REVIEW

It's a matter of time: the role of transcriptional regulation in the circadian clock-pathogen crosstalk in plants

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ABSTRACT

Most living organisms possess an internal timekeeping mechanism known as the circadian clock, which enhances fitness by synchronizing the internal timing of biological processes with diurnal and seasonal environmental changes. In plants, the pace of these biological rhythms relies on oscillations in the expression level of hundreds of genes tightly controlled by a group of core clock regulators and co-regulators that engage in transcriptional and translational feedback loops. In the last decade, the role of several core clock genes in the control of defense responses has been addressed, and a growing amount of evidence demonstrates that circadian regulation is relevant for plant immunity. A reciprocal connection between these pathways was also established following the observation that in *Arabidopsis thaliana*, as well as in crop species like tomato, plant-pathogen interactions trigger a reconfiguration of the circadian transcriptional network. In this review, we summarize the current knowledge regarding the interaction between the circadian clock and biotic stress responses at the transcriptional level, and discuss the relevance of this crosstalk in the plant-pathogen evolutionary arms race. A better understanding of these processes could aid in the development of genetic tools that improve traditional breeding practices, enhancing tolerance to plant diseases that threaten crop yield and food security all around the world.

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1. Introduction

In an ever-changing world, possessing the capability to adapt to, and even predict, daily and seasonal environmental changes is a valuable asset for living organisms. This has shaped the evolution, in most eukaryotic and some prokaryotic organisms, of an endogenous timekeeping mechanism known as the circadian clock. In plants, environmental signals such as light and temperature (referred to as clock “inputs”) are integrated by a core oscillator consisting of circadian clock proteins, which in turn modulates and coordinates a wide range of mechanisms known as “outputs” of the clock, that span from the regulation of metabolism and growth to hormone pathways and stress responses. Thus, the circadian clock orchestrates biological processes in concordance to daily and seasonal environmental conditions, allowing the allocation of resources during the most beneficial times of

the day and the year. This anticipation of daily and seasonal environmental cycles allows plants to optimize internal processes to match external conditions, providing a fitness advantage [1–6]. The molecular clock mechanism is based on transcriptional-translational feedback loops, comprising positive and negative elements that compose the core clock oscillator. The circadian clock regulates more than 30% of the transcriptome in *Arabidopsis*, rice, maize, papaya, poplar, and soybean [3,5,7–14]. Circadian-clock regulated genes are central to many relevant physiological processes such as flowering time, light signaling, growth control, metabolic activities, abiotic stress responses, and plant-pathogen interactions [8,15–23].

In *Arabidopsis thaliana*, the core of this clock is composed of a group of genes that act at different times of the day regulating the activity and

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expression of other members of the circadian network. In a simplified model, two partially redundant MYB transcription factors, *CIRCADIAN ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), show peak expression levels in the morning. These proteins directly repress the expression of all the members of the *PSEUDO-RESPONSE REGULATOR* (*PRRs*) gene family, which in turn act as repressors of *CCA1* and *LHY* and show sequential peaks in their expression starting with *PRR9* in the morning, followed by *PRR7*, *PRR5* and ending with *PRR1*, also known as *TIMING OF CAB2 EXPRESSION 1* (*TOC1*), in the evening. Additionally, the expression of another group of core clock genes, *EARLY FLOWERING 3* (*ELF3*), *ELF4*, and *LUX ARRHYTHMO* (*LUX*), peaks toward the end of the day, and their corresponding proteins act together in the so-called Evening Complex (EC), repressing the expression of morning genes such as *PRR*, and one other evening-expressed component of the clock, known as *GIGANTEA* (*GI*). The expression of *GI* is also repressed by *TOC1* and morning genes *CCA1/LHY*, which in turn are induced by *GI* itself, even though it isn't clear yet whether this activation is direct or not [24].

While all the above interactions are examples of transcriptional repression, transcriptional activators also play a key role in the regulation of the plant circadian network. *REVEILLE 8* (*RVE8*), *REVEILLE 6* (*RVE6*), and *REVEILLE 4* (*RVE4*), three members of the *CCA1/LHY/RVE* family of single MYB transcription factors, function as activators of several evening clock genes, such as *PRR5*, *TOC1*, and *ELF4* [25–29]. Along with the RVEs, four transcriptional activators termed *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED* (*LNKs*) have been described. Although these genes were proposed to act as transcriptional activators, they do not exhibit any known DNA binding domain [30]. Instead, it was described that both *LNK1* and *LNK2* interact with the MYB transcription factors *CCA1*, *LHY*, *RVE4*, and *RVE8* [31,32]. These activators (*RVE4*, *RVE6*, and *RVE8*) and co-activators (*LNK1* and *LNK2*) show peak expression at mid-morning and promote the expression of *PRR5* and *TOC1* in the afternoon, whose activity contributes not only to

complete the downregulation of *CCA1/LHY* expression at midnight but also to repress the expression of all the *LNK* genes, starting the cycle again [25,27,30,31].

The analysis of circadian regulated genes has led to the identification and characterization of several promoter motifs involved in phase-specific regulated gene expression. As mentioned above, transcriptional repression and activation underpin the molecular gear of the circadian network; efforts to understand these phenomena have focused on the identification and functional characterization of cis-acting elements required for circadian-regulated transcription. Two elements are well characterized as targets of *CCA1* and *LHY*, the *CCA1*-binding site (CBS; AAAAATCT) and the evening element (EE; AAAATATCT). Similarly, the single MYB transcription factor *RVE8* also shows EE binding activity [25]. Interestingly, the EE was shown to be overrepresented among the predicted promoters of genes with expression peaks in the evening [7]. On the other hand, G-box (CACGTG)-related elements are enriched among *PRR* target genes, indicating that these motifs mediate the association of transcriptional repressors toward the end of the day. Among the members of the evening complex (EC) [33,34], only *LUX* has been characterized as a transcription factor and shown to have direct DNA binding activity to motifs known as *LUX* Binding Sites (LBS; GATWCG where W indicates A or T) [35]. Nevertheless, in an interesting work performed by Ezer D. et al. it was shown that G-box motifs are highly enriched at EC binding sites. These elements are widely distributed among genes involved in plant stress, including pathogen responses [23,36–38].

