I. P. (2019). At the Interface of Lifestyle, Behavior, and Circadian Rhythms: Metabolic Implications. *Frontiers in Nutrition*, *6*, 132. https://doi.org/10.3389/fnut.2019.00132

Baker, C. L., Loros, J. J., & Dunlap, J. C. (2012). The circadian clock of Neurospora crassa. *FEMS Microbiology Reviews*, *36*(1), 95–110. https://doi.org/10.1111/j.1574-6976.2011.00288.x

Balcázar-López, E., Méndez-Lorenzo, L. H., Batista-García, R. A., Esquivel-Naranjo, U., Ayala, M., Kumar, V. V., Savary, O., Cabana, H., Herrera-Estrella, A., & Folch-Mallol, J. L. (2016). Xenobiotic compounds degradation by heterologous expression of a trametes sanguineus laccase in trichoderma atroviride. *PLoS ONE*, *11*(2), 1–13. https://doi.org/10.1371/journal.pone.0147997

Barik, S. (2019). Molecular interactions between pathogens and the circadian clock. *International Journal of Molecular Sciences*, *20*(23). https://doi.org/10.3390/ijms20235824

Barnett, H. L. (1963). The Nature of Mycoparasitism by Fungi. *Annual Review of Microbiology*, *17*(1), 1–14. https://doi.org/10.1146/annurev.mi.17.100163.000245

Bélanger, Dufour, Caron, & Benhamou. (1995). Chronological Events Associated with the Antagonistic Properties of Trichoderma harzianum against Botrytis cinerea: Indirect Evidence for Sequential Role of Antibiosis and Parasitism. *Biocontrol Science and Technology*, *5*(1), 41–54. https://doi.org/10.1080/09583159550040006

Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L., & Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature Reviews Genetics*, *6*(7), 544–556. https://doi.org/10.1038/nrg1633

Berrocal-Tito, G. M., Rosales-Saavedra, T., Herrera-Estrella, A., & Horwitz, B. A. (2000).

Characterization of Blue-light and Developmental Regulation of the Photolyase gene phr1 in Trichoderma harzianum. *Photochemistry and Photobiology*, *71*(5), 662. https://doi.org/10.1562/0031-8655(2000)071<0662:coblad>2.0.co;2

Bluhm, B. H., Burnham, A. M., & Dunkle, L. D. (2010). A circadian rhythm regulating hyphal melanization in Cercospora kikuchii. *Mycologia*, *102*(6), 1221–1228. https://doi.org/10.3852/09-041

Brunner, K., Omann, M., Pucher, M. E., Delic, M., Lehner, S. M., Domnanich, P., Kratochwill, K., Druzhinina, I., Denk, D., & Zeilinger, S. (2008). Trichoderma G protein-coupled receptors: Functional characterisation of a cAMP receptor-like protein from Trichoderma atroviride. *Current Genetics*, *54*(6), 283–299. https://doi.org/10.1007/s00294-008-0217-7

Canessa, P., Schumacher, J., Hevia, M. A., Tudzynski, P., & Larrondo, L. F. (2013). Assessing the effects of light on differentiation and virulence of the plant pathogen botrytis cinerea: Characterization of the white collar complex. *PLoS ONE*, *8*(12). https://doi.org/10.1371/journal.pone.0084223

Carreras-Villaseñor, N., Sánchez-Arreguín, J. A., & Herrera-Estrella, A. H. (2012). Trichoderma: Sensing the environment for survivaland dispersal. *Microbiology*, *158*(1), 3–16. https://doi.org/10.1099/mic.0.052688-0

Carvalho Cabral, P., Olivier, M., & Cermakian, N. (2019). The Complex Interplay of Parasites, Their Hosts, and Circadian Clocks. *Frontiers in Cellular and Infection Microbiology*, *9*, 425. https://doi.org/10.3389/fcimb.2019.00425

Casas-Flores, & Herrera-Estrella. (2013). The influence of light on the biology of Trichoderma. *Trichoderma: Biology and Applications*, *June*, 43–66. https://doi.org/10.1079/9781780642475.0043

Casas-Flores, Rios-Momberg, Bibbins, Ponce-Noyola, & Herrera-Estrella. (2004). BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in Trichoderma atroviride. *Microbiology*, *150*(11), 3561–3569. https://doi.org/10.1099/mic.0.27346-0

Casas-Flores, S., Rios-Momberg, M., Rosales-Saavedra, T., Martínez-Hernández, P., Olmedo-Monfil, V., & Herrera-Estrella, A. (2006). Cross talk between a fungal blue-light perception system and the cyclic AMP signaling pathway. *Eukaryotic Cell*, *5*(3), 499–506. https://doi.org/10.1128/EC.5.3.499-506.2006

Cascant-Lopez, E., Crosthwaite, S. K., Johnson, L. J., & Harrison, R. J. (2019). Homologues of key circadian clock genes present in Verticillium dahliae do not direct circadian programs of development or mRNA abundance. *BioRxiv*, 2019.12.20.883116. https://doi.org/10.1101/2019.12.20.883116

