

1 **Running Head:**
2 **Circadian rhythm of a downy mildew pathogen**

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1 **Synchronization of Circadian Clock Gene Expression in *Arabidopsis* and**
2 ***Hyaloperonospora arabidopsidis* and its Impact on Host-Pathogen**
3 **Interactions**

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30

1 **Abstract**

2 Organisms across all kingdoms have an internal circadian clock running in 24h cycles.
3 This clock affects a variety of processes, including innate immunity in plants. However,
4 the role of pathogen circadian clocks had not been extensively explored. We previously
5 showed that light can influence infection of the oomycete *Hyaloperonospora*
6 *arabidopsidis* (*Hpa*, downy mildew disease) on its natural host *Arabidopsis thaliana*.
7 Here, we identified *Hpa* orthologs of known circadian clock genes (CCGs) *Drosophila*
8 *TIMELESS* (*TIM*) and *Arabidopsis Sensitive to Red Light Reduced 1* (*AtSRR1*) genes.
9 Expression of both *HpaTIM* and *HpaSRR1* showed a circadian rhythm when *Hpa* was
10 exposed to constant light. Contrastingly, these two genes were negatively regulated by
11 constant dark exposure. Furthermore, the expression patterns of *HpaTIM* and *HpaSRR1*
12 correlate with those of *AtCCA1* and *AtLHY*, indicating a synchronisation of biological
13 clock genes between the host and the pathogen. In addition, screening mutants of
14 *Arabidopsis* Clock Regulated Genes (*AtCRGs*) with three virulent *Hpa* isolates revealed
15 that mutations in *AtCRGs* influenced *HpaTIM* and *HpaSRR1* expression and *Hpa*
16 development, indicating a functional link between the plant biological clock and
17 virulence. Moreover, sporulation of *Hpa* was reduced by targeting *HpaTIM* and
18 *HpaSRR1* with short synthesized small interfering RNAs, indicating that the pathogen
19 clock is also relevant to virulence. We propose that plant and pathogen clocks are
20 synchronized during infection and that proper regulation of both clocks are genetically
21 necessary for pathogen virulence.

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24

25 **Keywords: Circadian clock, downy mildew, *Arabidopsis*, compatibility, plant-**
26 **microbe interactions**

27

1 **Introduction**

2 Circadian clocks are endogenous subcellular machines that allow organisms to
3 anticipate predictable environmental changes. Changes in the expression of circadian
4 clock genes (CCGs) contribute to plant adaptation to environmental changes, including
5 growth regulation, photoperiodic control of flowering, and responses to biotic and abiotic
6 stresses (Creux & Harmer, 2019; Luklova *et al.*, 2019). Similarly, in fungi such as
7 *Neurospora crassa* and the plant-pathogen *Botrytis cinerea*, circadian rhythms affect
8 many processes, such as nutrient uptake, metabolism, conidial production and virulence
9 (Baker *et al.*, 2012; Hevia *et al.*, 2015). In addition, the circadian rhythms of plants and
10 microbes can influence the timing and outcome of their interactions, including the
11 establishment of beneficial or harmful relationships and defence responses (Bhardwaj
12 *et al.*, 2011; Hubbard *et al.*, 2018; Newman *et al.*, 2022).

13
14 The main external signals that affect circadian regulation are light and temperature
15 (Annunziata *et al.*, 2018; Wang *et al.*, 2020). The centre of all known circadian clocks
16 contains at least one internal autonomous circadian oscillator, with positive and negative
17 elements that create automatic regulatory feedback loops (Hevia *et al.*, 2015). In most
18 cases, these loops are used to create 24h timing circuits (Hennessey & Field, 1992).
19 Components of these loops can directly or indirectly receive environmental input to allow
20 entrainment of the clock to environmental time and transfer temporal information through
21 output pathways to regulate expression of rhythmic clock-regulated genes (CRGs) and
22 rhythmic biological activities (Panda *et al.*, 2002).

23
24 Studies with the model plant *Arabidopsis* and its obligate downy mildew pathogen
25 *Hyaloperonospora arabidopsidis* (*Hpa*) have illuminated many aspects of plant-
26 pathogen interactions (Holub, 2007; Herlihy *et al.*, 2019). For example, several disease
27 resistance genes against *Hpa* have been molecularly cloned from *Arabidopsis*. The *R*-
28 gene *RPP4* encodes a nucleotide-binding leucine-rich repeat protein with
29 Toll/interleukin-1 receptor domains and provides resistance to isolates *Hpa*-Emoy2 and
30 *Hpa*-Emwa1 (Van Der Biezen *et al.*, 2002). Recent studies on this gene identified a link
31 between *R*-gene-mediated defence and the circadian clock of the host plant (Wang *et al.*, 2011). This raises the question of whether *Hpa*'s clock influences virulence in
32 compatible host plants, and whether the host and pathogen clocks might influence each
33 other. Recently, we reported that all developmental stages of *Hpa* during the infection
34

1 cycle (germination, mycelial growth, and sporulation) are subject to photoregulation
2 (Telli *et al.*, 2020), prompting a search for oscillator and clock-related genes (CRGs) in
3 *Hpa*.

4
5 The TIMELESS (TIM) gene regulate circadian clocks across species (Myers *et al.*, 1995;
6 Lee *et al.*, 1996; Panda *et al.*, 2002). In *Drosophila*, TIM facilitates entrainment to light–
7 dark cycles by undergoing degradation induced by light, allowing adaptation to the 24h
8 environmental cycle (Allada & Chung, 2010; Rothenfluh *et al.*, 2000). TIM forms a
9 complex with the Period (PER) protein in the evening that represses clock gene
10 transcription. Degradation of this complex is initiated by a phosphorylation cycle at late
11 night (Rosato & Kyriacou, 2002). TIM homologs in other organisms like mice and
12 humans have varied functions, with roles in embryonic development and cell cycle
13 regulation (Unsal-Kaçmaz *et al.*, 2005; Gotter *et al.*, 2000; Young & Kay, 2001),
14 indicating evolutionary divergence while maintaining coordination with circadian
15 rhythms.

16
17 *SRR1* (*SENSITIVITY TO RED LIGHT REDUCED*) is a functional gene for clock-
18 regulated expression during day–night cycle (Staiger *et al.*, 2003). It was first identified
19 in *Arabidopsis* and its orthologs have been discovered in various organisms (Johansson
20 & Staiger, 2014). *Arabidopsis srr1* mutants display some disfunctions in hypocotyl
21 elongation, greening, petiole growth and flowering, indicating *SRR1* is multifunctional in
22 *Arabidopsis* (Staiger *et al.*, 2003). The mouse *SRR1* homologue plays a role in circadian
23 rhythms and cell proliferation (Adachi *et al.*, 2017). Similarly, the yeast *SRR1*-like protein
24 BER1 (Benomyl RESistant 1) is involved in microtubule stability and cell proliferation
25 (Fiechter *et al.*, 2008).

26
27 Considering the effects of light on *Hpa* virulence (Telli *et al.*, 2020) and the rich
28 information on circadian control in other organisms, we reasoned that it would be useful
29 to examine circadian clock regulation in *Hpa*. Here, we identified *HpaTIM* and *HpaSRR1*
30 in *Hpa*, and investigated their expression patterns under different light regimes.
31 Additionally, we investigated the expression pattern of three well-characterized
32 *Arabidopsis* circadian clock genes, *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*),
33 *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *LATE ELONGATED HYPOCOTYL*
34 (*LHY*) during infection by *Hpa* under different light regimes. We report here that *HpaTIM*

1 and *HpaSRR1* show rhythmic expression and synchrony with the expression of *CCA1*
2 and *LHY*, and we provide genetic evidence from the host and pathogen that supports
3 the biological relevance of this synchronization.

4

5 **Results**

6 ***Hpa* encodes clock-related genes**

7 Until now, no investigation on circadian-related genes has been reported for *Hpa*. We
8 addressed this knowledge gap by searching the *Hpa* genome for homologues of
9 important circadian genes from other organisms, using BLAST and domain-searches
10 using protein domains that are characteristic of the relevant proteins in model
11 organisms. Two putative CRGs were identified in the *Hpa* genome: *Timeless* (*Hpa*-
12 *G810921*, designated *HpaTIM*) and *Sensitive to Red Light Reduced 1* (*Hpa-G801448*,
13 designated *HpaSRR1*). Further bioinformatic analyses and EnsemblProtists gene
14 annotation revealed that *HpaTIM* exists as a single-copy gene with two introns that
15 encodes a predicted protein of 1175 amino acids with a molecular mass of 131.7 kDa.
16 Domain and motif searches of *HpaTIM* revealed two TIMELESS domains (N35-A592;
17 W688-T751) (Supplemental Figure 1). Amino acid sequences of TIMELESS proteins
18 from various species were aligned (Figure 1A) and a phylogenetic tree was constructed
19 (Figure 1C). *HpaTIM* showed a high amino-acid identity to TIM proteins from other
20 species (Figures 1A and C). *HpaTIM* orthologues were also found in other oomycete
21 pathogens (Supplemental Figure 2). Published transcriptome data in *Arabidopsis* Col-0
22 inoculated with the avirulent or virulent *Hpa* isolates Emoy2 or Waco9, respectively (Asai
23 *et al.*, 2018) indicates that *HpaTIM* is expressed in spores and during infection
24 (Supplemental Figure 3).

25

26 *HpaSRR1* exists as a single copy in the reference *Hpa* genome and has three predicted
27 introns. The open reading frame of *HpaSRR1* encodes a predicted protein of 335 aa
28 (molecular weight 37.013 kDa). Domain and motif searches revealed a SRR1-like
29 protein domain (H3-S304) (Supplemental Figure 4). Proteins from various species were
30 identified, amino-acid sequences were aligned (Figure 1B) and a phylogenetic tree was
31 constructed (Figure 1D). Additionally, orthologues of *HpaSRR1* were identified in other
32 oomycete pathogens, indicating the conserved nature of the gene (Supplemental Figure
33 5). Expression of this gene was not evident in the published transcriptome data (Asai *et*

1 *al.*, 2018). However, we were able to demonstrate *HpaSSR1* expression during
2 infection, as described in the following section.