It is well established that a major contribution of the circadian clock to optimize plant fitness is fine-tuning resource allocation between growth and stress responses. Stress responses are under circadian control and, in turn, feedback into the clock core itself [39]: several clock genes contain cis-elements responsive to environmental stresses in their promoter regions [14,23,40,41]. This feedback is essential to establish a timely gating of stress responses, thus minimizing the fitness costs associated with unspecific activation of defense pathways. As a consequence of the gating effect,

plant susceptibility and tolerance level to pathogens display a circadian behavior.

The fact that plants don't have specialized immune cells implies that each cell must regulate and balance the high energy-consuming stress responses with other cellular functions. Plants display a myriad of defense mechanisms; the first physical and/or chemical barriers are preformed while the second level of defense responses are triggered by the invaders. Plant cells can recognize pathogens using two interconnected layers of extracellular and intracellular high-affinity cell surface pattern-recognition receptors [42]. The extracellular receptor system is the first defense barrier, specialized in the recognition of pathogen-associated molecular patterns (PAMPs) [43]. This recognition activates a basal level of defense, the PAMP-triggered immunity (PTI). Induction of PTI leads to intracellular signaling cascades that result in a strong transcriptional reprogramming and the mounting of physiological responses that restrain pathogen colonization [42]. In turn, pathogens produce and deliver effector proteins that inhibit PTI. To counter the pathogen effectors, plants acquired through evolution an intracellular receptor system involving resistance proteins (R). This second surveillance system recognizes specific pathogen effectors and activates the effector-triggered immunity (ETI) resistance mechanism, also known as R gene-mediated resistance. The resistance associated with ETI responses is tightly linked to programmed cell death, in a process known as the hypersensitive response (HR) [44]. The ETI activation coordinates a rapid reboot and a high-amplitude increase in defense signaling pathways, mainly mediated by a reactive oxygen species (ROS) burst coupled to a tightly organized production of phytohormones [45,46]. Different defense hormones play diverse roles in immunity, and which hormone pathway is activated upon infection depends on the nature of the pathogen attacking the plant. According to their lifestyle and mode of infection, pathogens can be classified as biotrophic, which feed on living tissue and have developed strategies to exploit resources while keeping the host alive, or necrotrophic, which usually kill their host cells with toxins and then consume its contents. Some pathogens, like *Pseudomonas syringae* (Psa), follow

an intermediate strategy, in which they first present a biotrophic lifestyle but ultimately enter a necrotrophic mode. Biotrophic pathogens most commonly induce the Salicylic Acid (SA) pathway, and necrotrophic pathogens, along with herbivores, upregulate both Jasmonic Acid (JA) and Ethylene pathways [47]. In the final stage of defense activation, different kinds of systemic immune responses can also be activated to protect distal tissues of the plant. SA mediates the so-called systemic acquired resistance (SAR), while JA triggers the induced systemic resistance (ISR) [48]. Altogether, the fast reprogramming of the transcriptome and metabolism are key factors that help the plant prevent the colonization by pathogens.

In this review we discuss the latest findings associated with the transcriptional crosstalk between the circadian clock and plant defenses, focusing on the feedback that immune responses exert over clock function. We also extend our survey to relevant agronomic crops. Understanding the extent of the transcriptional reprogramming of the circadian network as a result of the plant-pathogen interaction could lead to a new dimension in biotic stress breeding targets. We apologize to colleagues whose work has not been included in this review due to space constraints.

2. Clock regulation of plant immunity

The “disease triangle”, one of the fundamental principles of plant pathology, establishes that any given disease caused by a pathogenic agent depends unequivocally on three factors: a susceptible host, a virulent pathogen, and a favorable environment [49]. In line with this notion, early studies in plant-pathogen interactions focused on the direct effect of abiotic factors, such as light and temperature, over immune responses. Many comprehensive reviews address the relevance of the integration of environmental cues in the modulation of pathogenic infection [50–52].

In the last decade, given the role of biological clocks as hubs for the interpretation of external information, many authors have focused on the study of the circadian system as a master regulator

of defense responses. Two main lines of evidence support this notion. On the one hand, reverse genetic studies demonstrated that defense responses are impaired in many core clock mutants. On the other hand, daily oscillations in susceptibility correlate with circadian regulation of expression of many defense-related genes.

2.1. Core clock components in defense responses

Studies on the role of clock genes in defense responses have mainly focused on the repressor components of the circadian network. Many interesting studies tackled the implication of components such as CCA1/LHY and TOC1, and several reviews cover this information [37,39,53].

Recently, our group conducted the first study regarding the role of clock activators. We found that mutations in members of the *LNK* family led to an enhanced disease susceptibility phenotype upon infection with *Psa* [54]. *lnk1-lnk2-lnk3-lnk4* quadruple mutants (*lnkQ*) [55], which present a long-period phenotype in leaf movement assays, were much more susceptible to *P.syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection than WT plants. This effect was also present, but to a lower extent, in *lnk1-lnk2* doubles mutants [30]. Interestingly, a significant effect of the *LNK* genes on susceptibility was observed regardless of the time of infection. Given that *LNK* genes act as co-activators that work in association with members of the REVEILLE (RVE) family of activators, it would be interesting to also assess the role of the RVE family in defense responses to have a better understanding of the role of clock activators in defense responses.