Cervantes-Badillo, M. G., Muñoz-Centeno, T., Uresti-Rivera, E. E., Argüello-Astorga, G. R., & Casas-Flores, S. (2013). The Trichoderma atroviride photolyase-encoding gene is transcriptionally regulated by non-canonical light response elements. *FEBS Journal*, *280*(15), 3697–3708. https://doi.org/10.1111/febs.12362

Contreras-Cornejo, H. A., Macías-Rodríguez, L., del-Val, E., & Larsen, J. (2020). *Interactions of Trichoderma with Plants, Insects, and Plant Pathogen Microorganisms: Chemical and Molecular Bases*. *8701*, 263–290. https://doi.org/10.1007/978-3-319-96397-6_23

Corrochano, L. M. (2019). Light in the Fungal World: From Photoreception to Gene Transcription and beyond. *Annual Review of Genetics*, *53*, 149–170. https://doi.org/10.1146/annurev-genet-120417-031415

Danielson, R. M., & Davey, C. B. (1973). Carbon and nitrogen nutrition of Trichoderma. *Soil Biology and Biochemistry*, *5*(5), 505–515. https://doi.org/10.1016/0038-0717(73)90040-0

Dattenböck, C., Tisch, D., Schuster, A., Monroy, A. A., Hinterdobler, W., & Schmoll, M. (2018). Gene regulation associated with sexual development and female fertility in different isolates of Trichoderma reesei. *Fungal Biology and Biotechnology*, *5*(1), 9. https://doi.org/10.1186/s40694-018-0055-4

de Bekker, C., Will, I., Hughes, D. P., Brachmann, A., & Merrow, M. (2017). Daily rhythms and enrichment patterns in the transcriptome of the behavior-manipulating parasite Ophiocordyceps kimflemingiae. *PLOS ONE*, *12*(11), 1–20. https://doi.org/10.1371/journal.pone.0187170

De Paula, R. M., Lamb, T. M., Bennett, L., & Bell-Pedersen, D. (2008). A connection between MAPK pathways and circadian clocks. *Cell Cycle*, *7*(17), 2630–2634. https://doi.org/10.4161/cc.7.17.6516

Delgado-Jarana, J., Sousa, S., González, F., Rey, M., & Llobell, A. (2006). ThHog1 controls the hyperosmotic stress response in Trichoderma harzianum. *Microbiology*, *152*(6), 1687–1700. https://doi.org/10.1099/mic.0.28729-0

Deng, Y. Z., Qu, Z., & Naqvi, N. I. (2015). Twilight, a Novel Circadian-Regulated Gene, Integrates Phototropism with Nutrient and Redox Homeostasis during Fungal Development. *PLoS Pathogens*, *11*(6), 1–22. https://doi.org/10.1371/journal.ppat.1004972

Díaz, R. D., & Larrondo, L. F. (2020). A circadian clock in Neurospora crassa functions during plant cell wall deconstruction. *Fungal Biology*, *124*(5), 501–508. https://doi.org/https://doi.org/10.1016/j.funbio.2020.03.003

Druzhinina, I., & Kubicek, C. P. (2005). Species concepts and biodiversity in Trichoderma and Hypocrea: From aggregate species to species clusters. *Journal of Zhejiang University: Science*, *6 B*(2), 100–112. https://doi.org/10.1631/jzus.2005.B0100

Druzhinina, I. S., Schmoll, M., Seiboth, B., & Kubicek, C. P. (2006). Global carbon utilization profiles of wild-type, mutant, and transformant strains of Hypocrea jecorina. *Applied and Environmental Microbiology*, *72*(3), 2126–2133. https://doi.org/10.1128/AEM.72.3.2126-2133.2006

Druzhinina, Seidl-Seiboth, Herrera-Estrella, Horwitz, Kenerley, Monte, Mukherjee, Zeilinger, Grigoriev, & Kubicek. (2011). Trichoderma: The genomics of opportunistic success. *Nature Reviews Microbiology*, *9*(10), 749–759. https://doi.org/10.1038/nrmicro2637

Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell*, *96*(2), 271–290. https://doi.org/10.1016/S0092-8674(00)80566-8

Dunlap, J. C., & Loros, J. J. (2017). Making Time: Conservation of Biological Clocks from Fungi to Animals. *The Fungal Kingdom*, *May 2020*, 515–534. https://doi.org/10.1128/9781555819583.ch24

EFSA European Food Safety. (2015). Conclusion on the peer review of the pesticide risk assessment of the active substance Trichoderma atroviride, strains IMI-206040, T11. *EFSA Journal*, *13*(4), 3056. https://doi.org/10.2903/j.efsa.2015.3056

El-Nagdi, W. M. A., Youssef, M. M. A., El-Khair, H. A., & Abd-Elgawad, M. M. M. (2019). Effect of certain organic amendments and Trichoderma species on the root-knot nematode, Meloidogyne incognita, infecting pea (Pisum sativum L.) plants. *Egyptian Journal of Biological Pest Control*, *29*(1). https://doi.org/10.1186/s41938-019-0182-0

Emerson, J. M., Bartholomai, B. M., Ringelberg, C. S., Baker, S. E., Loros, J. J., & Dunlap, J. C. (2015). Period-1 encodes an ATP-dependent RNA helicase that influences nutritional compensation of the Neurospora circadian clock. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(51), 15707–15712. https://doi.org/10.1073/pnas.1521918112