3

4 **Targeting *HpaTIM* and *HpaSRR1* with small dsRNA reduces *Hpa* sporulation**

5 Following identification of *HpaTIM* and *Hpa-SSR1*, we used a genetic approach to test
6 whether these genes are necessary for *Hpa* virulence on *Arabidopsis*. *Hpa* is an obligate
7 biotroph and cannot be genetically transformed by conventional approaches. However,
8 a small RNA-based approach was recently developed to reduce the expression of
9 targeted *Hpa* genes through transcriptional or translational silencing (Bilir *et al.*, 2019).
10 Short, synthetic, double-stranded RNAs (SS-dsRNAs) were designed to target *HpaTIM*
11 and *HpaSSR1*. These SS-dsRNAs were mixed with *Hpa-Emoy2* spores at 5 μ M
12 concentrations and used to drop-inoculate 7-day old seedlings of the disease-
13 susceptible mutant *Arabidopsis Ws-eds1*. At 7dpi, plants inoculated with spore
14 suspensions containing 5 μ M SS-dsRNA targeting *HpaTIM* and *HpaSRR1* showed
15 reduced sporulation (~50-70%) compared to plants inoculated with untreated spores
16 (Figure 2A). No sporulation was observed with the positive control targeting the essential
17 *Hpa-CesA3* gene as reported before (Bilir *et al.*, 2019). We then checked the relative
18 mRNA abundance of the targeted genes. While there was statistically significant
19 reduction in the expression level of *Hpa-CesA3*, we did not observe any significant
20 decrease in the expression level of *HpaTIM* and *HpaSRR1* (Figure 2B), suggesting that
21 the SS-dsRNAs interfered with translation of *HpaTIM* and *HpaSRR1* rather than
22 transcription.

23

24 ***HpaTIM* gene is influenced by light regime**

25 After establishing *HpaTIM* as a strong candidate CRG, with biologically relevant effects
26 on *Hpa* virulence, we wanted to investigate whether *HpaTIM* expression shows a
27 circadian rhythm during infection of *Arabidopsis*. Because Col-0 is resistant to *Hpa*-
28 Emoy2, we used a susceptible mutant (*Col-rpp4*) seedlings in the experiments. Seven-
29 day old seedlings were infected at 0 ZT hour with *Hpa* spores, allowed to grow under a
30 “normal” light regime (12h D / 12h L) for the first 3 days, and then were exposed to
31 constant light or constant dark for 24h between 3 to 4dpi. After the 24h exposure,
32 samples were taken every 6h from infected plants between 4-7dpi. Similarly, samples
33 were also taken from infected plants between 4-7dpi that were kept under the normal

1 light cycle to serve as controls. Abundance of the *HpaTIM* mRNA was quantified with
2 qRT-PCR.

3
4 *HpaTIM* displayed a rhythmic pattern with a 24h period under a normal 12h D / 12h L
5 (DL) cycle (Figure 3A and B). Expression peaked at the beginning of each light cycle
6 (dawn) and gradually decreased until the beginning of the dark cycle (Figure 3A). After
7 24h constant light exposure (between 3 and 4dpi), the expression pattern of the *HpaTIM*
8 was disrupted slightly: although the amplitude was not changed, the length of the period
9 shortened to around 18h, but was still rhythmic indicating that this gene has a circadian
10 pattern. At 60 hrs after constant light treatment (6 dpi), *HpaTIM* expression returned to
11 its normal 24h-period cycle (Figure 3A).

12
13 The amplitude of *HpaTIM* expression in tissues exposed to constant dark for 24 hours
14 was much lower compared to tissues exposed to normal or constant light. There was
15 also a shift in the expression cycle of *HpaTIM* in tissues exposed to 24h constant dark
16 between 3 and 4 dpi (Figure 3B). The period was shortened, however, by day 6 the
17 cycle of expression had almost returned to normal.

18
19 In a second set of experiments, inoculated plants were allowed to grow for 3 days under
20 a normal light regime (12h D / 12h L) and were then exposed to 4 days constant light or
21 constant dark (Figure 4). The expression of *HpaTIM* under constant light showed
22 reduced period and increased amplitude in comparison to the control. The expression
23 peaked at different times, such as into 4 and 5 dpi, peaks were observed at dusk,
24 however into 6 and 7dpi, peaks were observed at dawn. *HpaTIM* expression under
25 constant dark did not display a proper period and a clear peak (Figure 4), suggesting
26 that it may be totally suppressed.

27
28 ***HpaSRR1 shows rhythmic expression***

29 Similar to *HpaTIM*, we investigated *HpaSRR1* expression under different light regimes.
30 Inoculated plants were grown under normal light regimes for 3d (12h D / 12h L) and then
31 were exposed to constant light or constant dark for 24h. Samples were taken every 6h
32 for 3 days and expression pattern of *HpaSRR1* was determined. Under a normal DL
33 regime, expression of *HpaSRR1* showed a periodic cycle similar to that of *HpaTIM*.

1 Expression peaked at dawn for each day (Figure 5). Exposure to constant light at 3-4
2 dpi did not change the expression pattern of *HpaSSR1*; the gene exhibited a rhythmic
3 expression pattern under the constant light just as in the normal cycle. The amplitude of
4 *HpaSSR1* expression was slightly higher at the beginning but in general, it was very
5 similar to that of the control (Figure 5A).

6
7 *HpaSSR1* expression levels were observed with samples, which were exposed to
8 constant dark at 3-4 dpi (Figure 5B). Similar to the DL and 3-4 dpi constant light series,
9 all peaks in expression levels were observed at dawn for each day. However,
10 expression levels in samples exposed to constant dark were lower than that found with
11 constant light (4d/0h) and 4d/6h. These findings indicate that *HpaSSR1* expression may
12 have rhythmic oscillation, even under the irregular light regime for a short period where
13 expression patterns were not broken or shifted (Figure 5A).

14
15 ***Arabidopsis and Hpa timing systems are synchronised***
16 *Hpa* has an obligate biotrophic lifestyle and cannot exist apart from its host (Woods-Tör
17 *et al.*, 2018). Given this intimate relationship, it is plausible that the *Hpa*'s clock is
18 synchronized with the *Arabidopsis*' clock during infection. In the *Arabidopsis* circadian
19 clock system, expression of *CCA1* (*Circadian Clock Associated 1*), *LHY* (*Late Elongated*
20 *Hypocotyl*) and *TOC1* (*Timing of CAB expression 1*) genes are commonly used as
21 biomarkers for circadian regulation (McClung, 2006). Expression of *CCA1* and *LHY* has
22 been reported to peak at dawn, and *TOC1* expression peaks have been observed at
23 dusk. (McClung, 2006; De Caluwé *et al.*, 2016). Reciprocal regulation
24 between *CCA1*, *LHY*, and *TOC1* is thought to provide a feedback loop mechanism
25 which is essential for circadian rhythmicity in *Arabidopsis* (Alabadi *et al.*, 2001)

26
27 We wanted to determine whether there is a correlation between the expression pattern
28 of *HpaTIM* and *HpaSSR1* genes compared to *Arabidopsis* *CCA1*, *LHY* and *TOC1*
29 genes. First, we examined the expression patterns of *CCA1* and *LHY* over 3d between
30 4 and 7dpi. Expression of both *CCA1* and *LHY* peaked at dawn during this period (Figure
31 6A). *CCA1* expression levels were higher than that of *LHY*. With *TOC1* expressions,
32 peaks were observed at dusk, and were lowest at dawn (Figure 6A). These results were
33 in agreement with published data from uninfected plants (Alabadi *et al.*, 2001).
34 Secondly, we compared the rhythmic expression patterns of *HpaTIM* and *HpaSSR1*

1 with that of *CCA1* and *LHY*. We observed that the expression pattern of *HpaTIM* and
2 *HpaSRR1* were very similar to that of *CCA1* and *LHY* (Figure 6B). In all series,
3 expression levels were peaked at dawn. Expression levels increased during the dark
4 and decreased in light cycle. These findings suggest that *Arabidopsis-Hpa* pathosystem
5 has a synchronised circadian regulation.

7 **Mutations in *cca1* and *cca1/lhy* influence *HpaTIM* and *HpaSRR1* expressions**

8 If the *Arabidopsis* and *Hpa* clocks are synchronized, then *Arabidopsis* mutations that
9 affect circadian regulation might disrupt the regulation of CRGs in *Hpa* during the
10 infection cycle. To address this prediction, we investigated expression patterns of
11 *HpaTIM* and *HpaSRR1* during infection of Col-*cca1* single mutants and Col-*cca1/lhy*
12 double mutant lines.

13
14 *HpaTIM* and *HpaSRR1* expression were assayed between 4 and 6 dpi on the *cca1*
15 mutant line (Figure 7A). Although *HpaTIM* and *HpaSRR1* showed a circadian
16 expression pattern on the Col-*cca1* mutant, this pattern did not exactly match with that
17 of *HpaTIM* and *HpaSRR1* on wild-type Col-0 line (Figure 7A). When compared with the
18 normal pattern; the expression peaks on the *cca1* mutant line were observed not at
19 dawn, but in the middle of the day (4d.18h and 5d.18h), and the lowest points of the
20 expression were observed in the middle of the night (Figure 7A), indicating that the
21 *CCA1* gene influences the timing but not the amplitude of *HpaTIM* and *HpaSRR1*
22 expression.

