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

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

26 **microbe interactions**

27

Introduction

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

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

Studies with the model plant *Arabidopsis* and its obligate downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) have illuminated many aspects of plant-pathogen interactions (Holub, 2007; Herlihy *et al.*, 2019). For example, several disease resistance genes against *Hpa* have been molecularly cloned from *Arabidopsis*. The *R*-gene *RPP4* encodes a nucleotide-binding leucine-rich repeat protein with Toll/interleukin-1 receptor domains and provides resistance to isolates *Hpa*-Emoy2 and *Hpa*-Emwa1 (Van Der Biezen *et al.*, 2002). Recent studies on this gene identified a link 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 compatible host plants, and whether the host and pathogen clocks might influence each other. Recently, we reported that all developmental stages of *Hpa* during the infection

4

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 *Hpa*TIM 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). *Hpa*TIM 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*

6

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* viulence 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 5μM

12 concentrations and used to drop-inoculate 7-day old seedings of the disease-

13 susceptible mutant *Arabidopsis* Ws-*eds1*. At 7dpi, plants inoculated with spore

14 suspensions containing 5μM 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

7

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   *HpaSRR1* expression was slightly higher at the beginning but in general, it was very

5   similar to that of the control (Figure 5A).

6

7   *HpaSRR1* 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 *HpaSRR1* 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 *HpaSRR1* 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 *HpaSRR1*

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.

6

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.

33

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

10

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: *prr9* (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 *prr9* (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: *prr3* (74%), *prr7* (72%), *elf3* (71%), *cca1* (69%), *toc1* (61%), *prr5* (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 prr7 (155%), *prr5* (141%), *phyb* (138%), *lhy* (137%), *prr3* (133%) and *kat2* (117) had
34 more biomass than that obtained with control Col-0 (Figure 8D). Biomass production in

1   *phyb, prr3, prr7, prr5* 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.

15

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

16

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 × 10^4 spores/mL containing 5 µM siRNA. Six leaves in total were drop-inoculated with a

6 mixture of spores and siRNA (30µl 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 $2^{-\Delta\Delta Ct}$ 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 µl of SensiFAST™ SYBR® No-ROX One-Step mix, 2 µl of

17 template (RNA, 20-30ng/µl) from each sample, 0.5 µl of each primer (Supplemental

18 Table 1), 0.01 µl of reverse transcriptase, 0.002 µl RNA-inhibitor was prepared and

19 DEPC water was added to give a final reaction volume of 10 µl. 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-CRG*s 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   REDExtract-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 µl containing 50ng of gDNA, 12.5 µl 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^{TM}

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

18

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 a.,

11 2022) databases. To generate multiple sequence alignments against Pfam domains, we

12 used the hmmalign 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

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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Figure Legends

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

1

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

9

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

21

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

31

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

26

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 ± mean standard deviation.

1 **A)** Mutant lines inoculated with *Hpa*-Noks1 (CCA1ox, *cca1, pif3, tic1, toc1, prr9, prr5,*

2 *prr3, 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 (*prr9, 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, prr5, phyb, lhy, kat2, det1* ****p <

6 0.0001; *cca1* **p=0.0023; *prr7* **p=0.0069; *elf3* **p=0.0049; *prr3* *p=0.0190). **D)** Mutant

7 lines inoculated with *Hpa*-Maks9 (*det1, elf3, lhy, phyb, prr3, prr7, prr5, 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.**

