

## Microreview

# The circadian clock and defence signalling in plants

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## SUMMARY

The circadian clock is the internal time-keeping machinery in higher organisms. Cross-talk between the circadian clock and a diverse range of physiological processes in plants, including stress acclimatization, hormone signalling, photomorphogenesis and defence signalling, is currently being explored. Recent studies on circadian clock genes and genes involved in defence signalling have indicated a possible reciprocal interaction between the two. It has been proposed that the circadian clock shapes the outcome of plant–pathogen interactions. In this review, we highlight the studies carried out so far on two model plant pathogens, namely *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*, and the involvement of the circadian clock in gating effector-triggered immunity and pathogen-associated molecular pattern-triggered immunity. We focus on how the circadian clock gates the expression of various stress-related transcripts in a prolific manner to enhance plant fitness. An understanding of this dynamic relationship between clock and stress will open up new avenues in the understanding of endogenous mechanisms of defence signalling in plants.

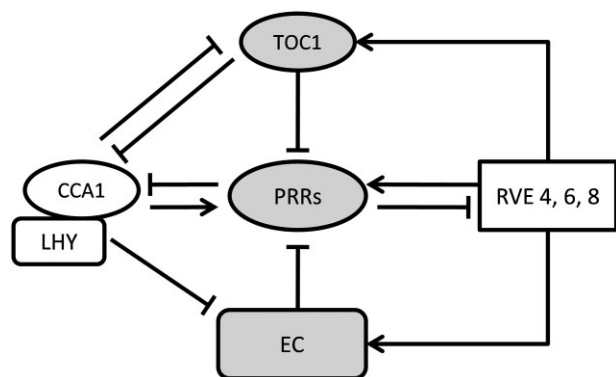
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## INTRODUCTION

The biological processes of an organism change greatly during the day and night, and continuous entrainment by environmental factors results in the establishment of a biological rhythm. An endogenous biological rhythm with a period of approximately 24 h is known as the circadian rhythm (McClung, 2001). These circadian rhythms persist even in the absence of external cues for a definite time period, known as the free running time (Jones, 2009). The circadian rhythms are maintained by a well-defined molecular machinery—the circadian clock. The circadian clock temporally coordinates biological processes with diurnal environmental changes, thus enhancing overall fitness. It is well documented that a diverse range of organisms, including

cyanobacteria, algae, fungi, plants, flies, birds and mammals, possess their own circadian clock (Bell-Pedersen *et al.*, 2005). Plants, being sessile in nature, are dependent on this endogenous clock machinery to acclimatize themselves with environmental variations and to prepare for future changes. Many of the plant physiological and developmental processes are regulated by this endogenous regulator (Yakir *et al.*, 2007). Since 1985, when the molecular study of the plant circadian clock began, the clock network has widened, and we are still trying to delineate it (Kloppstech, 1985; McClung, 2014). It has been documented that several abiotic factors, such as light and temperature, provide an input signal to the clock; in turn, the clock provides various output signals that influence the downstream processes (Kinmonth-Schultz *et al.*, 2013). In the model plant, *Arabidopsis thaliana*, the circadian clock framework is made up of various transcriptional–translational feedback loops (TTFLs). The circadian clock components constitute DNA-binding transcription factors. Two MYB domain-containing transcription factors, namely Circadian Clock Associated 1 (CCA1) and Late elongated HYpocotyl (LHY), and a pseudo-response regulator (PRR), Timing Of CAB2 expression 1 (TOC1), were initially attributed as core clock components, as mutations in these genes showed severe alterations in phase, amplitude and period of the circadian clock (McClung, 2006). These three components together form a negative feedback loop, thus allowing their phase of the day-specific expression. Negative feedback loops are formed by the majority of core clock proteins, where they inhibit their own expression at the transcriptional or translational level (Harmer, 2009). CCA1 and its close homologue LHY are morning-expressed genes. CCA1/LHY bind to evening element (EE) present in the promoter region of TOC1, an evening-expressed gene, and repress its expression (Alabadí *et al.*, 2001). In turn, TOC1 directly represses the expression of CCA1, thus closing the loop (Gendron *et al.*, 2012). The EE is a nine-nucleotide 'AAAATATCT' conserved motif and the target site for the binding of several clock proteins, including CCA1, LHY and REVEILLE (RVE). Advancements in the biology of the circadian clock during the last decade have revealed several other core components, namely PRRs, LUX Arrhythmia (LUX), Early Flowering 3 and 4 (ELF3, 4) and the RVE group of transcription factors. These components form different feedback loops interconnected with the CCA1–LHY–TOC1 network (Fig. 1). The circadian clock network is more complex and robust in plants relative to other eukaryotes. This