23
24 Similarly, expression levels of *HpaTIM* and *HpaSRR1* were also investigated in the Col-
25 *cca1/lhy* double mutants between 4 and 6 dpi for 2d (Figure 7B). The expression levels
26 of *HpaTIM* and *HpaSRR1* observed on the double mutant differed significantly from the
27 expression levels observed on the wild-type Col-0. *HpaTIM* and *HpaSRR1* expression
28 levels were peaked in the middle of the night (4d, 6h), at the beginning of the day (5d)
29 and in the middle of the day (5d,18h) (Figure 7B). The expression patterns of *HpaTIM*
30 and *HpaSRR1* were still similar and parallel to each other, and peaks were shifted
31 (Figure 7B), indicating that *HpaTIM* and *HpaSRR1* are regulated during infection by a
32 common mechanism that requires *Arabidopsis CCA1* and *LHY1* genes.

34 **Mutation in *Arabidopsis* CRGs alters *Hpa* sporulation and biomass production**

1 Observation of altered *Hpa* CR gene expression on *Arabidopsis* single and double clock
2 mutants prompted us to determine whether pathogen sporulation and biomass
3 production was affected by mutations in *Arabidopsis* CRGs. We screened 26 single-, 2
4 double- and 1 triple mutants, along with 3 overexpressors (ox) lines.

5
6 Each homozygous mutant line, and *CCA1ox* lines were inoculated with the compatible
7 isolate *Hpa*-Noks1, and the amount of sporulation was calculated and compared to the
8 control Col-0 line (Figure 8A). Overall, 10 lines supported less sporulation than that in
9 the control: *elf3* (75%), *phyb* (75%), *kat2* (73%), *lux* (73%), *cry1* (70%), *lcl5* (65%),
10 *CCA1ox* (60%), *lhy* (60%), *cca1*(47%) and *pif3* (47%) (Figure 8A), indicating that that
11 these CRGs contribute to compatibility. Contrastingly, three mutant lines supported
12 more sporulation compared to the Col-0 control: *prp9* (138%), *toc1*(135%) and *tic1*
13 (133%) (Figure 8A), suggesting that these genes could be essential for basal defence
14 in *Arabidopsis*.

15
16 The 13 mutant lines displaying a significant sporulation phenotype with *Hpa*-Noks1 were
17 evaluated for vegetative hyphal biomass at 3 dpi. Among the 13 lines, *cry1 phyb* (66%),
18 *cry1* (64%) and *elf3* (60%) produced less biomass production compared to the wild-type
19 control. Contrastingly more biomass was produced in *lux* (241%), *tic1* (226%),
20 *toc1*(173%), *cca1* (141%) and *prp9* (140%) (Figure 8B). Interestingly, null *lux* and *cca1*
21 mutant lines produced a smaller number of conidiophores despite increased hyphal
22 biomass amounts.

23
24 Inoculation of *Arabidopsis* CRG mutants with the compatible isolate *Hpa*-Maks9 was
25 carried out in the same way as with *Hpa*-Noks1. Reduced sporulation was observed in
26 seven lines: *prp3* (74%), *prp7* (72%), *elf3* (71%), *cca1* (69%), *toc1* (61%), *prp5* (56%) and
27 *phyb* (55%), suggesting the genes are required for compatibility. Enhanced sporulation
28 was observed in three lines (Figure 8C): *kat2* (177%), *det1* (163%) and *lhy* (150%),
29 indicating that these genes play a significant role for basal defence against *Hpa*-Maks9.

30
31 *Hpa*-Maks9 hyphal biomass was evaluated for these ten mutant lines: *cca1* (73%), *det1*
32 (70%), *toc1* (66%) and *elf3* (62%) produced less biomass than then control; by contrast,
33 *prp7* (155%), *prp5* (141%), *phyb* (138%), *lhy* (137%), *prp3* (133%) and *kat2* (117) had
34 more biomass than that obtained with control Col-0 (Figure 8D). Biomass production in

1 *phyb*, *prp3*, *prp7*, *prp5* were clearly higher than that in control at 3dpi, whereas sporulation
2 was reduced at 7dpi.

3

4 Result obtained with *Hpa*-Maks9 on *elf*, *phyb* and *cca1* mutant lines were similar to those
5 recorded with *Hpa*-Noks1, showing less sporulation than that on controls. Contrastingly,
6 *kat2* and *lhy* mutant lines supported less sporulation, and *toc1* showed more sporulation
7 with *Hpa*-Noks1 infection, giving totally opposite results to those obtained with Maks9.
8 These differences suggest that some mutants might have isolate-specific effects.

9

10 As *lhy/toc1* and *cca1/lhy* double mutants, *cca1/lhy/toc1* triple mutants were in Ws-0
11 background, the Ws-compatible isolate *Hpa*-Emco5 were used for both sporulation and
12 biomass production assays. While overexpressors including TOC1ox (113%), LHYox
13 (102%) showed results similar to that obtained from the control, *cca1/lhy* (40%), *lhy/toc1*
14 (34%) and a triple mutant line *cca1/lhy/toc1* (%3) showed statistically significant less
15 sporulation with *Hpa*-Emco5 compared to the Ws control (Figure 8E). *Hpa* biomass was
16 investigated 3 dpi and the results were consistent with the sporulation data (Figure 8F):
17 *lhy/toc1* 46%, *cca1/lhy* 49%, and the triple mutant line *cca1/lhy/toc1* showed the lowest
18 biomass production with 12% (Figure 8F).

19

20 **Discussion**

21

22 Circadian rhythms have long been known to govern various aspects of plant physiology,
23 from growth, differentiation, metabolism and flowering to responses to environmental
24 stresses (Annunziata *et al.*, 2018; Luklova *et al.*, 2019; Liang *et al.*, 2022; Zhu *et al.*,
25 2022). Similarly, microbial microorganisms including fungi and cyanobacteria have their
26 own circadian rhythms that influence processes such as metabolism, nutrient uptake
27 and virulence (Brody, 2019; Valim *et al.*, 2022). Previously we showed that light plays a
28 significant role in the development of *Hpa* (Telli *et al.*, 2020). In this study, our exploration
29 of the interplay between circadian rhythms and plant-microbe interactions in the
30 *Arabidopsis-Hpa* pathosystem revealed the circadian-related genes (CRGs) *HpaTIM*
31 and *HpaSRR1*. This current study extends our understanding to a biotrophic oomycete
32 pathogen, offering compelling evidence that *Hpa* possesses its own circadian-regulated
33 genes that are necessary for full virulence and provides evidence that circadian rhythms

1 in a biotrophic pathogen can be influenced, directly or indirectly, by CRGs in the plant
2 host.

3
4 The discovery of *HpaTIM*, and *HpaSRR1* genes in a plant-obligate oomycete pathogen
5 emphasizes the universal nature of circadian rhythms across diverse organisms.
6 *Drosophila TIM* (Myers *et al.*, 1995) plays a central role in entrainment to light-dark (LD)
7 cycles, an adaptation critical for organisms to synchronize with their environment. The
8 rhythmic expression of *HpaTIM* in response to different light conditions indicates its
9 involvement in regulating the pathogen's circadian clock. Similarly, *SRR1*, or
10 *SENSITIVITY TO RED LIGHT REDUCED 1*, is another key gene found to be essential
11 for circadian regulation (Staiger *et al.*, 2003). Its role in influencing various aspects of
12 plant growth and development, as well as its homologues in other organisms, highlights
13 its significance. The presence of *HpaSRR1* and its rhythmic expression further
14 underscores the similarity of biological clocks in both the host and pathogen.

15
16 Our recent work demonstrated the efficacy of SS-dsRNA targeting the *Hpa-CesA3* gene,
17 resulting in inhibited spore germination and plant infection (Bilir *et al.*, 2019). We used
18 this method to target *HpaTIM* and *HpaSRR1* and the results that both genes *HpaTIM*
19 and *HpaSRR1* are crucial for pathogen virulence. Interestingly, the mRNA levels of
20 *HpaTIM* and *HpaSRR1* remained unaltered, contrasting with complete suppression of
21 mRNA from positive control *Hpa-Ces3*. Two distinct gene silencing phenomena has
22 been reported: transcriptional and post-transcriptional gene silencing (TGS and PTGS,
23 respectively (Sijen *et al.*, 2001). Notably, small RNA studies predominantly implicate
24 PTGS, where they modulate gene expression by base pairing with mRNA targets,
25 leading to degradation or translational inhibition (Saxena *et al.*, 2003). Our findings with
26 SS-dsRNAs may be explained by PTGS that inhibits translation of *HpaTIM* and
27 *HpaSRR1* RNA.

28
29 One of the most intriguing findings of this study is the correlation of circadian rhythms
30 between *Arabidopsis* and *Hpa*. We observed that the expression patterns of *HpaTIM*
31 and *HpaSRR1* mirrored those of *Arabidopsis* circadian biomarkers *CCA1* and *LHY*. In
32 principle, this correlation could be coincident with no regulatory connection between host
33 and pathogen, due to both organisms' exposure to the same light regime during
34 infection. On the other hand, we demonstrated that expression of *HpaTIM* and

1 *HpaSRR1* are disrupted by mutations in *CCA1* and *LHY*. Based on this result, we
2 hypothesize that *Hpa* circadian rhythms are coordinated by the *Arabidopsis* clock. It is
3 well documented that host clock can influence rhizosphere microbial community
4 (Hubbard *et al.*, 2018; Lu *et al.*, 2021; Newman *et al.*, 2022). Similarly, the rhizosphere
5 microbial community affects the host clock function (Hubbard *et al.*, 2021). These
6 studies suggest there is a bidirectional rhythmic interaction between plants and their
7 rhizomicrobiome (Xu & Dodd, 2022). Investigations with *Drosophila* and its gut
8 microbiome led to the conclusion that microbiome stabilizes circadian rhythm in the host
9 gut to prevent rapid fluctuations with changing environmental conditions (Zhang *et al.*,
10 2023). In addition, the synchronization between the gut microbiome and the host in
11 humans involves intricate crosstalk influenced by diet, lifestyle, and host genetics. This
12 dynamic interaction impacts immune modulation, metabolism and overall health
13 (Thursby & Juge, 2017).