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30 **Tables**

31

32

Table 1. Putative *Hpa* circadian genes and their orthologues

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

1
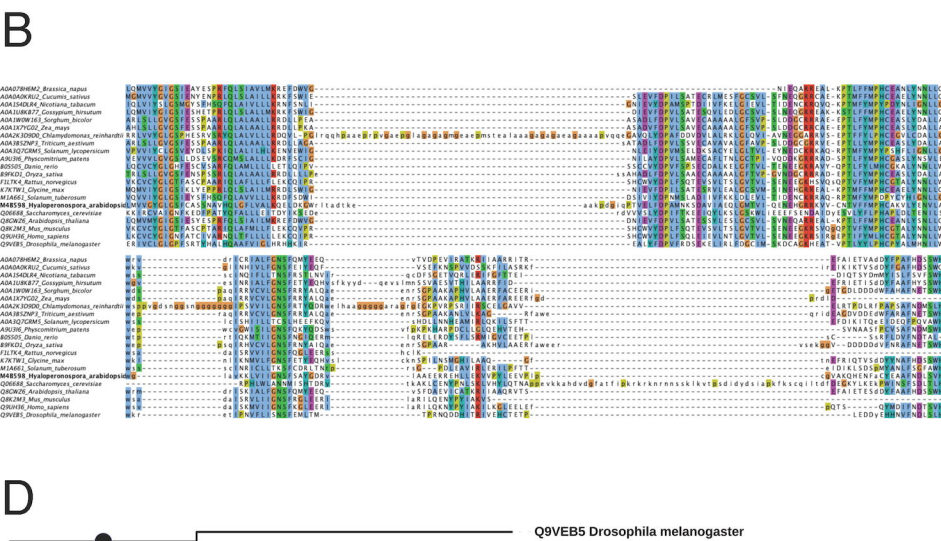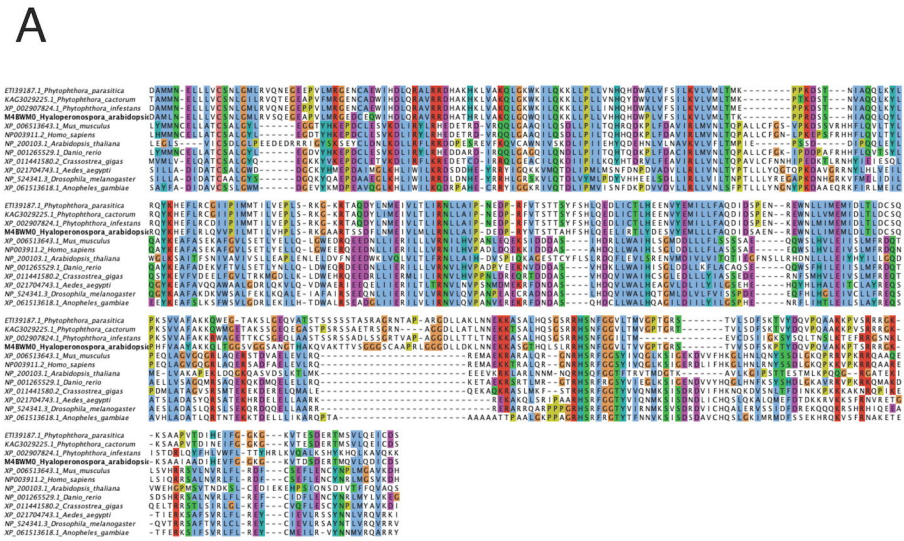
Figure 1
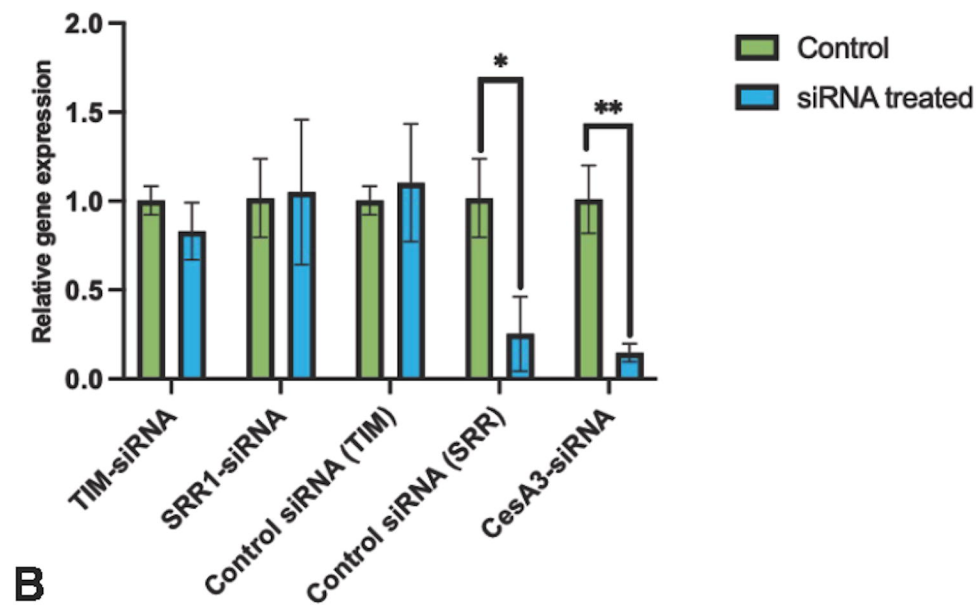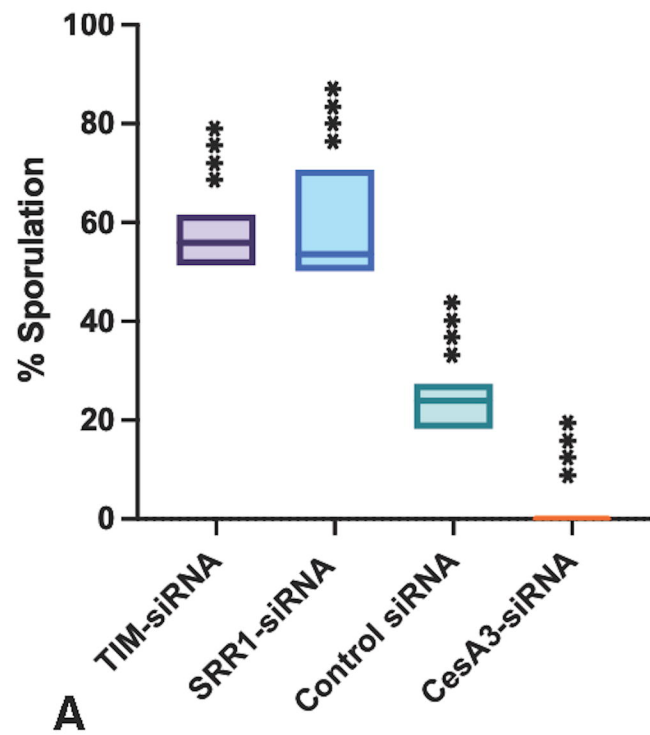
Figure 2