Esquivel-Naranjo, E. U., García-Esquivel, M., Medina-Castellanos, E., Correa-Pérez, V. A., Parra-Arriaga, J. L., Landeros-Jaime, F., Cervantes-Chávez, J. A., & Herrera-Estrella, A. (2016). A Trichoderma atroviride stress-activated MAPK pathway integrates stress and light signals. *Molecular Microbiology*, *100*(5), 860–876. https://doi.org/10.1111/mmi.13355

Francis, C. D., & Sargent, M. L. (1979). Effects of Temperature Perturbations on Circadian Conidiation in Neurospora . *Plant Physiology*, *64*(6), 1000–1004. https://doi.org/10.1104/pp.64.6.1000

Franco, D. L., Canessa, P., Bellora, N., Risau-Gusman, S., Olivares-Yañez, C., Pérez-Lara, R., Libkind, D., Larrondo, L. F., & Marpegan, L. (2017). Spontaneous circadian rhythms in a cold-adapted natural isolate of Aureobasidium pullulans. *Scientific Reports*, *7*(1), 13837. https://doi.org/10.1038/s41598-017-14085-6

Friedl, M. A., Kubicek, C. P., & Druzhinina, I. S. (2008a). Carbon source dependence and photostimulation of conidiation in Hpocrea atroviridis. *Applied and Environmental Microbiology*, *74*(1), 245–250. https://doi.org/10.1128/AEM.02068-07

Friedl, M. A., Schmoll, M., Kubicek, C. P., & Druzhinina, I. S. (2008b). Photostimulation of Hypocrea atroviridis growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress. *Microbiology*, *154*(4), 1229–1241. https://doi.org/10.1099/mic.0.2007/014175-0

Froehlich, A. C., Liu, Y., Loros, J. J., & Dunlap, J. C. (2002). White collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. *Science*, *297*(5582), 815–819. https://doi.org/10.1126/science.1073681

Froehlich, A. C., Loros, J. J., & Dunlap, J. C. (2003). Rhythmic binding of a White Collar-containing complex to the frequency promoter is inhibited by FREQUENCY. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(10), 5914–5919. https://doi.org/10.1073/pnas.1030057100

Galun and Gressel. (1966). Morphogenesis in Trichoderma: Suppression of photoinduction by 5-fluorouacil. *Science*, *151*.

García-Esquivel, M., Esquivel-Naranjo, E. U., Hernández-Oñate, M. A., Ibarra-Laclette, E., & Herrera-Estrella, A. (2016). The Trichoderma atroviride cryptochrome/photolyase genes regulate the expression of blr1-independent genes both in red and blue light. *Fungal Biology*, *120*(4), 500–512. https://doi.org/10.1016/j.funbio.2016.01.007

Goodspeed, D., Chehab, E. W., Min-Venditti, A., Braam, J., & Covington, M. F. (2012). Arabidopsis synchronizes jasmonate-mediated defense with insect circadian behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(12), 4674–4677. https://doi.org/10.1073/pnas.1116368109

Greene, A. V., Keller, N., Haas, H., & Bell-Pedersen, D. (2003). A circadian oscillator in Aspergillus spp. regulates daily development and gene expression. *Eukaryotic Cell*, *2*(2), 231–237. https://doi.org/10.1128/EC.2.2.231-237.2003

Gressel J and Galun E. (1967). Morphogenesis in Trichoderma: photoinduction and RNA. *Dev. Biol.*, *15*, 575–598.

Gutter, Y. (1957). Effect of light in sporulation of Trichoderma viride. *Bull. Res. Council*