14
15 The circadian system can be disturbed transiently or permanently by different factors
16 including light (Telli *et al.*, 2020) and temperature (Annunziata *et al.*, 2018). Such
17 disturbances are referred to as “circadian dysrhythmia (Bishehsari *et al.*, 2020) or
18 “circadian disruption” (Vetter, 2020). Circadian disruption could result in altered
19 microbiome communities and perturbed host metabolism in human health (Bishehsari
20 *et al.*, 2020), and changes in global responses in plant immune system (Wang *et al.*,
21 2011). We reported that altering light conditions delayed or inhibited *Hpa* development
22 and sporulation (Telli *et al.*, 2020). The data presented in this study represent another
23 step towards defining whether and how plant and pathogen clocks interact, as well as
24 whether circadian dysrhythmia is a factor in plant-pathogen interactions. It will be of
25 interest to assess bidirectional rhythmic interaction between *Hpa* and *Arabidopsis* using
26 gene silencing for *Hpa* and more detailed characterization of *Arabidopsis* CRG mutants.

27
28 A recent study on *Arabidopsis* demonstrated that disruption of the plant circadian clock
29 is associated with altered rhythmicity of rhizosphere bacteria and fungi (Newman *et al.*,
30 2022; (Xu & Dodd, 2022). Here we used *Arabidopsis* single or double clock mutants
31 *cca1* and *cca1/lhy* and investigated the expression of *HpaTIM* and *HpaSRR1*. There
32 was an alteration in the circadian expression pattern of these genes on single and
33 double mutants suggesting that the host clock could influence the pathogen clock.

34

1 As the influence of the host CRGs on the expression patterns of *HpaTIM* and *HpaSRR1*
2 was clear, we then used 26 singles, 2 double-, 1 triple mutant and also 3 overexpressors
3 (ox) of host clock genes to determine if host clock genes can influence infection and
4 development of the pathogen. We used relevant virulent isolates and measured
5 sporulation and vegetative growth (hyphae). Results of these investigations suggest that
6 CRGs could contribute to compatible interactions as well as basal defence. In addition,
7 double and triple mutants considerably reduced pathogen growth indicating the host
8 CRGs has a major role impact on compatibles interaction. The plant circadian genes
9 impact on large network of defence and development pathways, affecting multiple
10 aspects of biology (Hua, 2013; Luklova *et al.*, 2019). It is plausible that access to
11 nutrients by *Hpa* isolates, the amount of host metabolites in the infected tissues and the
12 host redox homeostasis will be altered by some of these CRG mutants resulting in the
13 influence on the pathogen development.

14
15 Effector- and PAMP-triggered immunity (ETI and PTI) have been studied in detail in
16 plant-microbe interactions. Control of the *R*-gene mediated defence responses to *Hpa*
17 has been shown to be regulated by CCA1 in *Arabidopsis* (Wang *et al.*, 2011). Similarly,
18 PTI against *Pseudomonas syringae* in *Arabidopsis* have been demonstrated to be
19 modulated by the circadian clock (Bhardwaj *et al.*, 2011). A recent study on *GIGANTIA*
20 (*GI*) in *Arabidopsis* and the wheat linked the circadian clock to plant susceptibility to
21 pathogens (Kundu & Sahu, 2021), indicating the compatibility may possibly be regulated
22 by the plant's clock function. Our study on *Arabidopsis* CRG mutants with different
23 virulent *Hpa* isolates clearly indicates the link between host circadian clock and the
24 pathogen in a compatible interaction. Some CRG mutants had different effects on
25 virulence of the two compatible isolates used in this study, suggesting that those isolates
26 might differ in their response to various CRG-regulated pathways that are relevant to
27 *Hpa* virulence. This is another avenue of potential interest in the future.

28
29 While we have identified circadian-regulated genes in *Hpa* and possible synchronization
30 with *Arabidopsis* clock genes, the specific mechanisms and the functional implications
31 of this coordination require further investigation. Future research could focus on
32 deciphering the molecular pathways and the specific genes involved in this
33 synchronization, as well as their impact on the development and pathogenicity of the
34 downy mildew pathogen.

1 **Materials and Methods**

2 **Plant lines, pathogen isolates and their propagation**

3 *H. arabidopsidis* isolates Emoy2, Noks1, Maks9 and Emco5 were maintained on
4 *Arabidopsis* accessions *Ws-eds1* (Parker et al., 1996) or *Col-rpp4* (Roux et al., 2011).
5 Inoculum preparation followed established protocols (Tör et al., 2002; Woods-Tör et al.,
6 2018)

7 **Identifying orthologues of two circadian clock genes in *Hpa* genome**

8 Important circadian genes published in model organisms for circadian clock studies
9 were identified through literature. Protein domain-searches through Pfam database
10 (<http://pfam.xfam.org>) revealed two putative CRGs in *Hpa* genome: *Timeless* (Pfam:
11 PF04821; *Hpa-G810921*, designated *HpaTIM*) and *Sensitive to Red Light Reduced 1*
12 (Pfam: PF07985; *Hpa-G801448*, designated *HpaSRR1*).

13 **Time course experiments**

14 *Col-rpp4* seedlings were infected with *Hpa*-Emoy2 as described previously in (Tör et al.,
15 2002). Samples were taken every 6h between 4 dpi to 7 dpi from infected seedlings.
16 Total RNA was extracted and analysed with Real-Time PCR. Real-Time PCR analysis
17 used *Hpa-Actin* or *At-Actin* genes as housekeeping genes. The results of samples were
18 analysed by Roche LightCycler 480 Real-Time software program. Each group of
19 experiment had three biological replicas and was repeated thrice. All samples were kept
20 under the 12h Dark/ 12h Light (D/L) cycle, referred to hereafter as “normal”. The
21 inoculated samples were exposed to 4 different light regimes 3 dpi; 1) exposed to
22 constant light between 3 dpi to 4 dpi, then normal 12h L/12h D cycle; 2) exposed to
23 constant dark between 3 dpi to 4 dpi, then normal 12h L/12h D cycle; 3) exposed to
24 constant light 3 dpi until 7 dpi; and 4) exposed to constant dark 3 dpi until 7 dpi. Similarly,
25 in these experiments, samples were taken every 6 hours 4 dpi, and they were analysed
26 with qRT-PCR.

27

28 **RNA extraction and gene expression analysis using qRT-PCR.**

29 An experiment was designed to confirm the expression pattern of *Arabidopsis* clock
30 genes *CCA1*, *TOC1* and *LHY1*. Uninfected *Col-rpp4* seedlings were used, only sprayed
31 with water, and placed into growth cabinet for a week at 16°C with a photoperiod of 12h

1 dark / 12h light. Samples were taken at 6h intervals 4 dpi and they were analysed using
2 qRT-PCR with the relevant primers (Supplemental Table 1).

3 To assess dsRNA-mediated gene silencing, gene expression analysis was conducted
4 on four-week-old *Ws-eds1* plants inoculated with a *Hpa-Emoy2* spore suspension of 5
5 $\times 10^4$ spores/mL containing 5 μ M siRNA. Six leaves in total were drop-inoculated with a
6 mixture of spores and siRNA (30 μ l per leaf), with two leaves serving as a biological
7 replicate. The *Arabidopsis* plants were then placed in a magenta box and subjected to
8 a 12-hour light/12-hour dark regime at 16°C for 3 days.

9 RNA extraction at different time points after inoculation was carried out. Total RNA
10 extraction protocol by TRIzol (Thermo Fisher) was adapted from (Chomczynski &
11 Sacchi, 2006). Quantitative Real-time PCR (qRT-PCR) was carried out using
12 SensiFAST™ SYBR® No-ROX One-Step Kit (Bioline) and the results were analysed
13 using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Primers for the housekeeping gene
14 for *Arabidopsis* or *Hpa*, *At-Actin* or *Hpa-Actin*, were included as a control to normalize
15 the results.

16 A master mix containing; 5 μ l of SensiFAST™ SYBR® No-ROX One-Step mix, 2 μ l of
17 template (RNA, 20-30ng/ μ l) from each sample, 0.5 μ l of each primer (Supplemental
18 Table 1), 0.01 μ l of reverse transcriptase, 0.002 μ l RNA-inhibitor was prepared and
19 DEPC water was added to give a final reaction volume of 10 μ l. qRT-PCR reactions
20 were carried out in 96-well plates using a Roche Light Cycler Real-Time PCR System.
21 *PCR conditions were as follows*, 45°C 10 min, 95°C for 2 min, followed by 9 cycles
22 touchdown procedure; 95°C for 5 s; 1°C in each annealing step of (68-60) °C for 10 s,
23 72°C 5 s, then 31 cycles of 95°C for 5 s, 60°C for 10 s, 72°C for 5 s.

24 **Selection of *At-CRGs* genes and their mutant lines**

25 *Arabidopsis* TAIR database (<https://www.arabidopsis.org>) were searched using the
26 “clock gene” keyword and 46 loci matching with 131 distinct gene models were
27 identified. These were then further evaluated for their function and involvement in the
28 circadian rhythm using the available literature. Twenty-six different genes that may play
29 a role in circadian regulation were then selected (Supplemental Table 2).

30 **Identification of homozygous T-DNA mutants**

31 Totally, 26 singles, 2 double and 1 triple *Arabidopsis* mutant lines along with 3
32 overexpressors (ox) were selected. Seed of the mutant lines were obtained from NASC

1 (<https://arabidopsis.info>). To confirm the identity and homozygosity of each mutant,
2 specific primers for each T-DNA line (Supplemental Table 2) were designed using SALK
3 site (<http://signal.salk.edu/tdnaprimers.2.html>) and were ordered from Sigma
4 (<https://www.sigmaaldrich.com/GB/en>). Ten seeds from each T-DNA mutant line were
5 sown and seedlings were picked and transferred to pots. DNA was extracted using the
6 REDEExtract-N-Amp Tissue PCR Kit protocol (Sigma-R4775) according to the
7 manufacturer's instruction. DNAs were then used for PCR amplifications. Using this
8 protocol, all lines were screened, and homozygous plants were propagated to obtain
9 seeds for subsequent analyses.