2.2. Circadian regulation of the defense transcriptome

The first evidence of circadian control of the immune system was the identification of the *Pathogen and Circadian Controlled 1* (*PCC1*) gene from *Arabidopsis thaliana*. As the name implies, this gene was found to be circadian-regulated, in part through CCA1, under non-infective conditions, and up-regulated in response to infection with *P.syringae*. The fact that plants overexpressing *PCC1* were also more resistant to

an oomycete demonstrated that this gene indeed plays a role in defense responses [56]. Similarly, other circadian-controlled genes were later identified and implicated in defense responses as well [57–].

The advent of high-throughput techniques for the analysis of gene expression consolidated this idea. In a study of the transcriptional responses of *Arabidopsis* to the biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*), a group of 22 deregulated genes whose promoters were enriched in the EE was identified. Given that CCA1 bound to this sequence can both negatively and positively regulate gene expression [7,61], this finding was the first evidence of circadian regulation in a large cluster of defense-related genes. This notion was further confirmed by the fact that the peak of expression of these genes largely overlapped with the expression of CCA1 [23].

In a thorough analysis of publicly available microarray datasets, Bhardwaj et al. found that almost half of a list of 121 defense-related genes in *Arabidopsis* oscillated in a circadian manner [62]. This included not only the flagellin receptor *FLS2* but also the whole downstream *MKK4/5-MAPK3/6-WRKY22* signaling module responsible for the activation of defense responses upon bacterial flagellum detection. This group of genes showed an expression peak in the morning. This result correlated with another major finding: the detection of a circadian oscillation in the susceptibility to *P.syringae*. By performing infections at different times of the subjective day in plants kept in free-running conditions (continuous white light after an entrainment period in light/dark cycles), the authors found increased resistance in the early hours, suggesting for the first time a circadian (rather than diurnal) “gating” of the immune system in *Arabidopsis*. This finding was later confirmed in several studies that also found a circadian regulation of *Arabidopsis* immunity when challenged with virulent and avirulent strains of *Pseudomonas* [36,63] and with the necrotrophic fungus *Botrytis cinerea* (*Bci*) [64,65].

A few years later, the number of defense-related genes under clock control significantly increased thanks to a study that analyzed the presence of CBS and EE in the promoter region of 464 genes,

that were either classified as defense-related based on microarray experiments or as empirical markers for various pathogen infections [36]. The authors found a mild enrichment (40%) in CBS sequences, but a much larger enrichment of EE sequences (200%) in the promoters of defense-related genes when normalized to non-defense genes. These results further proved a preferential regulation of immunity genes by CCA1 and LHY. Taken together, these observations support the idea of a regulation of defense genes by core clock components.

The circadian regulation of gene transcription is a widespread feature. The analysis of several global Arabidopsis expression datasets suggests that over 37% of the genes are circadian regulated in at least one experimental condition; these genes can be defined as circadian controlled genes (CCG) [8,66,67]. Interestingly, in a very recent dataset, Romanowski et al. found that biotic stress responses were enriched among the circadian genes identified by deep transcriptome sequencing [66]. Thus, this result confirms a specific transcriptional regulation of immune responses by the circadian clock.

To further explore which core clock components are involved in this preferential circadian regulation of defense genes, we mined available datasets from chromatin immunoprecipitation experiments followed by high-throughput sequencing (ChIPseq) of core clock components [68–75] and publicly available RNAseq data from Arabidopsis plants infected with *Pst* DC3000 [76]. We found that among the differentially expressed genes at different time points after infection, several are targets of core clock components under non-infective conditions (Table 1). Furthermore, when the list of genes differentially expressed after the infection is compared with the list of CCG, it seems that the vast majority of target genes display a circadian regulated transcription. These results show that, under normal non-stressing conditions, core clock components directly bind to promoters and regulate the expression of a wide range of genes that are involved in the massive transcriptional reprogramming that takes place after a pathogenic infection. It would be interesting to assess whether this physical interaction is indeed sustained under biotic stress conditions.

Table 1. Targets of core clock components are differentially expressed upon infection with *pseudomonas syringae*. For full datasets refer to Supplementary Table 1.

Core clock components	Targets under non-infective conditions (ChIPseq)	Differentially expressed targets after infection (RNAseq)		
		Circadian regulation	Non-circadian regulation	Total
CCA1	2224	660	137	797
PRR9	132	69	3	72
PRR7	1096	458	50	508
PRR5	6326	1712	467	2179
TOC1	334	116	31	147
GI	5532	1484	381	1865
LUX	67	20	3	23
ELF3	392	110	22	132
ELF4	111	32	4	36

3. Effect of plant-pathogen interactions over core clock regulation

In recent years, the study of the interconnection between clock and immunity has gained complexity due to the identification of a regulatory cross-talk between defense responses and the circadian system. A growing body of evidence indicates that the transcriptional reconfiguration triggered by pathogens includes core clock and clock-controlled genes. Nevertheless, untangling the pathways by which an infection affects core clock gene expression networks is not an easy task [76–80].

One of the first studies that addressed this issue found that the rhythmic expression of *CCA1*, but not *LHY*, was significantly perturbed by infection of Arabidopsis with *Hpa Emwa1* at dawn, and that *CCA1* expression levels increased after exposure in continuous light conditions. They also found that under cycling 12 h:12 h light/dark conditions, an infection performed at dawn led to an up-regulation of defense genes that are controlled in a circadian manner by *CCA1*. These results suggested that the infection, rather than disrupting the overall tick of the clock, could have just engaged *CCA1* [23].