Israel Sect, *5*, 273–286.
Guzmán-Guzmán, P., Porras-Troncoso, M. D., Olmedo-Monfil, V., & Herrera-Estrella, A. (2019). Trichoderma species: Versatile plant symbionts. *Phytopathology*, *109*(1), 6–16. https://doi.org/10.1094/PHYTO-07-18-0218-RVW
Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). Trichoderma species - Opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*, *2*(1), 43–56. https://doi.org/10.1038/nrmicro797
He, Q., Cheng, P., Yang, Y., He, Q., Yu, H., & Liu, Y. (2003). FWD1-mediated degradation of FREQUENCY in Neurospora establishes a conserved mechanism for circadian clock regulation. *The Embo Jpurnal*, *22*(17), 4421–4430.
Heintzen, C., & Liu, Y. (2007). The Neurospora crassa Circadian Clock. *Advances in Genetics*, *58*(06), 25–66. https://doi.org/10.1016/S0065-2660(06)58002-2
Hernández-Oñate, M. A., Esquivel-Naranjo, E. U., Mendoza-Mendoza, A., Stewart, A., & Herrera-Estrella, A. H. (2012). An injury-response mechanism conserved across kingdoms determines entry of the fungus Trichoderma atroviride into development. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(37), 14918–14923. https://doi.org/10.1073/pnas.1209396109
Herrera-Estrella, A., Goldman, G. H., & Montagu, M. Van. (1990). Notes High-efficiency transformation system for the biocontrol agents, Trichoderma spp. *Molecular Microbiology*, *4*(5), 839–843. https://doi.org/10.1111/j.1365-2958.1990.tb00654.x
Hevia, M. A., Canessa, P., & Larrondo, L. F. (2016). Circadian clocks and the regulation of virulence in fungi: Getting up to speed. *Seminars in Cell and Developmental Biology*, *57*, 147–155. https://doi.org/10.1016/j.semcdb.2016.03.021
Hevia, M. A., Canessa, P., Müller-Esparza, H., & Larrondo, L. F. (2015). A circadian oscillator in the fungus Botrytis cinerea regulates virulence when infecting Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(28), 8744–8749. https://doi.org/10.1073/pnas.1508432112
Howell, C. R. (1998). *The role of antibiosis in biocontrol in Trichoderma and Gliocladium 2*.
Hurley, J. M., Dasgupta, A., Emerson, J. M., Zhou, X., Ringelberg, C. S., Knabe, N., Lipzen, A. M., Lindquist, E. A., Daum, C. G., Barry, K. W., Grigoriev, I. V., Smith, K. M., Galagan, J. E., Bell-Pedersen, D., Freitag, M., Cheng, C., Lorosa, J. J., & Dunlap, J. C. (2014). Analysis of clock-regulated genes in Neurospora reveals widespread posttranscriptional control of metabolic potential. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(48), 16995–17002. https://doi.org/10.1073/pnas.1418963111
Hurley, J. M., Jankowski, M. S., De Los Santos, H., Crowell, A. M., Fordyce, S. B., Zucker, J. D., Kumar, N., Purvine, S. O., Robinson, E. W., Shukla, A., Zink, E., Cannon, W. R., Baker, S. E., Loros, J. J., & Dunlap, J. C. (2018). Circadian Proteomic Analysis Uncovers Mechanisms of Post-Transcriptional Regulation in Metabolic Pathways. *Cell Systems*, *7*(6), 613-626.e5. https://doi.org/10.1016/j.cels.2018.10.014
Hurley, J. M., Loros, J. J., & Dunlap, J. C. (2016). The circadian system as an organizer of metabolism. *Fungal Genetics and Biology : FG & B*, *90*, 39–43. https://doi.org/10.1016/j.fgb.2015.10.002
Ingle, R. A., Stoker, C., Stone, W., Adams, N., Smith, R., Grant, M., Carré, I., Roden, L. C., & Denby, K. J. (2015). Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen Botrytis cinerea. *Plant Journal*,

84(5), 937–948. https://doi.org/10.1111/tpj.13050

Kilani, J., Davanture, M., Simon, A., Zivy, M., & Fillinger, S. (2020). Comparative quantitative proteomics of osmotic signal transduction mutants in Botrytis cinerea explain mutant phenotypes and highlight interaction with cAMP and Ca2+ signalling pathways. *Journal of Proteomics*, *212*, 103580. https://doi.org/https://doi.org/10.1016/j.jprot.2019.103580

Kim, H., Ridenour, J. B., Dunkle, L. D., & Bluhm, B. H. (2011). Regulation of stomatal tropism and infection by light in cercospora zeae-maydis: Evidence for coordinated host/pathogen responses to photoperiod? *PLoS Pathogens*, *7*(7). https://doi.org/10.1371/journal.ppat.1002113

Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D. A., Druzhinina, I. S., Thon, M., Zeilinger, S., Casas-Flores, S., Horwitz, B. A., Mukherjee, P. K., Mukherjee, M., Kredics, L., Alcaraz, L. D., Aerts, A., Antal, Z., Atanasova, L., Cervantes-Badillo, M. G., Challacombe, J., Chertkov, O., … Grigoriev, I. V. (2011). Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. *Genome Biology*, *12*(4). https://doi.org/10.1186/gb-2011-12-4-r40

Kubicek, C. P., Steindorff, A. S., Chenthamara, K., Manganiello, G., Henrissat, B., Zhang, J., Cai, F., Kopchinskiy, A. G., Kubicek, E. M., Kuo, A., Baroncelli, R., Sarrocco, S., Noronha, E. F., Vannacci, G., Shen, Q., Grigoriev, I. V., & Druzhinina, I. S. (2019). Evolution and comparative genomics of the most common Trichoderma species. *BMC Genomics*, *20*(1), 1–24. https://doi.org/10.1186/s12864-019-5680-7

Kurosawa, G., Fujioka, A., Koinuma, S., Mochizuki, A., & Shigeyoshi, Y. (2017). Temperature–amplitude coupling for stable biological rhythms at different temperatures. *PLoS Computational Biology*, *13*(6), 1–16. https://doi.org/10.1371/journal.pcbi.1005501

Kusakina, J., Gould, P., & Hall, A. (2014). A fast circadian clock at high temperatures is a conserved feature across Arabidopsis accessions and likely to be important for vegetative yield. *Plant, Cell & Environment*, *37*(2), 327–340. https://doi.org/10.1111/pce.12152

Lakin-Thomas, P. L., Bell-Pedersen, D., & Brody, S. (2011). The genetics of circadian rhythms in Neurospora. *Advances in Genetics*, *74*, 55–103. https://doi.org/10.1016/B978-0-12-387690-4.00003-9

Lamb, T. M., Finch, K. E., & Bell-Pedersen, D. (2012). The Neurospora crassa OS MAPK pathway-activated transcription factor ASL-1 contributes to circadian rhythms in pathway responsive clock-controlled genes. *Fungal Genetics and Biology*, *49*(2), 180–188. https://doi.org/10.1016/j.fgb.2011.12.006