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**Fig. 1** A simplified schematic representation of transcriptional regulatory networks of the *Arabidopsis thaliana* circadian clock. Arrows indicate activation and perpendicular bars represent repression. Circadian Clock Associated 1/Late elongated HYpocotyl (CCA1/LHY) represses the expression of Timing Of CAB2 expression 1 (TOC1) and evening-expressed Early Flowering 3 and 4 (ELF4) and LUX Arrhythmia (LUX) via binding to evening element (EE). CCA1 also activates the transcription of *PRR9* and *PRR5*. PRRs are transcriptional repressors and RVEs (REVEILLE) function as transcriptional activators. TOC1, an evening-expressed component, represses the expression of other PRR's. EC, evening complex (ELF3, ELF4 and LUX together form an EC); PRRs, pseudo-response regulators. Adapted from Hsu and Harmer (2014).

network is tightly regulated at the post-transcriptional, post-translational and epigenetic levels (Cui *et al.*, 2013; Malapeira and Mas, 2013). The circadian clock model in higher plants is still evolving and is under developmental regulation (McClung, 2014).

The application of genome-wide 'omics' approaches has helped the scientific community to comprehend the clock-related output pathways. A genome-scale, time-course expression profiling of *A. thaliana* revealed that about one-third of global transcripts were circadian regulated (Covington *et al.*, 2008). These large-scale datasets provided a glance at the diversity of the circadian clock outputs. The clock output pathways vary from plant diurnal developmental processes, such as cotyledon and leaf movement (Engelmann and Johnsson, 1998), to biochemical and metabolic processes (Harmer *et al.*, 2000), including acclimatization to abiotic and biotic stresses (Roden and Ingle, 2009; Sanchez *et al.*, 2011) and hormone signalling (Robertson *et al.*, 2009).

Roles of light and temperature in the modulation of plant immunity have been elucidated extensively (Genoud *et al.*, 2002; Hua, 2013; Zeier *et al.*, 2004); nevertheless, the role of the clock in defence signalling was under shade (McClung, 2011; Molina *et al.*, 1997). Evidence from animal sciences has validated that the circadian clock plays an indispensable role in regulating the immune system (Scheiermann *et al.*, 2013). The clock mutants of *Drosophila* display enhanced susceptibility to pathogen attack

and a lower survival rate (Kuo *et al.*, 2010). Interaction of the clock and immune system is known to be bidirectional in animal systems (Duhart *et al.*, 2013). This reciprocal nature of clock–immune system interaction is now also evident in plants (Zhang *et al.*, 2013). Studies hitherto have been based on two core clock components of the model system *A. thaliana*: CCA1 and LHY. These components were examined for their involvement in plant defence signalling towards anticipating pathogen attack (Wang *et al.*, 2011; Zhang *et al.*, 2013). Several lines of evidence have supported the involvement of the circadian clock in the enhancement of organismal adaptation towards predictable changes in nature (Bhardwaj *et al.*, 2011; Goodspeed *et al.*, 2012, 2013a, b; Roden and Ingle, 2009). How this interaction is beneficial for plants is discussed below.

## POSSIBLE INTERACTIONS AND CIRCADIAN GATING OF DEFENCE GENES

The circadian clock limits a physiological or biochemical process to a specific portion of the circadian cycle; this is known as circadian gating (Yakir *et al.*, 2007). The circadian clock gates several signalling events to effectively employ resources. For instance, the expression of cold-responsive genes is higher in the early morning and late evening, when the temperature drops to a sudden low level (Bieniawska *et al.*, 2008). Likewise, the expression of defence-related genes is required to orchestrate with the peak time of pathogen attack during a subjective day. Extensive data, gathered over the past few years, have provided unambiguous evidence supporting the involvement of the circadian clock in the defence response by plants (Bhardwaj *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2013), and the studies indicate that the circadian clock is involved in the modulation of plant immunity.

The plant immune system can broadly be characterized at four levels. The presence of various physical barriers to restrict the entry of the pathogen to the inner core of plant tissues provides the first layer of defence (Campbell *et al.*, 2012). Second is pathogen-associated molecular pattern (PAMP)-triggered immunity, or PTI, which involves the identification of various conserved PAMPs via pattern recognition receptors present at the plant cell surface, thus activating downstream defence signalling. This results in a cascade of events, including stomatal closure, the production of reactive oxygen species and callose deposition in the cell wall (Ingle *et al.*, 2006; Nürnberger *et al.*, 2004), to prevent the entry of bacterial pathogens or effectors secreted by pathogens. PTI serves as a basal defence system and offers a primary shield on pathogen attack. Some pathogens have evolved to evade PTI and secrete virulence effector molecules inside the host cell to suppress the signalling events induced by PTI and to develop effector-triggered susceptibility (ETS) in the host (Jones and Dangl, 2006). Bacterial pathogens inject these effectors inside the host cell with the help