In contrast, infection of Arabidopsis with *Bci* did alter the expression of multiple core clock genes, suggesting a stress input to the whole central oscillator [79]. The authors found that infection with this necrotrophic fungus mildly reduced the expression amplitude of core clock genes such

as *CCA1*, *LHY*, *PRR9*, *PRR7*, *PRR5*, *TOC1*, *GI*, *LUX* and *ELF4*, 24 hours after infection in long-day conditions (16 h:8 h light/dark).

Although some aspects of the circadian regulation in this experiment could have been masked by the effect of environmental entrainment in light-dark cycles, over 60% of the 2404 genes with diurnal rhythmic oscillations were differentially expressed in response to *Bci* and, for most of them, expression in response to infection overrode rhythmic expression. Among the differentially expressed genes, the authors found a cluster of genes enriched in EE but that didn't show circadian oscillations, raising the question whether some clock components that bind to EE could function in a clock-independent manner.

A proper corroboration of the effect of *Bci* over circadian regulation was made a few years later, in an experiment in which Arabidopsis plants were inoculated under free-running conditions (LL) at either subjective dawn (CT24) or night (CT42) [64]. An analysis of Gene Ontologies (GO) showed enrichment in the circadian-rhythm category among the genes repressed in response to infection, including a general downregulation of morning clock genes such as *CCA1*, *LHY*, *PRR9*, *RVE8*, *LNK1*, *LNK2*, *LNK3*, and *LNK4* at both times of inoculation, which further validated the results reported by Windram et al. under cycling light conditions [79].

Different pathogens thus appear to have different effects on the regulation of core clock genes. In fact, seemingly contradictory results have arisen from the relatively small number of studies on this topic. In Table 2, we summarize the effects of different pathogens and infection protocols over core clock gene regulation.

One of the breaking points in the study of biotic stress effect over circadian clock function was achieved in a study by Zhang and collaborators [36], where a regulation of clock function by defense activation was identified for the first time: a shortening in the period of the clock reporter pCCA1:LUC after treatment with *P.syringae* pv. *maculicola* ES4326 either virulent (*PmaDG3*) or avirulent (*PmaDG6*) strains. The authors further explored which signaling pathways were involved in this feedback regulation of clock activity, and found that flg22 (a synthetic peptide that

elicits plant basal defenses) shortened the period of *CCA1* expression, but benzo (1,2,3) thiadiazole-7-carbothioic acid (BTH), which activates salicylic acid (SA) signaling, didn't. Thus, it appears that the activation of basal defense mechanisms, but not SA, could feedback to regulate the clock's pace.

In contrast, Zhou et al. later found that treatment with SA could indeed affect the clock regulatory network by increasing the amplitude and average expression of *TOC1* [81] and that this effect depended on the action of the master immune regulator NPR1 [82]. Interestingly, this alteration didn't affect *TOC1* expression period. Applying mathematical modeling, the authors attempted to explain these observations by predicting other possible NPR1-targeted clock genes. They identified that while *CCA1* did not respond to SA, the amplitude of *LHY* expression was significantly elevated by SA, as predicted by their model, and NPR1-controlled. Being *LHY* an antagonist of *TOC1* in the clock, the authors proposed that induction of *LHY* by SA could explain the delayed increase in *TOC1* after SA treatment at dawn, when *LHY* expression peaks. In microarray experiments 3 h after SA treatment either in the subjective morning (ZT24) or evening (ZT36) under constant light, the authors found that many defense-related genes were more induced in the morning, and that genes related to growth and development were more repressed after evening treatments. Interestingly promoters of these genes were significantly enriched in cis-elements bound by *CCA1*, *LHY* and *TOC1*.

A few years later, another study added a new level of complexity to this already intricate network. Li et al. found that a single-leaf *P.syringae* infection could systemically affect circadian rhythms in distal tissues [83]. Inoculation caused a significant reduction in amplitude and robustness of rhythms, and a period lengthening of ~0.5 h for *CCA1* and ~0.7 h for *LHY* and *TOC1* reporters. The authors also evaluated whether these clock phenotypes could be mediated by SA, and found that transient SA treatments could indeed phenocopy the amplitude reduction in the reporters, and introduce a delay in the phase of the clock. Thus, these studies suggested that SA could be one of the systemic signals that mediate the observed amplitude changes upon infection.

Table 2. Biotic stress signals alter the transcriptional regulation of core clock genes.