Larrondo, L. F., & Canessa, P. (2019). The Clock Keeps on Ticking: Emerging Roles for Circadian Regulation in the Control of Fungal Physiology and Pathogenesis. *Current Topics in Microbiology and Immunology*, *422*, 121–156. https://doi.org/10.1007/82_2018_143

Larrondo, L. F., Loros, J. J., & Dunlap, J. C. (2012). High-resolution spatiotemporal analysis of gene expression in real time: In vivo analysis of circadian rhythms in Neurospora crassa using a FREQUENCY-luciferase translational reporter. *Fungal Genetics and Biology*, *49*(9), 681–683. https://doi.org/10.1016/j.fgb.2012.06.001

Larrondo, L. F., Olivares-Yañez, C., Baker, C. L., Loros, J. J., & Dunlap, J. C. (2015). Decoupling circadian clock protein turnover from circadian period determination. *Science*, *347*(6221). https://doi.org/10.1126/science.1257277

Lillo, C. (1993). Light-induced circadian rhythms in NADP+-glyceraldehyde-3-phosphate dehydrogenase mRNA in corn seedlings. *Journal of Interdisciplinary Cycle Research*, *24*(1), 65–71. https://doi.org/10.1080/09291019309360196

Lin, Y.-R., Lo, C.-T., Liu, S.-Y., & Peng, K.-C. (2012). Involvement of Pachybasin and Emodin in Self-Regulation of Trichoderma harzianum Mycoparasitic Coiling. *Journal of Agricultural and Food Chemistry*, *60*(9), 2123–2128. https://doi.org/10.1021/jf202773y

Liu, Y., & Bell-Pedersen, D. (2006). Circadian rhythms in Neurospora crassa and other filamentous fungi. *Eukaryotic Cell*, *5*(8), 1184–1193. https://doi.org/10.1128/EC.00133-06

Liu, Y., Merrow, M., Loros, J. J., & Dunlap, J. C. (1998). How temperature changes reset a circadian oscillator. *Science*, *281*(5378), 825–829. https://doi.org/10.1126/science.281.5378.825

Loros, J. J. (2020). Principles of the animal molecular clock learned from Neurospora. *European Journal of Neuroscience*, *51*(1), 19–33. https://doi.org/10.1111/ejn.14354

Martínez-Jarquín, S., Moreno-Pedraza, A., Guillén-Alonso, H., & Winkler, R. (2016). Template for 3D Printing a Low-Temperature Plasma Probe. *Analytical Chemistry*, *88*(14), 6976–6980. https://doi.org/10.1021/acs.analchem.6b01019

Martínez-Jarquín, S., & Winkler, R. (2013). Design of a low-temperature plasma (LTP) probe with adjustable output temperature and variable beam diameter for the direct detection of organic molecules. *Rapid Communications in Mass Spectrometry*, *27*(5), 629–634. https://doi.org/10.1002/rcm.6494

Mattern, D. L., Forman, L. R., & Brody, S. (1982). Circadian rhythms in Neurospora crassa: a mutation affecting temperature compensation. *Proceedings of the National Academy of Sciences of the United States of America*, *79*(3), 825–829. https://doi.org/10.1073/pnas.79.3.825

Medina-Castellanos, E., Esquivel-Naranjo, E. U., Heil, M., & Herrera-Estrella, A. (2014). Extracellular ATP activates MAPK and ROS signaling during injury response in the fungus trichoderma atroviride. *Frontiers in Plant Science*, *5*(NOV), 1–11. https://doi.org/10.3389/fpls.2014.00659

Michael, T. P., Salomé, P. A., Yu, H. J., Spencer, T. R., Sharp, E. L., McPeek, M. A., Alonso, J. M., Ecker, J. R., & McClung, C. R. (2003). Enhanced Fitness Conferred by Naturally Occurring Variation in the Circadian Clock. *Science*, *302*(5647), 1049–1053. https://doi.org/10.1126/science.1082971

Minter, D. W., Sutton, B. C., & Brady, B. L. (1983). What are phialides anyway? *Transactions of the British Mycological Society*, *81*(1), 109–120. https://doi.org/10.1016/s0007-1536(83)80210-1

Montenegro-Montero, A., Canessa, P., & Larrondo, L. F. (2015). Around the Fungal Clock: Recent Advances in the Molecular Study of Circadian Clocks in Neurospora and Other Fungi. *Advances in Genetics*, *92*, 107–184. https://doi.org/10.1016/bs.adgen.2015.09.003

Moreno-ruiz, D., Fuchs, A., Missbach, K., Schumacher, R., & Zeilinger, S. (2020). *Influence of Different Light Regimes on the Mycoparasitic Activity and t he Production of the Secondary Metabolite 6-Pentyl- α -Pyrone in Two Strains of Trichoderma Atroviride*. *1*(September), 1–16. https://doi.org/10.20944/preprints202009.0251.v1

Mukherjee, P. K., Horwitz, B. A., & Kenerley, C. M. (2012). Secondary metabolism in Trichoderma - A genomic perspective. *Microbiology*, *158*(1), 35–45.