of a well-defined type III secretion system (T3SS), whereas oomycete pathogens secrete these effectors via the formation of invasion hyphae or haustoria (Büttner and He, 2009; Petre and Kamoun, 2014). A third level of the plant immune system has evolved to combat ETS, and is known as effector-triggered immunity, or ETI. Specific resistance (R) proteins recognize cognate effectors and trigger a stronger defence response leading to, not strictly, programmed cell death (PCD) (Chisholm *et al.*, 2006; Jones and Dangl, 2006). This basal and R-mediated defence response sometimes induces a higher echelon of defence response, known as systemic acquired resistance (SAR) (Ryals *et al.*, 1996). SAR is the fourth level of plant defence, producing whole-plant resistance to a broad range of pathogens. The resistance is systemic in the sense that a local encounter leads to the activation of resistance to the rest of the plant organs via intra-plant communication. SAR usually involves the accumulation of salicylic acid (SA) and leads to the induction of various SA-mediated signalling events (Fu and Dong, 2013; Gaffney *et al.*, 1993).

During PTI, PAMP perception by receptors at the cell surface initiates a mitogen-activated protein kinase (MAPK) signalling cascade, resulting in the downstream activation of various physiological processes. Bhardwaj *et al.* (2011) analysed, *in silico*, the public microarray repository of the model system *Arabidopsis* and demonstrated that a leucine-rich repeat receptor kinase, encoding a flagellin (bacterial PAMP) receptor FLS2 (FLagellin-Sensitive 2), and downstream MAPK signalling components (MKK4/5–MAPK3/6–WRKY22 module) are circadian regulated, as they show peak expression in the subjective morning during a circadian cycle. This concurs with stomatal opening in the morning, when stomata are initially unable to prevent bacterial invasion. This implies that the basal defence level should be sufficiently adequate to counter pathogen attack whenever the physical barriers, i.e. stomata, are unable to prevent pathogen entry.

One of the earliest studies to provide a clue on clock–defence connection was of a pathogen-responsive gene coding for glycine-rich protein (GRP) in barley (Molina *et al.*, 1997). Two GRPs (*Hvgrp2* and *Hvgrp3*) were shown to be rapidly induced on attack by fungal pathogens. The expression level of *Hvgrp3*, an RNA-binding GRP, was variable during different time courses in a light/dark cycle of 16 h/8 h. In *Arabidopsis*, a member of the GRP protein family, *AtGRP7*, is involved in the regulation of stomatal movement, stress responsiveness, floral transition and the circadian clock (Mangeon *et al.*, 2010; Streitner *et al.*, 2008; Zhang *et al.*, 2013). GRP7 is also associated with PTI in plants. The evidence indicates the direct binding of GRP7 with several pattern recognition receptors encoding transcripts in response to pathogen attack. GRP7 is also one of the targets of a pathogen effector that inhibits its binding with pattern recognition receptor transcripts and results in the suppression of PTI (Nicaise *et al.*, 2013). Although the expression of *AtGRP7* is circadian regulated, it cannot affect the expression of core clock genes, and hence is

designated as a 'slave oscillator' (Heintzen *et al.*, 1997). A slave oscillator has a circadian output, but is dependent on a master or core oscillator for its function. *AtGRP7* acts downstream of a core oscillator CCA1 via a putative interaction with an EE present in its promoter region.

Promoters enriched in EE could be hypothesized to act downstream of clock genes. Subsequently, to identify the set of defence-related genes regulated by the circadian clock, we performed *in silico* analysis of *Arabidopsis* circadian-regulated genes. A list of the circadian-regulated genes was obtained from supporting information submitted with Covington *et al.* (2008). We further refined the list by selecting only those circadian-regulated genes that were redundant in two independent experiments of Covington *et al.* (2008). The online web server Plant Gene Set Enrichment Analysis Toolkit (<http://structuralbiology.cau.edu.cn/PlantGSEA/>) was used for gene set enrichment analysis of the circadian-regulated genes. Initially, 1595 circadian-regulated genes were requested for analysis. After analysis of the enriched gene set, we identified 185 biotic stress- and defence-related genes involved in various signalling pathways. We further screened their promoter sequences for the presence of EE of the CCA1-binding site (CBS). We used the Athena program (<http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl>) to analyse the 1000-bp upstream region up to the adjacent gene of these 185 genes (cut-off  $P < 10^{-4}$ ). Of these 185 genes, 75 were identified to be significantly enriched with either EE (26) or CBS (42), or both (7) (Table 1). This analysis identified a number of putative defence-related genes that could be the direct targets of CCA1/LHY and might be circadian regulated. The list includes genes involved in various signalling pathways which are triggered to anticipate pathogen attack. There is a possibility that some of the genes might act as slave oscillators that work on the framework in a similar manner to *AtGRP7*, although a more robust biochemical analysis is needed to support this hypothesis.