10 **Sporulation and biomass production assays on T-DNA mutant lines**

11 T-DNA lines were screened with *Hpa-Maks9*, *Hpa-Noks1* and *Hpa-Emco5*. The samples
12 were taken at 3 dpi and their DNAs extracted for biomass production. DNA was isolated
13 using CTAB method (Doyle, 1987) and the quantitative PCR was performed in a total of
14 25 μ l containing 50ng of gDNA, 12.5 μ l of SyberGreen Mastermix (ABI,
15 Carlsbad, California), *Hpa-Actin* or *At-Actin* primers (Supplemental Table 1) and water
16 on a Roche LightCycler 480 device. PCR conditions were as follows, 95°C for 4 min,
17 then 10 cycles touchdown of 95°C for 30 s, annealing temperature of 65°C, decreasing
18 1°C every cycle to 56°C, and extension at 72°C for 30 s. After 10 cycles of touchdown,
19 a further 25 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final extension
20 at 72°C for 5 min were carried out. Biomass production and sporulation were assessed
21 using quantitative PCR and established protocol as described (Telli *et al.*, 2020).

22

23 **Application of SS-dsRNAs to pathogen spores and plant inoculations**

24 Silencing of *HpaTIM* and *HpaSRR1* were performed using 30nt-long dsRNA as
25 described previously (Bilir *et al.*, 2019). *Hpa-CesA3* were used as a positive control in
26 the silencing experiments. RNA duplexes (Supplemental Table 3) were obtained as
27 synthesised ribonucleotides from Merck.

28

29 **Statistical analysis**

30 Statistical analyses were performed using MiniTab Express™
31 (<https://www.minitab.com/en-us/>) and GraphPad prism version 10.1.1 (GraphPad
32 software, Inc. USA) computer software was used for the statistical analysis. All tests in
33 this study were performed in triplicate. The significant differences among the means

1 were analysed by one-way analysis of variance (ANOVA) complemented by Dunnett's
2 test at the $p < 0.05$ level.

3

4 **Bioinformatics and phylogenetics**

5 Primer design was performed using Geneious (v10.0) (Kearse *et al.*, 2012).

6 We used the EnsemblProtist (Kersey *et al.*, 2016) and InterPro (Quevillon *et al.*, 2005)
7 databases to identify candidate *Hpa* clock genes. Reciprocal BLASTN and BLASTX
8 (Altschul *et al.*, 1997) were used to perform similarity-searches of nucleotide and amino
9 acid sequences, respectively, between *Hpa* clock genes and oomycete and *Arabidopsis*
10 sequences in the UniProt (The UniProt Consortium, 2023) and GenBank (Sayers *et al.*,
11 2022) databases. To generate multiple sequence alignments against Pfam domains, we
12 used the hmalign tool from the HMMER package version 3.4 (Eddy, 2011) and profile-
13 HMMs downloaded from Pfam 36.0 (Mistry *et al.*, 2021). Alignments were trimmed,
14 visualised and rendered using JalView (Waterhouse *et al.* 2009).

15

16 Evolutionary histories of protein sequences were inferred by using the maximum-
17 likelihood method and JTT matrix-based model (Jones *et al.*, 1992) conducted in
18 MEGA11 (Tamura *et al.*, 2021). The trees with the highest log likelihoods are shown.
19 Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-
20 Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT
21 model, and then selecting the topology with superior log likelihood value. All positions
22 with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps,
23 missing data, and ambiguous bases were allowed at any position (partial deletion
24 option). Bootstrapping (*i.e.* random sampling with replacement) was performed to
25 generate 1000 bootstrapped trees (Felsenstein, 1985). Phylogenetic trees were
26 rendered and visualised using iTOL (Letunic and Bork, 2021). The trees are drawn to
27 scale, with branch lengths measured in the number of substitutions per site.

28

29 **Conflict of Interest**

30 The authors declare that there is no conflict of interests.

31

32 **Author contributions**

1 MT and OT planned and designed the research. OT and DG conducted the laboratory
2 work. OT, WJ and DJS performed bioinformatic research, OT, BC-K, YH, JMM, DJS and
3 MT were involved in the analysis of data and wrote the manuscript.

4

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9

10 **Data availability statement**

11 The data that support the findings of this study are available from the corresponding
12 author on reasonable request.

13

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23

24 **Figure Legends**

25

26 **Figure 1. Phylogenies of HpaTIM and Hpa-SSR1.** Panel A shows a multiple sequence
27 alignment of the Timeless protein domain (Pfam: PF04821) of HpaTIM against
28 homologues in model organisms. Panel B shows a multiple sequence alignment of the
29 SRR1 domain (Pfam: PF07985) of HpaSRR1 against homologues in plants and model
30 organisms. Panel C shows evolutionary analysis of HpaTIM. Panel D shows the
31 evolutionary history of HpaSRR1. The sizes of the black circles indicate the proportions
32 of 1000 bootstrapped trees in which the associated taxa clustered together. The trees
33 are drawn to scale, with branch lengths measured in the number of substitutions per
34 site.

1
2 **Figure 2. Sporulation of *Hpa* and expression level of *HpaTIM* and *HpaSRR1***
3 **targeted with SS-dsRNA.** Sporulation was assessed 7dpi (A), and gene expression
4 was determined using qRT-PCR at 3dpi (B). *Hpa-CesA3* was targeted as a positive
5 control. An SS-dsRNA that does not inhibit pathogen growth was also used. One-way
6 ANOVA has been performed on data to compare treated samples with control samples.
7 Data expressed as mean \pm mean standard deviation. ****p < 0.0001, **p = 0.0039, *p =
8 0.0102

9
10 **Figure 3. Expression analyses of *HpaTIM* in different light regimes. A.** Expression
11 of *HpaTIM* gene in a normal light cycle (black line) and constant light exposure between
12 3dpi to 4dpi followed by a normal light cycle (red line). **B.** Expression pattern of *HpaTIM*
13 normal cycle (black line) and subsequently exposed to constant dark between 3dpi-4dpi
14 followed by normal cycle (blue line). For the first three days post inoculation (dpi), all
15 samples were kept under the 12h Dark/ 12h Light (D/L) cycle, referred to hereafter as
16 “normal”. After 4dpi, ZT time (Zeitgeber Time) started, and samples were taken every
17 6h over 3 days. Experiments started at dusk. While black blocks represent dark periods,
18 yellow blocks represent light periods. *Hpa-Actin* was used as a standard. The
19 experiment was repeated 3 times and similar results were obtained. Standard error of
20 mean for 3 biological replicas are indicated.

21
22 **Figure 4. Expression of *HpaTIM* under constant light and constant dark exposure.**
23 After an initial 3-day normal D/L conditions, one group was exposed to continuous light
24 for the following 4d while the other group exposed to continuous darkness for the
25 following 4d. Normal D/L cycle time-course served as the control. Samples were taken
26 every 6h and the expression levels of the *HpaTIM* gene were investigated. Experiments
27 started at dusk. While black blocks represent dark periods, yellow blocks represent light
28 periods. *Hpa-Actin* was used as a standard. The experiment was repeated 3 times and
29 similar results were obtained. Standard error of mean for 3 biological replicas are
30 indicated.

31
32 **Figure 5. Expression of *HpaSRR1* in different light regimes. A.** Expression of
33 *HpaSRR1* in normal D/L cycle (black line) and then constant light exposure after 3 dpi
34 (blue line). Expression showed rhythmic expression pattern just as observed with a

1 normal cycle. **B.** Expression of *HpaSRR1* in a normal light cycle (black line) followed by
2 exposure to constant dark between 3 dpi to 4 dpi, followed by a normal light cycle (blue
3 line). Experiments started at dusk. Black bars represent dark periods, yellow bars are
4 light periods. *Hpa-Actin* was used as a standard. The experiment was repeated 3 times
5 and similar results were obtained. Standard error of mean for 3 biological replicas are
6 indicated.

7
8 **Figure 6. Expression of *HpaTIM* and *HpaSRR1* compared with *CCA1* and *LHY***
9 **during infection.** **A)** Expression of *CCA1* (black line), *LHY* (dashed line) and *TOC1*
10 (grey line) in D/L cycle. Samples were taken every 6h into 4 dpi-7 dpi. *At-Actin* was used
11 as a standard. **B)** Comparison of the expression of *HpaTIM* (red line) and *HpaSRR1*
12 (green line) with *CCA1* (black line), *LHY* (purple line). Samples were taken every 6h
13 from 4 dpi-7 dpi. This experiment started at dusk. Black bars represent dark period,
14 yellow bars are light period. Experiment was repeated 3 times and similar results were
15 obtained. Standard error of mean for 3 biological replicas are indicated.

16
17 **Figure 7. Expressions of *HpaTIM* and *HpaSRR1* on *Arabidopsis* single or double**
18 **clock mutants.** Each graph displays expression of these genes in *Hpa* growing on the
19 mutant (solid lines) or wild-type Col-0 (dashed lines). **A)** *HpaTIM* and *HpaSRR1*
20 expressions on Col-*cca1* mutant line. **B)** *HpaTIM* and *HpaSRR1* expression in *cca1/lhy*
21 double mutant line. Col-*cca1* or *cca1/lhy* mutants were inoculated with *Hpa-Maks9*.
22 Samples were taken from inoculated seedlings between 4 and 6 dpi and expression
23 patterns of *HpaTIM* and *HpaSRR1* were quantified with RT-qPCR. Experiments started
24 at dusk. Black bars represent dark periods, yellow bars are light periods. *Hpa-Actin* was
25 used as a normalization control. This experiment repeated 3 times with consistent
26 results. Standard error of mean for 3 biological replicas are indicated.