https://doi.org/10.1099/mic.0.053629-0
Naglot, Goswami, Rahman, Shrimali, Yadav, Gupta, Rabha, Gogoi, & Veer. (2015). Antagonistic potential of native Trichoderma viride strain against potent tea fungal pathogens in north east India. *Plant Pathology Journal*, *31*(3), 278–289. https://doi.org/10.5423/PPJ.OA.01.2015.0004
Nolan, R. A., & Nolan, W. G. (1972). Elemental Analysis of Vitamin-Free Casamino Acids1. *Applied Microbiology*, *24*(2), 290–291. https://doi.org/10.1128/aem.24.2.290-291.1972
Ode, K. L., & Ueda, H. R. (2018). Lost in clocks: non-canonical circadian oscillation discovered in Drosophila cells . *Molecular Systems Biology*, *14*(9), 2–4. https://doi.org/10.15252/msb.20188567
Olivares-Yañez, C., Emerson, J., Kettenbach, A., Loros, J. J., Dunlap, J. C., & Larrondo, L. F. (2016). Modulation of circadian gene expression and metabolic compensation by the RCO-1 corepressor of Neurospora crassa. *Genetics*, *204*(1), 163–176. https://doi.org/10.1534/genetics.116.191064
Oliveira, A. G., Stevani, C. V., Waldenmaier, H. E., Viviani, V., Emerson, J. M., Loros, J. J., & Dunlap, J. C. (2015). Circadian control sheds light on fungal bioluminescence. *Current Biology*, *25*(7), 964–968. https://doi.org/10.1016/j.cub.2015.02.021
Omann, M. R., Lehner, S., Rodríguez, C. E., Brunner, K., & Zeilinger, S. (2012). The seven-transmembrane receptor Gpr1 governs processes relevant for the antagonistic interaction of Trichoderma atroviride with its host. *Microbiology*, *158*(1), 107–118. https://doi.org/10.1099/mic.0.052035-0
Oosterman, J. E., Kalsbeek, A., la Fleur, S. E., & Belsham, D. D. (2015). Impact of nutrients on circadian rhythmicity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *308*(5), R337–R350. https://doi.org/10.1152/ajpregu.00322.2014
Pang Z, Chong J, Li S, X. J. (2020). MetaboAnalystR 3.0: Toward an Optimized Workflow for Global Metabolomics. *Metabolites*, *10*(5), 186.
Pregueiro, A. M., Price-Lloyd, N., Bell-Pedersen, D., Heintzen, C., Loros, J. J., & Dunlap, J. C. (2005). Assignment of an essential role for the Neurospora frequency gene in circadian entrainment to temperature cycles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(6), 2210–2215. https://doi.org/10.1073/pnas.0406506102
Rahman, M. A., Begum, M. F., & Alam, M. F. (2009). Screening of Trichoderma Isolates as a Biological Control Agent against Ceratocystis paradoxa Causing Pineapple Disease of Sugarcane . *Mycobiology*, *37*(4), 277. https://doi.org/10.4489/myco.2009.37.4.277
Ratnayake, L., Adhvaryu, K. K., Kafes, E., Motavaze, K., & Lakin-Thomas, P. (2018). A component of the TOR (Target Of Rapamycin) nutrient-sensing pathway plays a role in circadian rhythmicity in Neurospora crassa. *PLoS Genetics*, *14*(6), 1–29. https://doi.org/10.1371/journal.pgen.1007457
Reino, J. L., Guerrero, R. F., Hernández-Galán, R., & Collado, I. G. (2008). Secondary metabolites from species of the biocontrol agent Trichoderma. *Phytochemistry Reviews*, *7*(1), 89–123. https://doi.org/10.1007/s11101-006-9032-2
Reithner, B., Schuhmacher, R., Stoppacher, N., Pucher, M., Brunner, K., & Zeilinger, S. (2007). Signaling via the Trichoderma atroviride mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. *Fungal Genetics and Biology*, *44*(11), 1123–1133. https://doi.org/10.1016/j.fgb.2007.04.001
Rocha-Ramírez, V., Omero, C., Chet, I., Horwitz, B. A., & Herrera-Estrella, A. (2002).

Trichoderma atroviride G-protein α-subunit gene tga1 is involved in mycoparasitic coiling and conidiation. *Eukaryotic Cell*, *1*(4), 594–605. https://doi.org/10.1128/EC.1.4.594-605.2002

Rodriguez-Romero, J., Hedtke, M., Kastner, C., Müller, S., & Fischer, R. (2010). Fungi, Hidden in Soil or Up in the Air: Light makes a difference. *Annual Review of Microbiology*, *64*, 585–610. https://doi.org/10.1146/annurev.micro.112408.134000

Rosas-Román, I., Ovando-Vázquez, C., Moreno-Pedraza, A., Guillén-Alonso, H., & Winkler, R. (2020). Open LabBot and RmsiGUI: Community development kit for sampling automation and ambient imaging. *Microchemical Journal*, *152*(July), 1–11. https://doi.org/10.1016/j.microc.2019.104343

Salichos, L., & Rokas, A. (2010). The diversity and evolution of circadian clock proteins in fungi. *Mycologia*, *102*(2), 269–278. https://doi.org/10.3852/09-073