A firm connection between the two diverse physiological processes clock and defence signalling has been demonstrated by Wang *et al.* (2011). A study of the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), with regard to the delineation of the molecular basis of R gene-mediated defence, revealed that R gene-mediated resistance shares some common components with the basal defence mechanism. A leucine-rich repeat receptor-like kinase (LRR-RLK, At1g35710) was identified as a connecting link between the two. Plants defective in Atg35710 were insensitive to a microbe-associated molecular pattern (MAMP), EF-Tu (elf18), and were highly susceptible to *Hpa* Emwa1 infection, demonstrating the functional inactivation of both R gene-mediated and basal defence. The analysis of *Arabidopsis* mutants defective in R gene-mediated defence (*rrp4*), to avirulent *Hpa* isolate Emwa1 (recognized by R protein RRP4), resulted in the identification of a unique set of 22 defence-related

**Table 1** List of *Arabidopsis* defence-related genes enriched with clock regulatory elements, identified from Covington *et al.* (2008).

Locus	Gene name	Description	Clock element
At1g10760	SEX1 (Starch Excess 1)	Encodes an $\alpha$ -glucan, water dikinase	EE
At1g11260	STP1 (Sugar Transporter 1)	H <sup>+</sup> /hexose co-transporter	CBS
At1g18570	MYB51	Involved in indoleglucosinolate biosynthesis	EE
At1g20020	LFNR2	Encodes a leaf-type ferredoxin:NADP(H) oxidoreductase	CBS
At1g20620	CAT3 (Catalase 3)	Catalyses the breakdown of hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> )	EE
At1g22410	Class-II DAHP synthetase	3-Deoxy-7-phosphoheptulonate synthase activity	CBS
At1g24100	UGT74B1	Involved in glucosinolate biosynthesis	CBS
At1g33590	Leucine-rich repeat (LRR) family protein	Signal transduction, defence response	CBS
At1g33600	Leucine-rich repeat (LRR) family protein	Signal transduction	CBS
At1g33970	P-loop-containing NTP hydrolases	GTP binding	EE
At1g37130	ATNR2 (Nitrate Reductase 2)	Encodes nitrate reductase structural gene	CBS
At1g53230	TCP3	Involved in leaf differentiation	CBS
At1g53320	TLP7 (Tubby Like Protein 7)	Phosphoric diester hydrolase activity	CBS
At1g55210	Disease resistance-responsive protein	Defence response	CBS
At1g59870	PDR8 (Pleiotropic Drug Resistance 8)	ATP-binding cassette transporter	CBS
At1g61740	Sulphite exporter TauE/SafE family protein	Regulation of plant-type hypersensitive response	CBS
At1g64780	AMT 1;2 (Ammonium Transporter 1;2)	Act as a high-affinity transporter	CBS
At1g66760	MATE efflux family protein	Antiporter activity, drug transmembrane transport	CBS
At1g66980	SNC4 (Suppressor Of Npr1-1 Constitutive 4)	An atypical receptor-like kinase	CBS
At1g74710	ICS1 (Isochorismate Synthase 1)	Mutants fail to accumulate salicylic acid	CBS
At1g75800	PR thaumatin superfamily protein	Pathogenesis-related protein	CBS
At1g78600	LZF1 (Light-Regulated Zinc Finger protein 1)	Negative regulation of defence response	CBS, EE
At2g02100	PDF2.2 (Plant Defensin 2.2)	Predicted to encode a PR (pathogenesis-related) protein	EE
At2g15890	MEE14 (Maternal Effect Embryo Arrest 14)	Defence response to fungus	EE
At2g18290	APC10 (Anaphase Promoting Complex 10)	Ubiquitin-dependent protein catabolic process	CBS
At2g21660	ATGRP7 (Glycine-rich protein 7), CCR2	Slave oscillator, promotes stomatal opening	EE
At2g22240	MIP52 (Inositol 3-Phosphate Synthase 2)	Response to phosphate starvation	EE
At2g22540	AGL22 (Agamous-Like 22)	Nuclear protein that acts as a floral repressor	EE
At2g24550	Unknown	Regulation of plant-type hypersensitive response	CBS
At2g26170	CYP711A1 (Cytochrome P450 Family)	Involved in flavonoid pathway	CBS
At2g29630	PY (Pyrimidine Requiring)	Involved in thiamine biosynthesis	EE
At2g29650	PHT4;1 (PHOSPHATE TRANSPORTER 4;1)	Inorganic phosphate transporter	CBS
At2g34690	ACD11 (ACCELERATED CELL DEATH 11)	Transports the glycolipid precursor sphingosine	CBS