27
28 **Figure 8. Mutations in *Arabidopsis* clock regulated genes influence sporulation**
29 **and biomass production.** T-DNA null mutants in Col-0 or Ws-0 background and
30 overexpressor (ox) were inoculated with a compatible *Hpa* isolate and the amount of
31 sporulation was calculated at 7 dpi. The biomass of vegetative hyphae was investigated
32 with selected mutant lines 3 dpi using q-PCR. Overexpressors, *TOC1ox* and *LHYox*
33 were also included. One-way ANOVA has been performed on data to compare mutant
34 lines with control samples. Data expressed as mean \pm mean standard deviation.

1 **A)** Mutant lines inoculated with *Hpa*-Noks1 (*CCA1ox*, *cca1*, *pif3*, *tic1*, *toc1*, *prp9*, *prp5*,
2 *prp3*, *phya*, *lcl5*, *lhy*, *cry1* **** $p < 0.0001$; *lux*, *kat2*, *** $p = 0.0003$; *phyb*, *elf3* ** $p = 0.0013$,
3 *lwd1* * $p = 0.0301$). **B)** Mutant lines inoculated with *Hpa*-Noks1 (*prp9*, *lux*, *toc1*, *tic1* ****
4 $p < 0.0001$; *cry1* *** $p=0.0007$; *elf3* *** $p=0.0002$; *cca1* *** $p=0.0001$; *phyb* ** $p=0.0015$).
5 **C)** Mutant lines inoculated with *Hpa*-Maks9 (*toc1*, *prp5*, *phyb*, *lhy*, *kat2*, *det1* **** $p <$
6 0.0001 ; *cca1* ** $p=0.0023$; *prp7* ** $p=0.0069$; *elf3* ** $p=0.0049$; *prp3* * $p=0.0190$). **D)** Mutant
7 lines inoculated with *Hpa*-Maks9 (*det1*, *elf3*, *lhy*, *phyb*, *prp3*, *prp7*, *prp5*, *toc1*, *cca1* **** p
8 < 0.0001 ; *kat2* ** $p=0.0067$). **E)** Mutant lines were inoculated with *Hpa*-Emco5 (*cca1/lhy*,
9 *cca1/lhy/toc1*, *lhy/toc1* **** $p < 0.0001$). **F)** Mutant lines were inoculated with *Hpa*-Emco5
10 (*lhy/toc1*, *cca1/lhy/toc1*, *cca1/lhy* **** $p < 0.0001$; LHYox * $p=0.0232$).
11
12

13 **Supplemental Figure Legends**

14 **Supplemental Figure 1. Domain structure of *HpaTIM*.**

15

16 **Supplemental Figure 2. Amino-acid multiple sequence alignment of Timeless**
17 **proteins from *Hpa* and other oomycete pathogens.**

18

19 **Supplemental Figure 3. Expression pattern of *HpaTIM* in Waco9 and Emoy2**
20 **isolates.** Expression levels were represented as TPM (tags per million) of total reads
21 mapped to *Hpa* genome. Data was acquired from Asai et al. (2018). Cs, conidiospore,
22 dpi, days post inoculation.

23

24 **Supplemental Figure 4. Domain structure of *HpaSRR1*.**

25

26 **Supplemental Figure 5. Amino-acid multiple sequence alignment of SRR1**
27 **proteins from *Hpa* and other oomycete pathogens.**

28

29

30 **Tables**

31

32

Table 1. Putative <i>Hpa</i> circadian genes and their orthologues

Gene and Gene ID	Orthologue from	Domain name and Domain ID	Function
<i>HpaTIM</i> (<i>HpaG810921</i>)	<i>Drosophila</i> , <i>Arabidopsis</i> , <i>Homo sapiens</i>	TIMELESS (PF04821) TIMELESS_C (PF05029)	Essential protein that regulates circadian rhythm (Sehgal et al, 1994).
<i>HpaSRR1</i> (<i>HpaG801448</i>)	<i>Arabidopsis</i>	SRR (PF07985)	Mediates phyB signalling and is required for normal circadian clock function (Staiger et al, 2003).

1

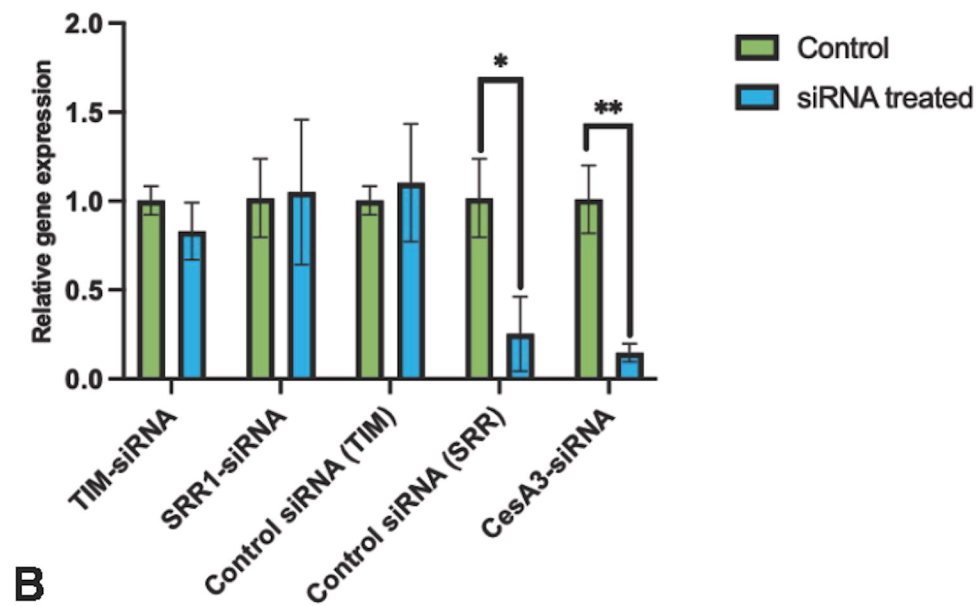
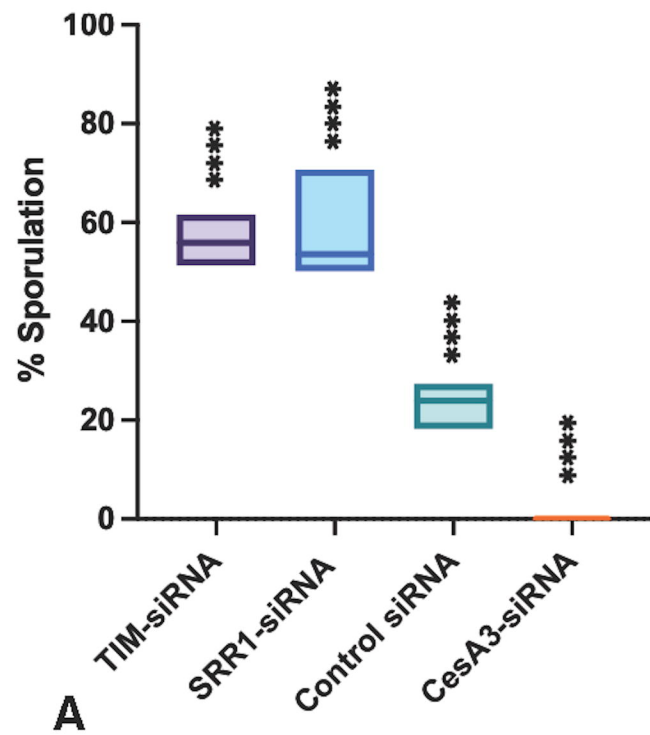
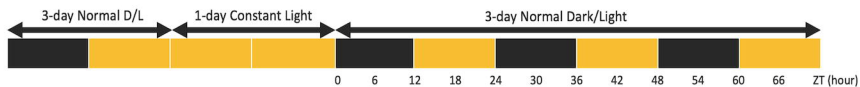
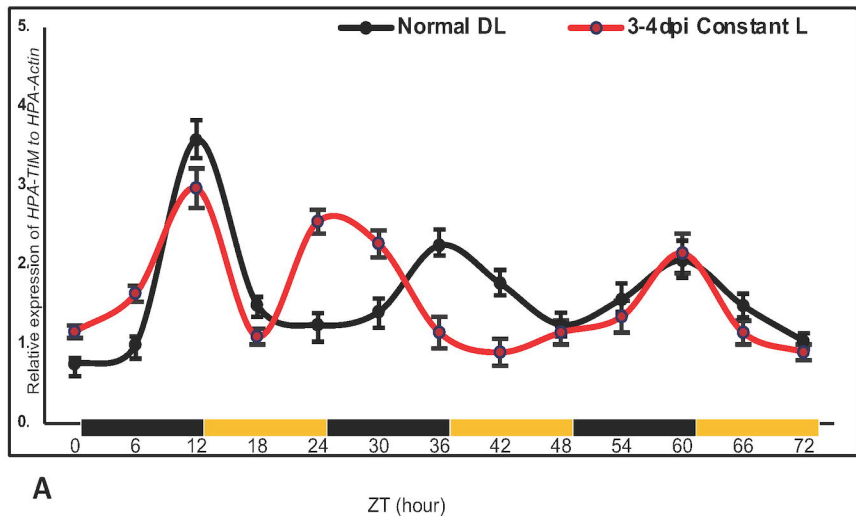
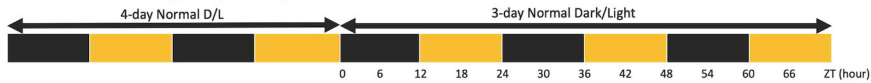