Biotic stress signal		Effect in core clock components	Measurement	Reference
Biotrophic oomycete	<i>Hyaloperonospora arabidopsidis Emwa1</i>	Alteration of the rhythmic expression pattern of <i>CCA1</i> . Unchanged <i>LHY</i> expression. Induced pCCA1:LUC expression that becomes arrhythmic. Unaltered pLHY:LUC expression.	RASL-seq time course in LD Bioluminescence time course in LL	[23]
	<i>Botrytis cinerea</i>	Reduction in the amplitude of expression of <i>CCA1</i> , <i>LHY</i> , <i>PRR9</i> , <i>PRR7</i> , <i>PRR5</i> , <i>TOC1</i> , <i>GI</i> , <i>LUX</i> and <i>ELF4</i> around 24 h.p.i. Reduced expression levels in <i>CCA1</i> , <i>LHY</i> , <i>PRR9</i> , <i>RVE8</i> , <i>LNK1</i> , <i>LNK2</i> , <i>LNK3</i> , and <i>LNK4</i> at 18 and 22 h.p.i.	qPCR time course in LD Microarrays in LL	
Pseudomonas syringae	Virulent <i>PmaDG3</i> and avirulent <i>PmaDG6</i> strain.	Shortening in the period of pCCA1:LUC expression.	Bioluminescence time course in LL	[36]
	Flg22 peptide Pathovar tomato DC3000	Downregulation of morning clock genes: <i>CCA1</i> , <i>LHY</i> , <i>PRR9</i> , <i>LNK1</i> , <i>LNK2</i> , <i>LNK3</i> , <i>LNK4</i> and <i>RVE8</i> 1 h.p.i. Upregulation of evening clock genes: <i>PRR5</i> , <i>TOC1</i> , <i>GI</i> , <i>ELF3</i> and <i>ELF4</i> 1 h.p.i. Downregulation of <i>PRR7</i> , <i>PRR5</i> , <i>LNK1</i> , <i>LNK2</i> , <i>LNK4</i> , <i>GI</i> , <i>LUX</i> , <i>ELF4</i> 6 h.p.i. Upregulation of <i>ELF4</i> 6 h.p.i. Upregulation of <i>LNK1</i> , <i>LNK3</i> , <i>LNK4</i> and <i>GI</i> 12 h.p.i.	RNAseq time course in LL	[54,76] and this review
Defense hormones	Virulent <i>PmaDG3</i> and avirulent <i>Pma avrRpm1</i> strain	Lower amplitude and ~0.5 hr longer period of pCCA1:LUC in systemic tissues. Amplitude and robustness reduction and ~ 0.7 hr longer period for pLHY:LUC and pTOC1:LUC in systemic tissues. Reduced <i>CCA1</i> and <i>LHY</i> mRNA levels 96 h.p.i. Unaltered <i>TOC1</i> mRNA levels. Reduced <i>LNK1</i> , <i>LNK2</i> , <i>LNK3</i> and <i>LNK4</i> mRNA levels 1 h.p.i. Induced <i>LUX</i> mRNA levels 24 and 48 h.p.i.	Bioluminescence time course in LL qPCR time course in LL qPCR qPCR in LL	[83] [54] [38]
	Salicylic acid (SA) long-term exposure	Increases in amplitude and average expression of <i>TOC1</i> mRNA levels. Increase in the amplitude of <i>LHY</i> mRNA levels. Unchanged expression levels in <i>CCA1</i> and <i>ELF3</i> . Increases in amplitude and average expression, but not in period, of pTOC1:LUC levels, when treated at subjective dawn or dusk. Increases in amplitude and average expression, but not in period, of pLHY:LUC levels, when treated at subjective dawn.	qPCR time course in LL Bioluminescence time course in LL	[81]
	Transient SA treatment	Low-amplitude and delayed phases in pCCA1:LUC, pLHY:LUC and pTOC1:LUC rhythms, without affecting period. Robustness only mildly perturbed in pLHY:LUC. Delayed phase in <i>CCA1</i> , <i>LHY</i> and <i>TOC1</i> mRNA levels.	Bioluminescence time course in LL qPCR time course in LL	[83]
	Methyl Jasmonate (MeJA)	Induced <i>LUX</i> and <i>CCA1</i> mRNA levels one and six hours after treatment (h.a.t) respectively. Suppression of <i>LUX</i> and <i>CCA1</i> , and <i>GRP7</i> transcripts at 24 and 48 h.a.t. Dampened amplitude of pCCA1:LUC. No effect in period nor phase.	qPCR in LL qPCR in LL	[38]
	Jasmonic Acid (JA) derivative JA-isoleucine (JA-Ile)	Amplitude dampening and mild period lengthening (<1 h) in pCCA1:LUC expression, without any phase change.	Bioluminescence time course in LL Bioluminescence time course in LL	

Interestingly, using the *npr1* null mutant, the authors also revealed an unexpected role for this gene antagonizing SA treatment and *Pst* DC3000 effect over clock regulation. Strikingly, at first glance, several contradictions arise from the comparison of these results with previous works. The authors attributed these discrepancies to differences in infection protocols, such as culture media, bacterial strains or SA concentrations and exposure time, raising the possibility that multiple

variables may influence the outcome of clock regulation upon infection. Differences aside, one common result is that exposure to pathogens, along with SA signaling, appears to have a significant effect on circadian clock regulation. These results confirm the role of biotic stress as a new regulatory layer of the plant circadian system.

Even though SA signaling is central for the deployment of defense responses against

biotrophic pathogens, these are only part of the range of biotic agents than can threaten a plant. Under necrotrophic-pathogens and insects attack, JA instead is responsible for the promotion of resistance [84]. The SA- and JA-response pathways are interdependent and act in complex networks, and the crosstalk between these two hormones fine-tunes transcriptional programs, which ultimately determine the balance of plant defense against invaders with different lifestyles [85]. Recently, Zhang and collaborators explored the interplay between the circadian clock and SA- and JA-mediated responses, and found that LUX, a component of the EC previously implicated in the control of antibacterial defenses [63], modulates key defense signaling mediated by SA and JA [38]. The authors further proved a prominent role of core clock components in the transcriptional regulation of defense-related genes, by performing a transcriptome profiling of the *lux-1* mutation. 1618 genes were differentially affected in *lux-1* mutants, with GO terms for abiotic and biotic stimuli significantly enriched. Furthermore, the activation of JA signaling was found to influence clock activity, as treatment of plants with different jasmonates dampened the expression profile and/or lengthened the period of pCCA1:LUC reporter. These results, combined with the upregulation of *LUX* expression 24–48 h after JA treatment, led the authors to propose LUX as a potential key node mediating the crosstalk between the circadian clock and defense signaling involving JA.