At2g37040	PAL1 (Phenylalanine ammonia-lyase)	L-Phenylalanine catabolic process	CBS
At2g37220	A chloroplast RNA-binding protein	Involved in innate immune response	CBS,EE
At2g40460	Major facilitator superfamily protein	Involved in oligopeptide transport	CBS
At2g41430	LSR1 (Light Stress-Regulated 1)	Hydrophilic protein lacking cysteine residues	EE
At2g42530	COR15b (Cold Regulated 15B)	Response to cold and defence to fungus	CBS,EE
At2g46450	CNGC12 (Cyclic Nucleotide-Gated Channel)	Cation channel activity, RESPONSE TO FUNGUS	CBS
At3g05800	AIF1 (AtBS1 Interacting Factor 1)	Brassinosteroid-mediated signalling pathway	CBS,EE
At3g12580	HSP70 (Heat Shock Protein 70)	Response to heat, ER stress, bacteria	CBS
At3g17020	Adenine nucleotide $\alpha$ hydrolases-like	Response to cold, molecule of fungal origin	EE
At3g21690	MATE efflux family protein	Antiporter activity	EE
At3g27210	Unknown protein	N-terminal protein myristoylation	CBS
At3g46530	RPP13 (Recognition of <i>P. parasitica</i> 13)	A nucleotide-binding site leucine-rich repeat (NBS-LRR)-type R protein	CBS
At3g58750	CSY2 (CITRATE SYNTHASE 2)	Peroxisomal citrate synthase	EE
At4g16950	RPP5 (Recognition of <i>P. parasitica</i> 5)	Putative TIR-NB-LRR receptors	EE
At4g16990	RLM3 (Resistance To Leptosphaeria mac. 3)	Toll-Interleukin receptor, ATP binding	CBS
At4g26850	VTC2 (Vitamin C Defective 2)	GDP-D-glucose phosphorylase activity	CBS
At4g30650	Low temperature and salt-responsive protein	Defence response to fungus	EE
At4g32770	SDX1 (Sucrose Export Defective 1)	Tocopherol synthase involved in tocopherol synthesis	CBS
At4g33300	ADR1 like 1	Nucleotide-binding leucine-rich repeat (NB-LRR)	CBS
At4g34590	BZIP11	Encodes a basic domain leucine zipper (bZip)	CBS
At4g37870	PEPCK1	Encodes a phosphoenolpyruvatecarboxylase	CBS
At4g39980	DHS1 (Phosphate Synthase 1)	Aromatic amino acid family biosynthetic process	CBS,EE
At5g02500	HSP70-1 (Heat Shock Protein 70-1)	Member of heat shock protein 70 family	EE
At5g08330	TCP11 (TCP Domain Protein 11)	Negative regulation of sequence-specific DNA binding	CBS
At5g11670	NADP-ME2 (NADP-Malic Enzyme 2)	Involved in malate metabolism	CBS
At5g15410	DN1 (Defence No Death 1)	Mutated cyclic nucleotide-gated cation channel	EE
At5g20630	GLP3 (Germin-Like Protein 3)	Cellular cation homeostasis	CBS,EE
At5g24530	DMR6 (Downy Mildew Resistant 6)	Encodes a putative 2OG-Fe(II) oxygenase	CBS
At5g35735	Auxin-responsive family protein	Negative regulation of defence response	CBS
At5g36910	THIONIN 2.2	Encodes a thionin, toxin receptor binding	EE
At5g40170	RLP54 (Receptor Like Protein 54)	Kinase activity	EE
At5g42650	CYTOCHROME P450 74A (CYP74A)	Allene oxide synthase	EE
At5g42810	Inositol-pentakisphosphate 2-kinase 1	Involved in the biosynthesis of phytic acid	CBS
At5g47220	ERF2 (Ethylene Response Factor-2)	Ethylene-mediated signalling pathway	CBS
At5g48590	DUF760 (Unknown function)	Starch biosynthetic process, systemic acquired resistance	CBS
At5g49450	BZIP1 (Basic Leucine-Zipper 1)	Positive regulator of plant tolerance to abiotic stresses	CBS
At5g51770	Serine/threonine kinase family protein	Kinase activity, systemic acquired resistance	CBS
At5g51820	PGM1 (Phosphoglucomutase 1)	Involved in controlling photosynthetic carbon flow	EE
At5g56280	CSN6A	Subunit 6 of COP9 signalosome complex	CBS
At5g57110	Autoinhibited Calcium ATPase, Isoform 8	ATP biosynthetic process	CBS
At5g63780	SHA1 (Shoot Apical Meristem Arrest 1)	Putative E3 ligase	CBS,EE
At5g66570	OEE33 (Oxygen Evolving Complex 33)	An extrinsic subunit of photosystem II	CBS

CBS, *Circadian Clock Associated 1* (CCA1)-binding site; EE, evening element.



genes critically involved in *R* gene-mediated plant defence in response to *Hpa*. Many of these genes were rhythmic in terms of expression. Promoters of these 22 genes were also enriched with either EE and/or CBS, implicating their circadian regulation via CCA1/LHY.