Sancar, C., Sancar, G., Ha, N., Cesbron, F., & Brunner, M. (2015). Dawn- and dusk-phased circadian transcription rhythms coordinate anabolic and catabolic functions in Neurospora. *BMC Biology*, *13*(1), 17–19. https://doi.org/10.1186/s12915-015-0126-4

Sancar, G., Sancar, C., & Brunner, M. (2012). Metabolic compensation of the Neurospora clock by a glucose-dependent feedback of the circadian repressor CSP1 on the core oscillator. *Genes and Development*, *26*(21), 2435–2442. https://doi.org/10.1101/gad.199547.112

Schmoll, M., Esquivel-Naranjo, E. U., & Herrera-Estrella, A. (2010). Trichoderma in the light of day - Physiology and development. *Fungal Genetics and Biology*, *47*(11), 909–916. https://doi.org/10.1016/j.fgb.2010.04.010

Schmoll, M., & Wang, T.-F. (2016). 17 Sexual Development in Trichoderma. *Growth, Differentiation and Sexuality*, 457–474. https://doi.org/10.1007/978-3-319-25844-7_17

Seidl, V., Song, L., Lindquist, E., Gruber, S., Koptchinskiy, A., Zeilinger, S., Schmoll, M., Martínez, P., Sun, J., Grigoriev, I., Herrera-Estrella, A., Baker, S. E., & Kubicek, C. P. (2009). Transcriptomic response of the mycoparasitic fungus Trichoderma atroviride to the presence of a fungal prey. *BMC Genomics*, *10*, 1–13. https://doi.org/10.1186/1471-2164-10-567

Serrano-Carreon, L., Hathout, Y., Bensoussan, M., & Belin, J.-M. (1993). Metabolism of Linoleic Acid or Mevalonate and 6-Pentyl-α-Pyrone Biosynthesis by Trichoderma Species. *Applied and Environmental Microbiology*, *59*(9), 2945 LP – 2950. http://aem.asm.org/content/59/9/2945.abstract

Sharma, P., Vignesh Kumar, P., Ramesh, R., Saravanan, K., Deep, S., Sharma, M., Mahesh, S., & Dinesh, S. (2011). Biocontrol genes from Trichoderma species: A review. *African Journal of Biotechnology*, *10*(86), 19898–19907. https://doi.org/10.5897/AJBX11.041

Shi, M., Larrondo, L. F., Loros, J. J., & Dunlap, J. C. (2007). A developmental cycle masks output from the circadian oscillator under conditions of choline deficiency in Neurospora. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(50), 20102–20107. https://doi.org/10.1073/pnas.0706631104

Shinohara, M. L., Correa, A., Bell-Pedersen, D., Dunlap, J. C., & Loros, J. J. (2002). Neurospora clock-controlled gene 9 (ccg-9) encodes trehalose synthase: Circadian regulation of stress responses and development. *Eukaryotic Cell*, *1*(1), 33–43. https://doi.org/10.1128/EC.1.1.33-43.2002

Sood, M., Kapoor, D., Kumar, V., Sheteiwy, M. S., Ramakrishnan, M., Landi, M., Araniti, F., & Sharma, A. (2020). Trichoderma: The "secrets" of a multitalented biocontrol agent. *Plants*, *9*(6), 1–25. https://doi.org/10.3390/plants9060762

Speckbacher, V., Ruzsanyi, V., Martinez-Medina, A., Hinterdobler, W., Doppler, M., Schreiner, U., Böhmdorfer, S., Beccaccioli, M., Schuhmacher, R., Reverberi, M., Schmoll, M., & Zeilinger, S. (2020). The Lipoxygenase Lox1 Is Involved in Conidiation , and Volatile Organic Compound Biosynthesis in the Mycoparasitic Fungus Trichoderma atroviride. *Frontiers in Microbiology*, *11*(August), 1–18. https://doi.org/10.3389/fmicb.2020.02004

Speckbacher, V., Ruzsanyi, V., Wigger, M., & Zeilinger, S. (2020). The Trichoderma atroviride Strains P1 and IMI 206040 Differ in Their Light-Response and VOC Production. *Molecules*, *25*(1). https://doi.org/10.3390/molecules25010208

Stecca Steindorff, A., Soller Ramada, M. H., Siqueira, A., Coelho, G., Neil, R., Miller, G., Joannis, G., Júnior, P., Ulhoa, C. J., & Ferreira Noronha, E. (2014). Identification of mycoparasitism-related genes against the phytopathogen Sclerotinia sclerotiorum through transcriptome and expression profile analysis in Trichoderma harzianum. *BMC Genomics*, *15*, 1–14.

Steyaert, J. M. (2007). Studies on the regulation of conidiation in specios of Trichoderma. *PhD Thesis, Lincoln University*.