A common feature to most of the results discussed thus far is the fact that exposure to pathogenic bacteria, fungi, or defense-related hormones, lead to alterations in the transcriptional regulation of the circadian core clock several hours after the stress signal [36,38,64,79,83]. In contrast, hallmark responses of the defense activation in plants such as Ca^{+2} and ion fluxes, extracellular alkalization, ROS burst, MAPK activation, ethylene production, and even defense gene expression are triggered within the first hour upon PAMP recognition [86]. In fact, in a recent paper of our own, we found that an effect over the core clock transcriptional regulation can be detected as soon as one hour after bacterial infection [54]. By analyzing publicly

available RNAseq data from Arabidopsis infected with *Pst* DC3000 [76], we detected a significant enrichment in clock genes among those differentially expressed as early as one hour post-infection (1 h.p.i). Strikingly, this significant reconfiguration included most of the core clock gene network, resulting in an upregulation of evening genes and a downregulation of morning genes, including the four members of the *LNK* gene family. This effect required a functional nuclear pore complex (NPC), as in the nucleoporin mutant *ENHANCED DISEASE SUSCEPTIBILITY 4* (*eds4*) the effect of infection on *LNK* transcriptional regulation was attenuated. As a result, we proposed that an early event following pathogenic infection in plants includes a very rapid reconfiguration of the core clock-gene expression network and that the signal that triggers this transcriptional reprogramming must travel from the cytoplasm to the nucleus through the NPC.

To further dissect the transcriptional reconfiguration of the circadian gene network after pathogen infection, we meta-analyzed the transcriptomic data obtained 1, 6, and 12 h.p.i with *Pst* DC3000 [76]. PPR9 targets peak at 1 h.p.i, but don't display major changes at later times. TOC1 and PRR7 targets instead peak at 6 h.p.i and remain stable thereafter, whereas the EC targets peak at 6 h.p.i but decrease after 12 h.p.i. CCA1, PRR5 and GI targets, in turn, increase all along the infection time course, peaking at 12 h.p.i. Significantly, among the direct clock targets that are differentially expressed upon infection, the highest percentage of circadian controlled genes (CCG) are found 1 h.p.i, and this proportion tends to diminish at later times post-infection (Figure 1(a)).

Using statistical significance enrichment analyses, we studied the abundance of gene sets related to defense responses and the circadian function across the post-infection time course. Not unexpectedly, we found a significant enrichment (representation factor (RF) > 1; $p < 0.05$) in all post-infection time points for GO categories corresponding to defense responses, response to bacterium and response to SA and JA stimulus. Categories such as innate immune responses,

Figure 1. Time course of the circadian transcriptional reconfiguration after pathogen infection. (a) Meta-analysis of the Howard et al. Arabidopsis RNAseq dataset 1, 6 and 12 hours post infection (h.p.i) with *Pst* DC3000 [76]. At each time point, the differentially expressed genes (DEG) previously categorized as core clock targets in ChIPseq experiments were classified as circadian controlled genes (CCG) or non-circadian controlled genes (non-CCG) as per [66]. (b) Enrichment analysis of DEG along the post-infection time course. The representation factor (RF) was estimated as in [87]. The probability of each overlapping was determined using the hypergeometric test. The color gradient represents adjusted p values and the differences in bubble size correlate with the representation factor. (c) Expression level of the core circadian clock components. For full datasets and analysis refer to Supplementary Table 1.

systemic acquired resistance mediated through SA, hypersensitive response, and SA metabolic processes, as well as lignin metabolic processes, were enriched significantly at later times. These observations fit with the chronological evolution of Arabidopsis responses to bacteria, where higher-order ontologies are significantly enriched across all the time-course and lower order ontologies related to late physiological pathogen responses are enriched 6 and 12 h.p.i. Circadian clock-controlled genes, in contrast, are more enriched early after infection, and their RF decreases as the response evolves (RF = 5 1 h.p.i, RF = 3.1 5 h.p.i, and RF = 2.7 12 h.p.i). Interestingly, clock-core

genes display an acute enrichment 1 hpi (RF = 36.4 1 h.p.i, RF = 6.4 6 h.p.i and are not significantly enriched 12 h.p.i), and their targets, except for TOC1's, follow a similar pattern (Figure 1(b)). In fact, the expression levels of morning and evening core clock components were severely disturbed as fast as 1 h.p.i and this effect dampened as the infection proceeded, with only three members of the *LNK* family and *GI* differentially expressed 12 h.p.i (Figure 1(c)). Taken together these observations expose the dynamic nature of the transcriptional reconfiguration that underpins defense responses, whereby core clock genes and their targets, as well as genes that display

a circadian control, are rapidly and deeply affected by the pathogen challenge.

4. Implications of the clock-pathogen crosstalk

A growing body of evidence shows that various defense-related processes fluctuate in diurnal rhythms and that these are partially controlled by the circadian clock through transcriptional regulation. This observation leads to the questioning of the biological and evolutionary meaning attached to these phenomena.

Given their sessile nature, plants rely on continuous and directional growth programs, in which they allocate significant energetic resources. Simultaneously, their inability to escape danger, expose them to a wide variety of biotic and abiotic threats, which led them to evolve sophisticated defense and tolerance mechanism to cope with suboptimal environmental conditions. Nevertheless, the limited nature of energetic resources imposes growth-defense tradeoffs, so that resources can either be allocated to one or another of these vital processes [88]. Plants deploy several mechanisms to ameliorate the negative effect of this tradeoff [89]. One such strategy seems to be the spatial restriction of defense resources through plant tissues. This notion was proposed more than 50 years ago in the “Hypothesis of the Optimum Defense” (ODH) by McKey and collaborators [90]. This hypothesis establishes that plants preferentially assign resources to the defense of tissues that are either more likely to be attacked or more valuable, resulting in tissue-specific “defense on demand” through the integration of developmental and defense signaling pathways [90,91].