The circadian clock therefore seems to modulate the outcome of plant–pathogen interactions via its core components. The precise mechanism is largely unknown. Several lines of evidence have demonstrated that the interaction of clock and immunity in plants is reciprocal. In other words, the pathogen attack may result in the modulation of circadian clock output and vice versa. An alteration in the rhythmicity of *CCA1* expression was observed in plants on infection with *Pseudomonas syringae* and *Hpa* Emwa1. Even a 22-amino-acid synthetic peptide flg22 (mimics bacterial PAMP) could induce basal defence in plants with an altered rhythm and shortened period of *CCA1* expression (Zhang *et al.*, 2013). However, in the case of *Hpa* infection, the expression of another clock gene *LHY* remained unchanged (Zhang *et al.*, 2013), implying different modes of regulation of the plant defence response via these core oscillators.

#### TIMING OF PLANT–PATHOGEN INTERACTION

Various stages in the life cycle of an oomycete pathogen, starting from sporulation, spore dissemination with successive spore germination, formation of hyphae and establishment of primary haustoria, occur at a specific time in a subjective day (Donofrio and Delaney, 2001). Sporulation occurs mainly at night and spores are disseminated at dawn, soon after the clearance of dew (Slusarenko and Schlaich, 2003). The formation of penetration hyphae occurs approximately 12 h after spore germination and the development of primary haustoria starts approximately 24 h after spore germination (Donofrio and Delaney, 2001). Studies carried out on avirulent *Hpa* Emwa1 provided further evidence for a role of circadian component *CCA1* (Wang *et al.*, 2011) in defence signalling. The clock-defective mutant of *Arabidopsis*, *cca1*, was more susceptible towards pathogen attack relative to the wild-type, whereas *CCA1*-overexpressing lines displayed enhanced resistance (Wang *et al.*, 2011), as indicated by the extent of pathogen growth and spread. Defence genes show peak expression at midnight and at dawn, the time periods concurrent with the time of sporulation and spore dissemination, respectively. Similarly, plants infected at dusk were more susceptible to pathogen attack relative to those infected at dawn. Collectively, the data indicate that the circadian clock temporally coordinates the expression of defence-related genes corresponding to the predictable time of pathogen attack.

In an analysis, wild-type *Arabidopsis* plants and lines defective in *RRP4* were infected with *Hpa* Emwa1, and the expression level of a cluster of defence genes, involved in *R* gene-mediated PCD,

was measured. In the first case, without a functional *RRP4*, the genes involved in basal defence showed a peak expression at 6, 16 and 24 h time points of pathogen attack, strengthening the plant defence against three major cyclic events of *Hpa* infection, namely spore germination, hyphal penetration and the formation of primary haustoria, respectively (Wang *et al.*, 2011). Contrastingly, in the case of wild-type infected plants that carry a functional *RRP4*, the peak expression of the defence genes at 6 h was abolished. Instead, prolonged expression of these genes was observed with peaks at 16 and 24 h. Based on the above observations, it could be hypothesized that early signalling events in the host, on encountering a pathogen, modulate the circadian clock and, in turn, the circadian clock gates the downstream defence signalling events. However, further investigation is required to delineate this complex signalling network and to answer how clock genes and *RRP4* interact to shape the outcome of this interaction.

Studies on *P. syringae*–*A. thaliana* interaction have indicated that the extent of host susceptibility is dependent on the time of infection during a diurnal or circadian cycle. Initial studies on the effect of the light/dark (LD) cycle on the severity of pathogen attack indicated that plants infected during the light period are less susceptible than those infected in the dark. However, this was alleged to be caused by the sole effect of light conditions (Griebel and Zeier, 2008). Later experiments performed under constant light conditions provided the same results (Bhardwaj *et al.*, 2011), indicating a role for the circadian clock. Circadian rhythms are endogenous and sustained for a free running period of approximately 24 h under constant conditions. Therefore, the constant conditions are best to study the outputs of the circadian clock. In order to understand the role of the circadian clock in the modulation of plant immunity against biotrophic pathogens, plants were infected with the bacterial pathogen *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) at specific time intervals and, later, the growth and spread of the pathogen were observed (Bhardwaj *et al.*, 2011; Zhang *et al.*, 2013). The results of two independent studies, carried out under constant and variable light conditions, provided an insight into the preferred time of bacterial pathogen–plant interaction. In one study, *Arabidopsis* leaves were infiltrated with bacterial solution at definite time intervals to infect plants. The wild-type displayed greatest susceptibility when infected at subjective midnight (Bhardwaj *et al.*, 2011). In another study, where spray infection was preferred to infect plants, the greatest susceptibility was observed in plants infected in the subjective morning (Zhang *et al.*, 2013). Different modes of infection resulted in different outcomes. Spray infection may mimic the mode of bacterial infection in nature; therefore, in plants subjected to spray infection, stomata provide a barrier to restrict pathogen entry. In contrast, for bacterial infiltration, the extent of infection largely depends on the strength of the basal level of defence of the host. Variability in the degree of

basal defence extensively correlates with stomatal movement. As a result, when stomata remain closed during the night, the intensity of basal defence remains lower. This temporal variation in the basal level of defence provides a clue that helps to confirm the phenomenon of clock modulation of the immune system.