Steyaert, J. M., Ridgway, H. J., Elad, Y., & Stewart, A. (2003). Genetic basis of mycoparasitism: A mechanism of biological control by species of Trichoderma. *New Zealand Journal of Crop and Horticultural Science*, *31*(4), 281–291. https://doi.org/10.1080/01140671.2003.9514263

Steyaert, J., Weld, R. J., Loguercio, L. L., & Stewart, A. (2010b). Rhythmic conidiation in the blue-light fungus Trichoderma pleuroticola. *Fungal Biology*, *114*(2–3), 219–223. https://doi.org/10.1016/j.funbio.2010.01.001

Steyaert, Johanna M., Weld, R. J., Mendoza-Mendoza, A., & Stewart, A. (2010a). Reproduction without sex: Conidiation in the filamentous fungus Trichoderma. *Microbiology*, *156*(10), 2887–2900. https://doi.org/10.1099/mic.0.041715-0

Steyaert, Johanna M., Weld, R. J., & Stewart, A. (2010c). Isolate-specific conidiation in Trichoderma in response to different nitrogen sources. *Fungal Biology*, *114*(2–3), 179–188. https://doi.org/10.1016/j.funbio.2009.12.002

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., & Schuhmacher, R. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus Trichoderma atroviride by HS-SPME-GC-MS. *Journal of Microbiological Methods*, *81*(2), 187–193. https://doi.org/10.1016/j.mimet.2010.03.011

Talora, C., Franchi, L., Linden, H., Ballario, P., & Macino, G. (1999). Role of a white collar-1-white collar-2 complex in blue-light signal transduction. *EMBO Journal*, *18*(18), 4961–4968. https://doi.org/10.1093/emboj/18.18.4961

Tisch, D., & Schmoll, M. (2010). Light regulation of metabolic pathways in fungi. *Applied Microbiology and Biotechnology*, *85*(5), 1259–1277. https://doi.org/10.1007/s00253-009-2320-1

Topolovec-Pintaric, S. (2019). *Trichoderma: Invisible Partner for Visible Impact on Agriculture*. https://doi.org/10.5772/intechopen.83363

Traeger, S., & Nowrousian, M. (2015). Analysis of circadian rhythms in the basal filamentous ascomycete Pyronema confluens. *G3: Genes, Genomes, Genetics*, *5*(10), 2061–2071. https://doi.org/10.1534/g3.115.020461

Vaze, K. M., & Sharma, V. K. (2013). On the adaptive significance of circadian clocks for their owners. *Chronobiology International*, *30*(4), 413–433. https://doi.org/10.3109/07420528.2012.754457

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., Woo, S. L., & Lorito, M. (2008). A novel role for Trichoderma secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathology*, *72*(1–3), 80–86. https://doi.org/10.1016/j.pmpp.2008.05.005

Vinale, Francesco, Sivasithamparam, K., Ghisalberti, E. L., Woo, S. L., Nigro, M., Marra, R., Lombardi, N., Pascale, A., Ruocco, M., Lanzuise, S., Manganiello, G., & Lorito, M. (2014). Trichoderma Secondary Metabolites Active on Plants and Fungal Pathogens. *The Open Mycology Journal*, *8*(1), 127–139. https://doi.org/10.2174/1874437001408010127

Vitalini, M. W., De Paula, R. M., Goldsmith, C. S., Jones, C. A., Borkovich, K. A., & Bell-Pedersen, D. (2007). Circadian rhythmicity mediated by temporal regulation of the activity of p38 MAPK. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(46), 18223–18228. https://doi.org/10.1073/pnas.0704900104

Vos, C. M. F., De Cremer, K., Cammue, B. P. A., & De Coninck, B. (2015). The toolbox of Trichoderma spp. in the biocontrol of Botrytis cinerea disease. *Molecular Plant Pathology*, *16*(4), 400–412. https://doi.org/10.1111/mpp.12189

Weirauch, M. T., Yang, A., Albu, M., Cote, A. G., Montenegro-Montero, A., Drewe, P., Najafabadi, H. S., Lambert, S. A., Mann, I., Cook, K., Zheng, H., Goity, A., van Bakel, H., Lozano, J. C., Galli, M., Lewsey, M. G., Huang, E., Mukherjee, T., Chen, X., … Hughes, T. R. (2014). Determination and inference of eukaryotic transcription factor sequence specificity. *Cell*, *158*(6), 1431–1443. https://doi.org/10.1016/j.cell.2014.08.009

Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K., & Johnson, C. H. (2004). The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. *Current Biology : CB*, *14*(16), 1481–1486. https://doi.org/10.1016/j.cub.2004.08.023

Yerushalmi, S., & Green, R. M. (2009). Evidence for the adaptive significance of circadian rhythms. *Ecology Letters*, *12*(9), 970–981. https://doi.org/10.1111/j.1461-0248.2009.01343.x

Yoshida, Y., Maeda, T., Lee, B., & Hasunuma, K. (2008). Conidiation rhythm and light entrainment in superoxide dismutase mutant in Neurospora crassa. *Molecular Genetics and Genomics*, *279*(2), 193–202. https://doi.org/10.1007/s00438-007-0308-z

Zeilinger, S., Reithner, B., Scala, V., Peissl, I., Lorito, M., & Mach, R. L. (2005). Signal Transduction by Tga3, a Novel G Protein ⎵ Subunit of. *Applied and Environmental Microbiology*, *71*(3), 1591–1597. https://doi.org/10.1128/AEM.71.3.1591

Zielinski, T., Moore, A. M., Troup, E., Halliday, K. J., & Millar, A. J. (2014). Strengths and Limitations of Period Estimation Methods for Circadian Data. *PLOS ONE*, *9*(5), e96462. https://doi.org/10.1371/journal.pone.0096462