Figure 2

1-day constant light experiment

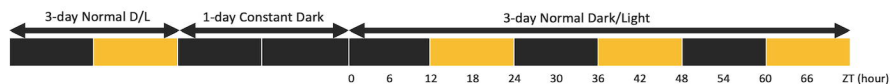


Normal Dark/Light cycle experiment

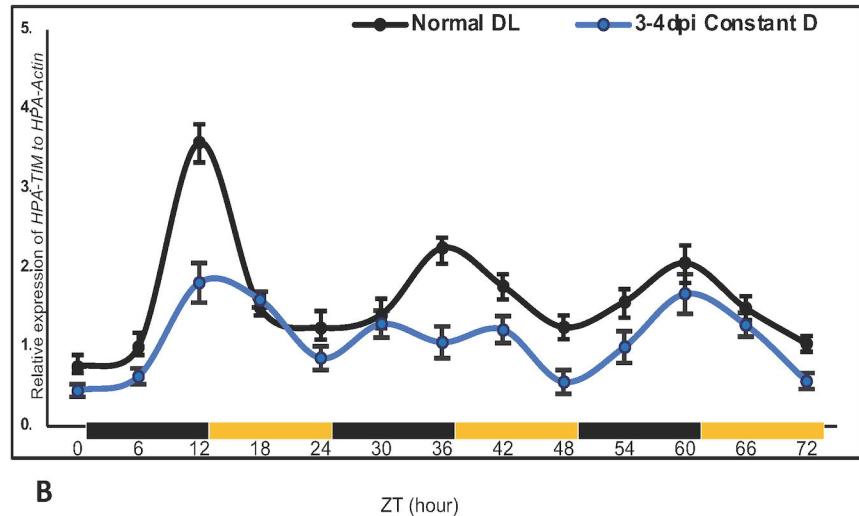
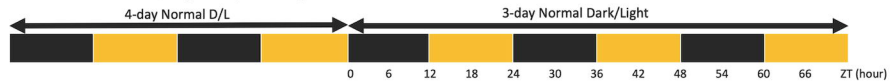


A

1-day constant dark experiment



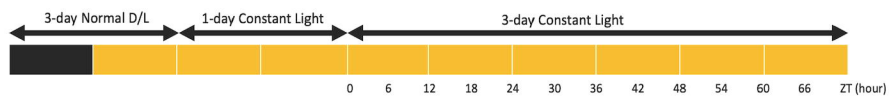
Normal Dark/Light cycle experiment



B

Figure 3

Constant Light Exposure Experiment



Constant Dark Exposure Experiment



Normal Dark/Light cycle experiment

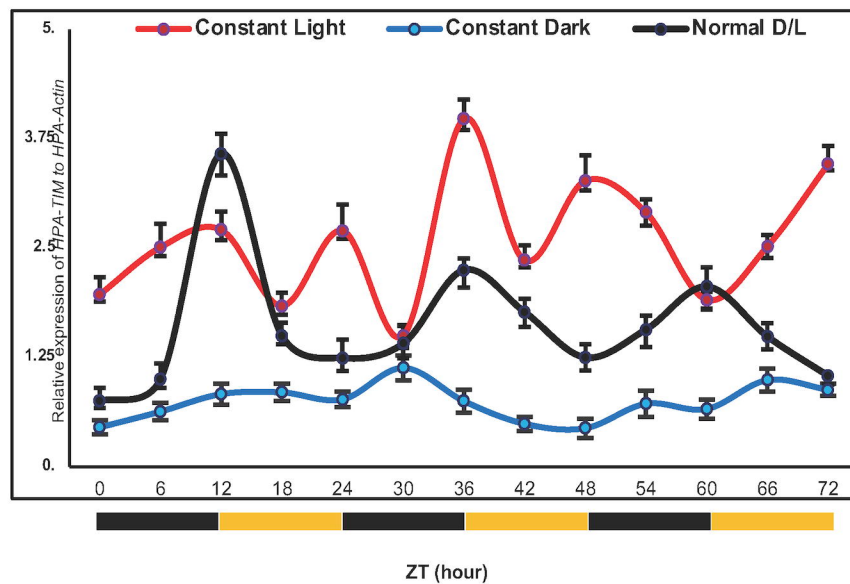
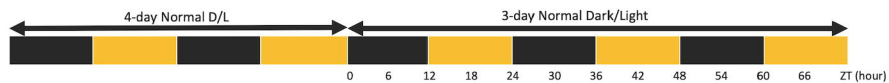


Figure 4

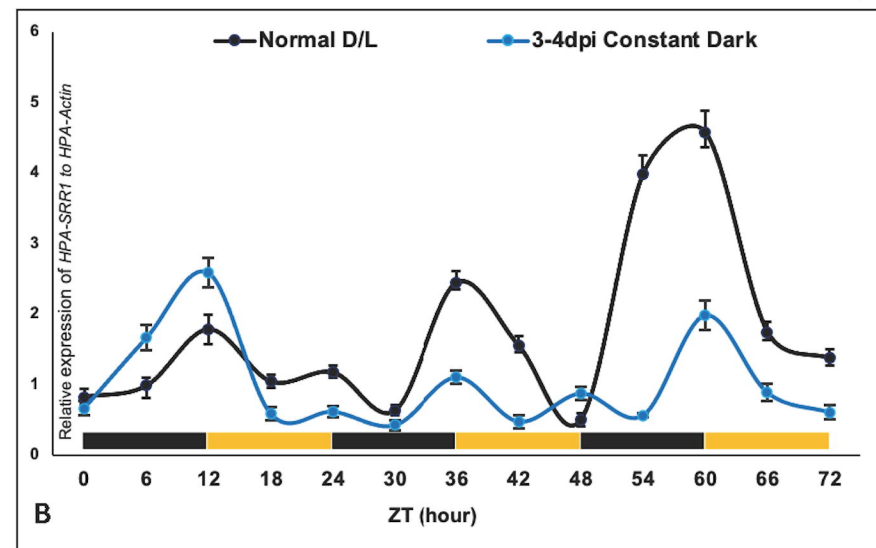
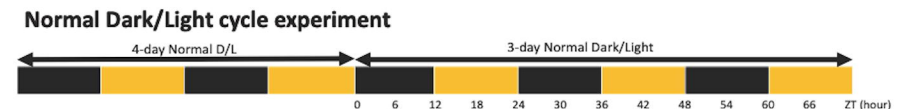
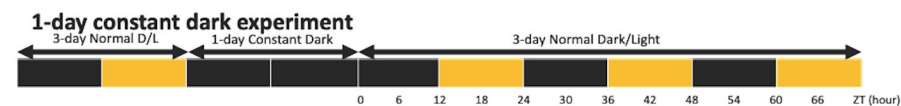
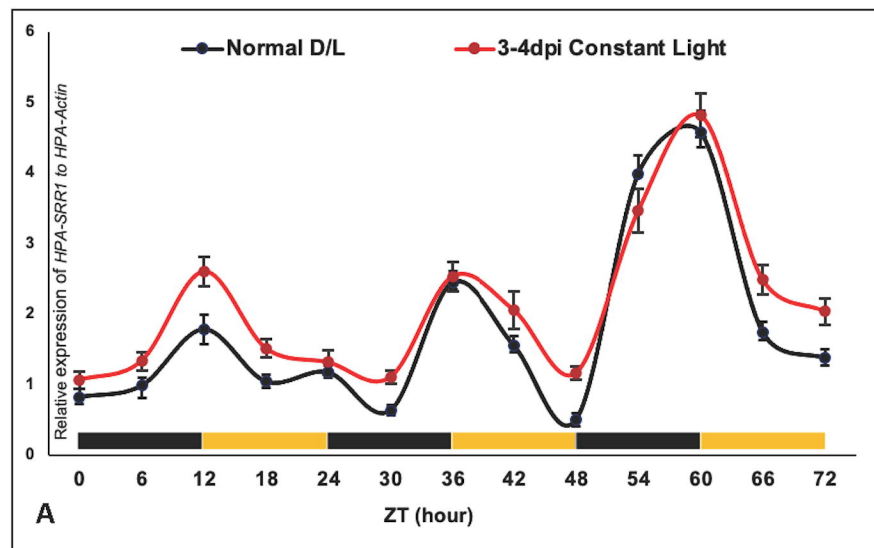
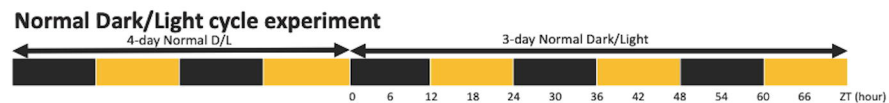
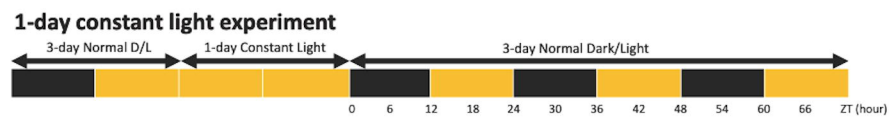
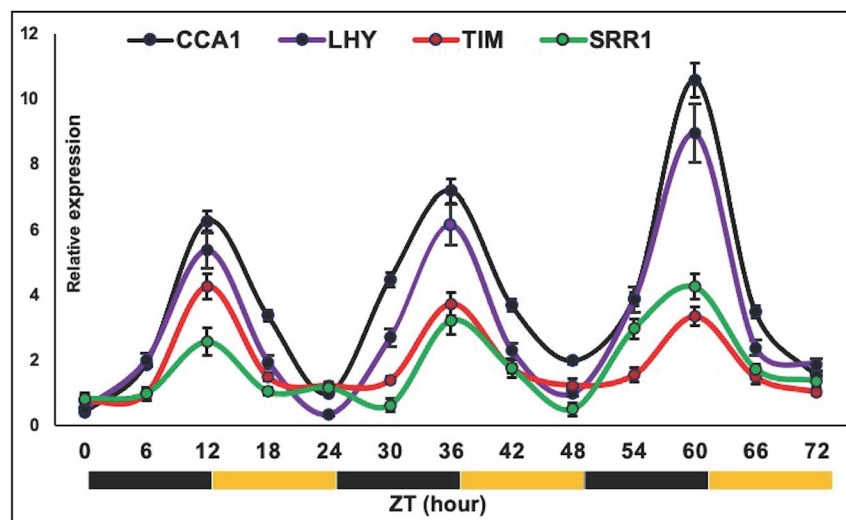
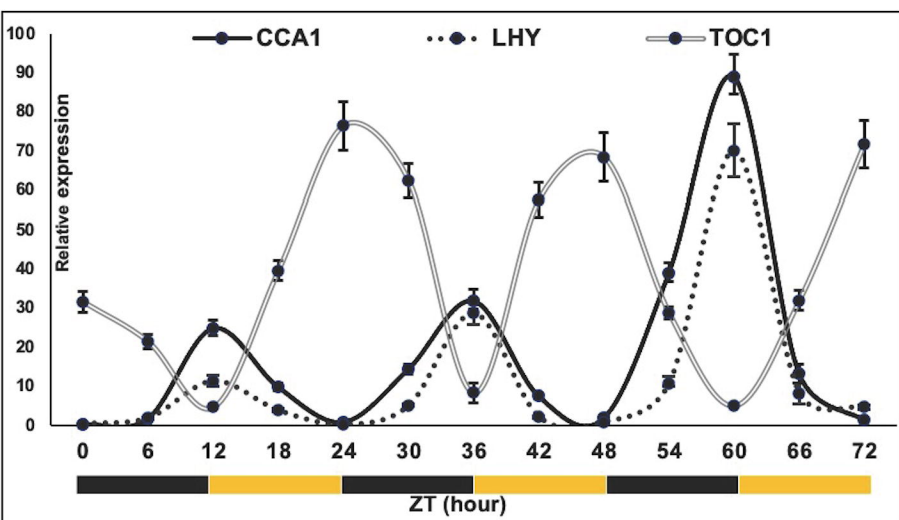
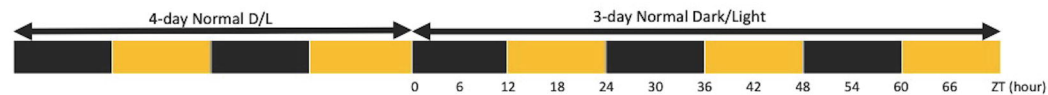