Together, the above data indicate that the outcome of the interaction largely depends on light and the circadian clock. CCA1, in conjunction with LHY (as shown by Zhang *et al.*, 2013), acts as a major link to establish the circadian gating of plant immunity and is also involved in the modulation of plant defence. Double mutants (*cca1-lhy*) displayed severe alteration in rhythmicity of defence genes and were comparatively more susceptible towards pathogen attack than the single mutants. The extent of susceptibility was quantified as bacterial growth and the number of sporangiophores per cotyledon in the case of oomycete pathogens (*Hpa*). A two- to five-fold increase in sporangiophore count and a significantly higher bacterial growth were observed in double mutants (Zhang *et al.*, 2013). This suggests that both of the core clock components (CCA1 and LHY) act synergistically to modulate defence signalling in plants. In addition, two further clock components, namely ELF3 and ZTL-4 (ZEITLUPE-4), have also been reported to modulate plant immunity (Wang *et al.*, 2011; Zhang *et al.*, 2013).

## PLANT GROWTH REGULATORS, DEFENCE AND CLOCK

Plant hormones are known to be an integral part of the plant immune system (Robert-Seilanianz *et al.*, 2011). Plant growth regulators involved in defence signalling mainly include SA, jasmonic acid (JA), auxin and ethylene. SA is a plant hormone involved in several different disease resistance mechanisms (Vlot *et al.*, 2009). SAR in plants is associated with higher levels of SA accumulation. SA plays a central role in the establishment of SAR (Gaffney *et al.*, 1993). SA accumulation in plants has been shown to be rhythmic, with a peak accumulation at subjective night (from 25 to 35 ng/g fresh weight) (Goodspeed *et al.*, 2012). The time of peak SA accumulation is a few hours before the time of *Hpa* infection, hence preparing the plant for a predictable pathogen encounter. However, it is unknown how this small increase in SA level strengthens the plant defence against biotrophic pathogens (Goodspeed *et al.*, 2012). SA biosynthesis is also regulated by the circadian clock. A clock component CHE (CCA1 HIKING EXPEDITION) has been shown to bind with the promoter element of *ICS1* (ISOCHORISMATE SYNTHASE 1), a major gene involved in SA biosynthesis, and positively regulates its expression. Another gene *NPR1* (NONEXPRESSER OF *PR1*) has been established as a connecting link between SA-mediated defence signalling and the circadian clock (Zhou, 2014). Although SA accumulation is modulated by the circadian clock,

clock-mediated modulation of plant immunity by CCA1/LHY has been shown to be independent of SA (Zhang *et al.*, 2013). This has been demonstrated by experiments carried out with *A. thaliana* defective in the *ACD6* (*accelerated cell death 6-1*) gene. The mutant line, *acd6-1*, displays constitutive defence against the pathogen and accumulates higher levels of SA (Rate *et al.*, 1999). Therefore, to analyse whether the higher level of SA accumulation complements the loss of disease resistance in clock-defective mutants, the clock-defective lines *cca1* and *lhy* were crossed with *acd6-1* to generate double (*acd6-1-cca1*, *acd6-1-lhy*) and triple (*acd6-1-cca1-lhy*) mutants. The double and triple mutants displayed a similar dwarf phenotype to *acd6-1* and a similar SA level, but plants were unsuccessful in recovering the loss of disease resistance because of the absence of a functional circadian clock (Zhang *et al.*, 2013). These results indicate that an SA-independent mechanism also exists in plant cells that links the circadian clock to disease resistance. A hormonal cross-talk, activated in response to inputs from pathogens, results in a pathogen-specific defence signalling cascade. Another plant growth regulator, JA, functions as an agonist to SA (Niki *et al.*, 1998). JA is synthesized on pathogen perception and mediates defence signalling to encounter pathogen attack. JA is involved in the production of anti-herbivore metabolites that protect plants against herbivore attack. JA is also involved in defence signalling against necrotrophic fungal pathogens (Antico *et al.*, 2012). A clock component TIC (Time for Coffee) function as an intermediate between JA and circadian clock signaling (Shin *et al.*, 2012). JA accumulation peaks at the subjective day and is known to be circadian regulated (Goodspeed *et al.*, 2012). The peak accumulation of JA corresponds to the peak time of herbivore attack. Similarly, a third plant hormone, ethylene, is also known to be involved in biotic stress signalling (Abeles *et al.*, 1992; Thain *et al.*, 2004). Ethylene plays a dual role in plant defence signalling. In some cases, ethylene is used by pathogens as a virulence factor and promotes pathogenesis, whereas, in other cases, it aids in stress alleviation (Van der Ent and Pieterse, 2012; van Loon *et al.*, 2006). Broadly, the ethylene-regulated defence responses depend on the outcome of interactions between multiple signals. Ethylene biosynthesis is also known to be circadian regulated (Thain *et al.*, 2004). Another plant growth regulator, auxin, involved in stomatal movement, fluctuates in a circadian manner (Robertson *et al.*, 2009). Auxin also regulates the clock as an input (Covington and Harmer, 2007).