1-day constant light experiment

3-day Normal D/L | 1-day Constant Light | 3-day Normal Dark/Light

ZT (hour): 0 6 12 18 24 30 36 42 48 54 60 66

Normal Dark/Light cycle experiment

4-day Normal D/L | 3-day Normal Dark/Light

ZT (hour): 0 6 12 18 24 30 36 42 48 54 60 66

1-day constant dark experiment

3-day Normal D/L | 1-day Constant Dark | 3-day Normal Dark/Light

ZT (hour): 0 6 12 18 24 30 36 42 48 54 60 66

Normal Dark/Light cycle experiment

4-day Normal D/L | 3-day Normal Dark/Light

ZT (hour): 0 6 12 18 24 30 36 42 48 54 60 66

A

Legend: ● Normal DL ● 3-4 dpi Constant L

Y-axis: Relative expression of *HPA-TIM to HPA-Actin*

X-axis: ZT (hour)

B

Legend: ● Normal DL ● 3-4 dpi Constant D

Y-axis: Relative expression of *HPA-TIM to HPA-Actin*

X-axis: ZT (hour)

Figure 3

Constant Light Exposure Experiment

3-day Normal D/L   1-day Constant Light   3-day Constant Light

0   6   12   18   24   30   36   42   48   54   60   66   ZT (hour)
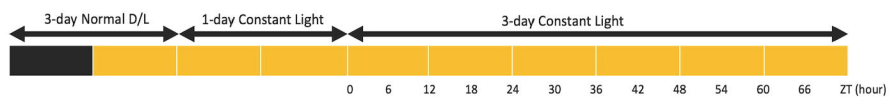
Constant Dark Exposure Experiment

3-day Normal D/L   1-day Constant Dark   3-day Constant Dark

0   6   12   18   24   30   36   42   48   54   60   66   ZT (hour)

Normal Dark/Light cycle experiment

4-day Normal D/L   3-day Normal Dark/Light

0   6   12   18   24   30   36   42   48   54   60   66   ZT (hour)

Figure 4

1-day constant light experiment

3-day Normal D/L — 1-day Constant Light — 3-day Normal Dark/Light

0 6 12 18 24 30 36 42 48 54 60 66   ZT (hour)

Normal Dark/Light cycle experiment

4-day Normal D/L — 3-day Normal Dark/Light

0 6 12 18 24 30 36 42 48 54 60 66   ZT (hour)

1-day constant dark experiment

3-day Normal D/L — 1-day Constant Dark — 3-day Normal Dark/Light

0 6 12 18 24 30 36 42 48 54 60 66   ZT (hour)

Normal Dark/Light cycle experiment

4-day Normal D/L — 3-day Normal Dark/Light

0 6 12 18 24 30 36 42 48 54 60 66   ZT (hour)

Figure 5

Figure 6

Figure 7

Figure 8