Another way of reducing the cost associated with immunity is to mount defense responses only upon infection. A clear example of this strategy is the massive transcriptional reconfiguration that takes place after the identification of stress signals [77]. Still, inducing the whole defense machinery *de novo* would take a considerable amount of time, in which fast-growing pathogens could gain an advantage in colonization. As a consequence, plants have evolved a temporal regulation of defense responses to anticipate

possible pathogenic attacks. In line with this notion, in 2013 Baldwin et al. [92] proposed that the diurnal regulation of defense responses may be considered another dimension of the ODH.

From the results obtained in different studies performed with diverse pathogens and insects, circadian regulation of plant defense could have evolved as a response to the integration of daily environmental cues combined with the circadian oscillation of biotic threats. Almost a decade ago Wang and collaborators proposed that the CCA1-mediated pulse expression of defense genes correlated with the time of *Hpa* sporulation at night and spore dissemination at dawn, suggesting that plants could anticipate the oomycete infectiousness according to a circadian schedule [23]. In line with this, it was found that, when sprayed with *P.syringae*, plants display a higher resistance during the night, when stomata are mostly closed and physical barriers limit colonization [36]. Instead, when the infection is performed by pressure infiltration (bypassing the natural entrance via stomata), plants present a higher resistance at dawn [62]. This could mean that during the day, when stomata are wide open to favor gas exchange, plants reinforce nonphysical barriers. Thus, these observations position the circadian regulation as a mean to optimize defense responses. This notion is further supported by feeding essays of cabbage loopers (*Trichoplusia ni*) in *Arabidopsis* under free-running conditions after entrainment in 12 h light/12 h dark cycles [18]. Plants are more resistant when entrained under the same conditions as the loopers, but more susceptible when entrained in light/dark cycles with opposite phase to that of the loopers, and this effect requires an intact JA pathway and a functional circadian clock. Also, JA levels peak in the middle of the subjective day, a few hours earlier than *T. ni* maximal feeding activity, but SA levels peak in the middle of the subjective night, prior to *Hpa* spore dissemination at dawn, suggesting a circadian regulation of hormonal fluctuations in tune with distinct pathogen behaviors.

Although these studies were performed under controlled growth conditions, quite distant from the complexity of plants’ natural environment, they reinforce the idea of a prominent role for the circadian clock in the orchestration of plant

growth and defense. Nevertheless, a complex landscape arises from recent findings regarding the effect of pathogenic attack and subsequent defense responses over circadian clock regulation. Different authors propose different explanations for the transcriptional rewiring of core clock components upon infection. Many authors have considered that the deregulation of core clock gene expression could be part of the defense-response mechanisms deployed by plants to resist infection. As proposed by Zhang and collaborators, a feedback regulation of the circadian clock by defense activation could be useful for the host to balance resource assignation between defense and other vital processes such as growth and development [36,38]. In line with this notion, Zhou and collaborators proposed that the upregulation of *TOC1* expression in the evening and of *LHY* in the morning could be a way of reinforcing the circadian function in plants, after SA treatment. Given that *TOC1* was identified as a negative regulator of defense responses, and that *LHY* was already characterized as a partially redundant positive regulator of defenses [36,64], the authors hypothesized that increasing the expression of both positive and negative regulators of immune responses could balance resource assignation under pathogenic attack. In this way plants could prevent a bias toward defense at the expense of other functions [81]. An alternative hypothesis presented by Li et al. concurs with the idea that a transcriptional reprogramming of the circadian network could be part of defense responses, but instead proposes that the main goal of this reconfiguration could be instead the attenuation of circadian rhythms. Both local *Pst DC3000* infection and transient SA treatment induced a period and/or phase change in core clock components such as *CCA1*, *LHY*, and *TOC1*, which would lead to a desynchronization of the endogenous cycles from external environmental cues. The authors propose that this effect could anticipate a suboptimal photosynthetic capacity, which could prevent the detrimental consequences of ROS hyper-accumulation, and weaken the circadian function after infection to ensure a continuous immunity that can restrain bacterial growth as well as prevent future re-infections [83].

In contrast to this “plant-centered strategy”, it has been suggested that the effects on the function of the clock could instead be part of a “pathogen strategy” to gain an advantage during colonization. When Windram and collaborators found a dampening in the expression of core clock components 24 h after infection with *Bci*, they proposed that this could be an attempt of the pathogen to interfere with the rhythmic defense response [79]. Through the evolutionary arms race between hosts and pathogens, the latter have evolved an arsenal of effector proteins that aid in the process of host colonization. A wide range of proteins secreted by diverse pathogenic species can function either in the apoplast or inside host cells, facilitating pathogen entry into the plant’s body, suppressing defense responses, and even altering host physiology for their benefit [93]. Pathogen strategies include targeting of transcription factors [94,95], induction of the expression of host genes that lead to susceptibility [96,97] and interference with host gene silencing [98–100]. In a recent work performed by our group, we proposed that this could be an explanation for the early transcriptional reconfiguration of *Arabidopsis* core clock genes 1 hour after infection with *Psa* [54]. We found that infection in the morning down-regulated morning clock genes and upregulated evening genes, which antagonizes the fact that basal defense responses are circadian gated toward the morning. Also, we found that the *eds4* mutant, well known for its susceptible phenotype to *Pseudomonas* [101], presented a similar deregulated profile of core clock transcripts under non-infective conditions. That led us to hypothesize that such re-configuration of clock gene expression could be detrimental for defense responses. Still, much work must be done to elucidate the evolutionary meaning and the molecular mechanisms behind the crosstalk between the circadian clock and immunity. The detection of a nearly immediate effect over the transcriptional regulation of the circadian clock after a bacterial challenge [54], combined with sustained alterations after SA treatment [81,83], could expose different stages during pathogenic infection, in which circadian regulation could be manipulated either by the host or the pathogen to influence the interaction

outcome. It is worth noting that even though SA plays an important role in both PTI and ETI [102], it represents only a portion of the signaling mechanisms activated during infection. Given that many plant pathogens implement different kinds of strategies to interfere with defense-responses, the use of whole pathogens instead of hormones could provide a more realistic representation of the wide range of processes at work in the plant-pathogen interaction.