Figure 5



A

B

Figure 6

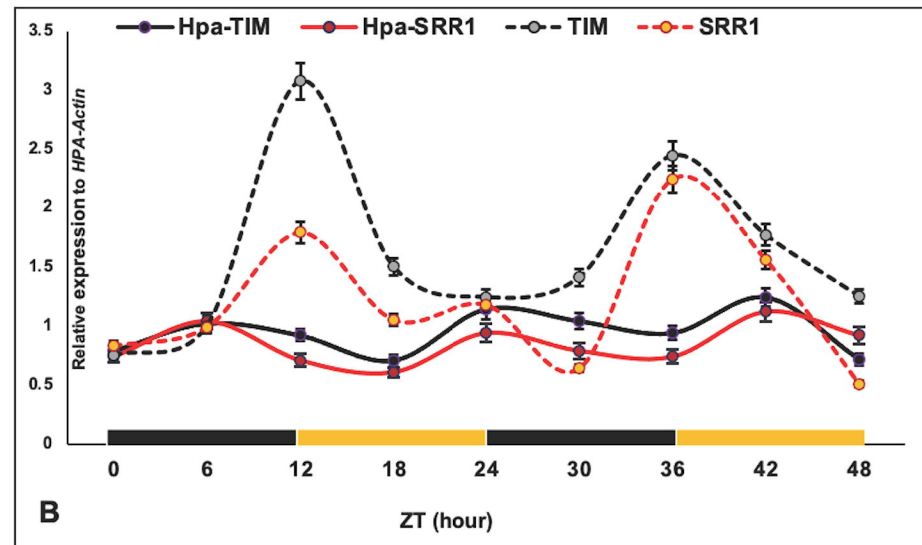
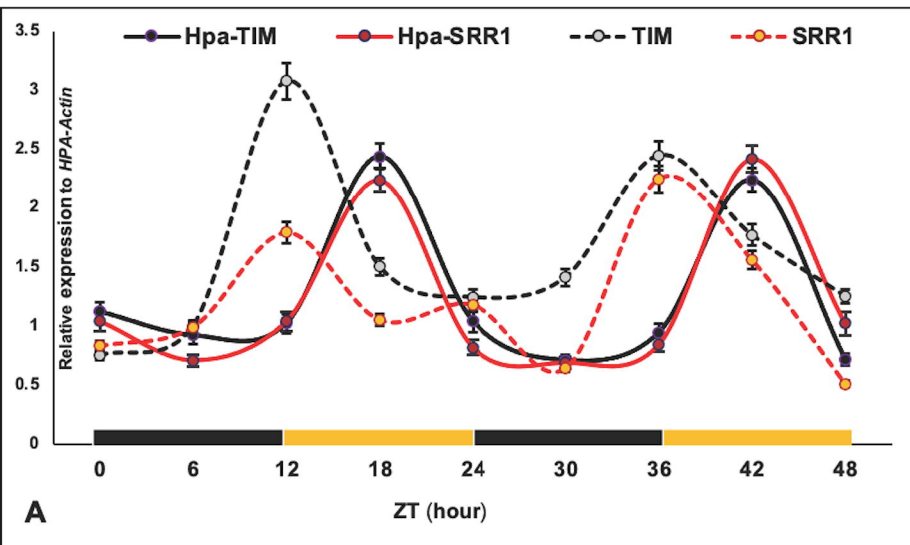
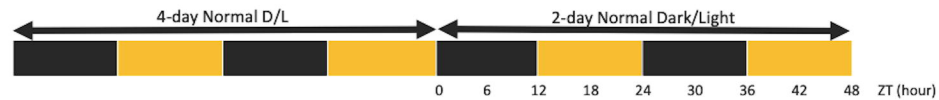


Figure 7

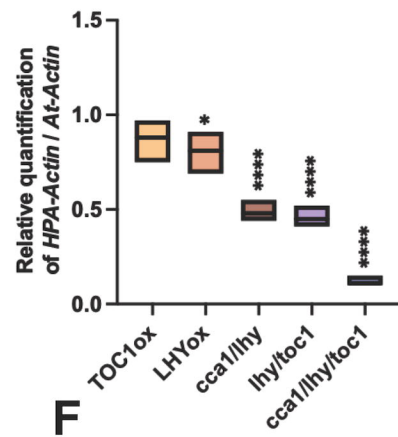
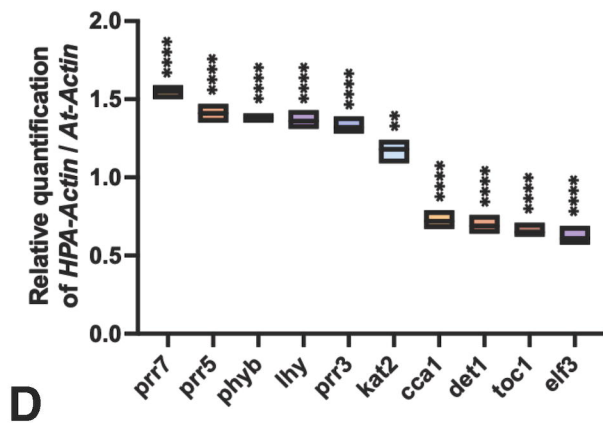
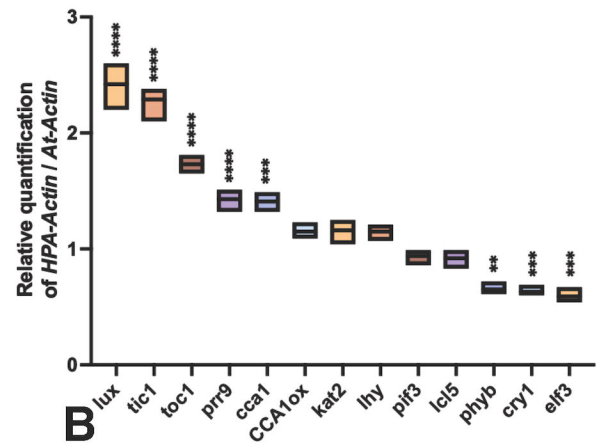
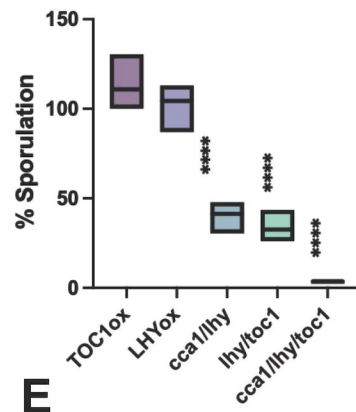
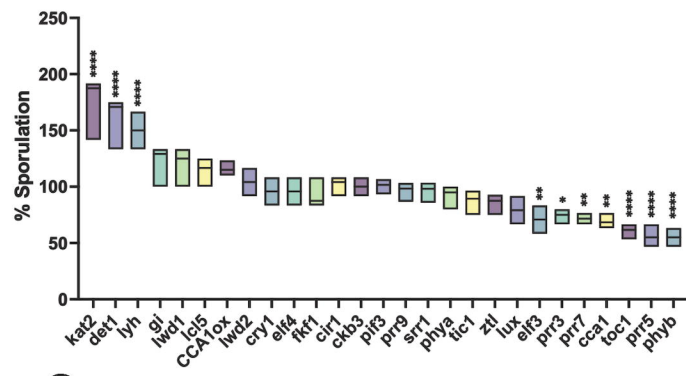
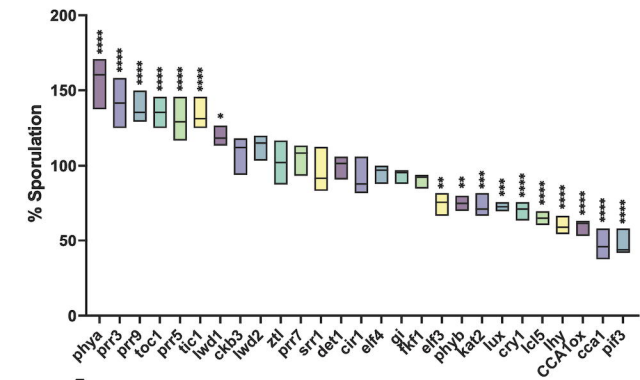


Figure 8