5. Circadian clock and defense responses in crops

Given the status of *Arabidopsis* as a model organism, it is not unexpected that most of the circadian clock studies have been carried on this plant. It is well known that many fitness-related agronomical traits in crops are controlled by the circadian clock, and even more knowledge is available regarding the negative impact of pathogen infections over plant fitness [103–105]. Nevertheless, while there is abundant evidence linking the circadian clock function with abiotic stress tolerance in crops, there is only a handful of studies addressing the role of the circadian clock in biotic stress responses. As proposed by Rathinavel S. et al. [106] “the principles of chronobiology have gone unrecognized and thus under-exploited in the study of plant diseases” [107,108].

After a small number of studies at the end of the ‘80s reported alterations in circadian processes upon pathogen infection, the first contemporary study regarding circadian clock and pathogens in crops was performed in rice. The authors showed that the rice susceptibility to *Helminthosporium oryzae* displayed a circadian behavior, evidencing more damage when the infection takes place at night [107,109,110]. Ten years later, Goodspeed et al. demonstrated that the circadian clock of harvested cabbage (*Brassica oleracea*) is responsive to light-dark cycles, and influences postharvest physiology in concordance with environmental cues, which leads to enhanced herbivore resistance probably due to the cyclical accumulation of defensive metabolites. Furthermore, they showed that the phenomena of postharvest entrainment and enhanced herbivore resistance are widespread among diverse crops [111]. The work by Soenegas

et al. also showed that the concentration period and amplitude of the herbivore deterrent glucosinolates in *Brassica* are cultivar-dependent [112]. In line with these findings, it was reported that the expression of two important defense resistance genes in crops (*TSN1* in wheat and *ZmLOX10* in maize) as well as *DEA1*, a gene of unknown function in tomato induced by arachidonic acid and *Phytophthora infestans* infection, are tightly controlled by the circadian clock [113–115]. These evidences strongly suggests that several crops keep track of the time of the day to coordinate their defenses.

The advent of new technologies made possible the analysis of global transcriptional effects of pathogen infection in plants of agronomic interest. To date, there are only three analyses that exploit RNAseq technology to this end. The infection of *Paulownia fortune* (an ornamental tree whose use in the wood industry is increasing) was characterized to unveil the molecular mechanisms behind the *Paulownia* witches’ broom (PaWB) phytoplasma infection. The authors reported that, as expected, genes that encoded key enzymes in plant-pathogen interaction processes were significantly up-regulated. Nevertheless, interesting genes involved in circadian rhythms were also altered after PaWB infection [116]. Another RNAseq analysis compared the alterations in the transcriptome of sweet orange trees (*Citrus sinensis* “Valencia”) when challenged with three different pathogens: CaLas-B232 (*Candidatus Liberibacter asiaticus*, CaLas), CTV-B6 and CTV-B2 (*Citrus tristeza virus*, CTV); causing agents of Huanglongbing and tristeza disease. The transcriptome profiles revealed 611, 404 and 285 DEG after infection with CaLas-B232, CTV-B6 and CTV-B2 respectively. Strikingly the circadian system was affected by all three pathogens [117]. However, there is so far only one high-throughput study specifically aimed at analyzing the interplay between circadian physiology and defense responses in plants of agronomic interest. In this study, tomato plants grown in 12 h light- 12 h dark photoperiod were infected with *Pst* DC3000 at different time points (0a.m. – 4a.m. – 8a.m. – 12p.m. – 4p.m. – 8p.m.). Interestingly, the authors reported that the highest level of susceptibility was observed when the plants were challenged at

midnight. An RNAseq analysis of plants infected in the morning revealed that the tomato orthologues of the morning genes *CCA1* and *LHY* were down-regulated, while the evening expressed gene *SLTOC1* was up-regulated [118]. Although studies addressing the interaction between the circadian clock and pathogen infections are sparse, an increasing amount of evidence is pointing out that the circadian network plays an important role in the plant-pathogen cross-talk, either as targeted by the pathogen to guarantee infection success or as a master regulator of plant immunity responses.

6. Perspectives

More than two decades of investigation have positioned the circadian clock and the circadian transcriptional regulation as a central feature of defense programs in plants. As it happens with many clock-controlled processes, this regulation isn't unidirectional. Nowadays, we are just beginning to understand the implications of the plant-pathogen interaction over circadian regulation and much work is needed to untangle the true meaning of the clock-defense crosstalk. A better understanding of the intricate network that connects the circadian system to immunity would represent a great advance for basic science, but also could pave the way for future applications in the improvement of crops. In humans, the study of the therapeutic implications of circadian rhythms over immunity has led to the development of a field termed chronotherapeutic. This field aims to study how the time of day conditions the metabolism, efficacy, toxicity, and specificity of therapeutics [119]. In plants, the concept of agricultural chronotherapy was recently introduced in a study with *Arabidopsis*, in which the authors identified differential effectiveness of glyphosate-based herbicide depending on the time of the day of application [120]. It would be worth studying whether this effect can also be found in crops in the field and if similar oscillations are detectable for other crop responses that could boost the effectiveness of pest control. Thus, it seems possible that in the future a better understanding of the interplay between clock and defenses could help improve

disease resistance, crop management, and productivity in agricultural relevant species.

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Supplementary information

Supplementary Table 1. Datasets used and analysis performed in this review.

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