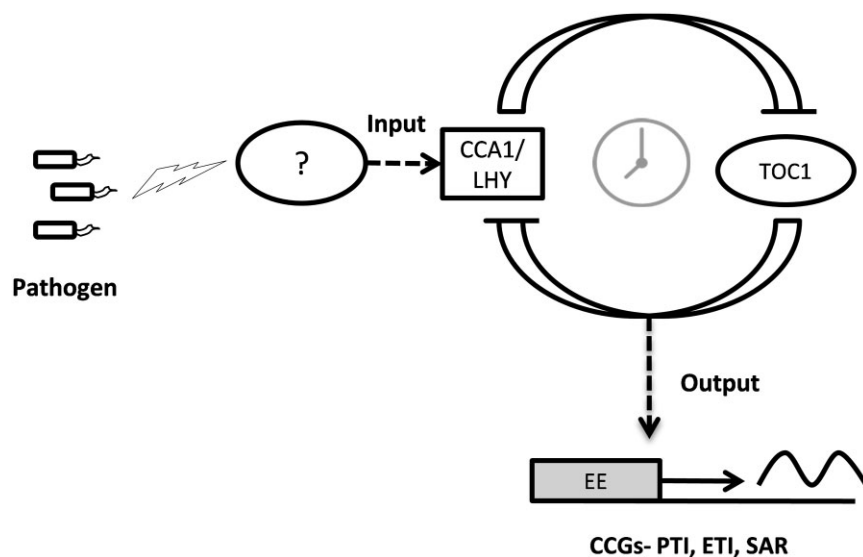
## PERSPECTIVES

The plant circadian clock is known to perform a wide variety of functions. Earlier, only abiotic factors, mainly light and temperature, were attributed as input signals to the clock. Recent advancements in clock–defence signalling in plants have

indicated that biotic factors, such as pathogens, might also act as input signals to the circadian clock (Fig. 2). Consistent host–pathogen interactions in nature set down a circadian rhythm in the expression of related genes in both host and pathogen. This rhythmicity makes organisms more adaptive and comparatively fitter to encounter these routine interactions. As the defence response in plants is an energy-consuming process, an appropriate synchronization of defence signalling events with other physiological processes is essential for the judicious utilization of energy resources. Therefore, according to the peak time of infection, the plant circadian rhythm orchestrates defence signals in such a way that it should be able to anticipate attacks in an enhanced manner. Studies carried out so far have not provided clear-cut evidence to support CCA1-mediated regulation of defence genes. As defence genes are not the only targets of CCA1 and a large proportion of global transcripts are speculated to be under the regulation of core clock components, a comprehensive biochemical and mechanistic approach is required to establish this cross-talk. It is notable that, to determine the phenotypic outcome of the circadian clock, a comparative study under both light/dark and a constant free-running period should be performed simultaneously. A time-course genome-wide profile of plant–pathogen interactions under different light conditions [constant light (LL), light/dark (LD) and constant dark (DD)] could help to identify those candidate defence genes which are solely regulated by the circadian clock and not light conditions. However, studies on clock–defence signalling hitherto have not covered these aspects. Abiotic factors, especially light and temperature, provide an input to the circadian clock via various receptors, such as phytochromes, that help

to transmit signals from stimuli to clock, whereas, in the case of biotic factors, the connecting link between pathogen receptors and clock components is largely unknown (Fig. 2).

So far, all studies have been restricted to the model system *Arabidopsis*, and so it would be interesting to explore other plant species as well to identify the conservative nature of clock–defence cross-talk. The circadian clock connections are vast and it is difficult to connect the loops without mathematical modelling. The mining of meaningful information from a large set of genome-wide data could be easier if we used systems biological approaches coupled with biochemical analysis. A model built after utilizing the systems approach could help us to predict the phenotypic changes on modification of one of the components of this network. A delineated network might provide us with better candidate genes for crop improvement. Clock–defence cross-talk is in its preliminary stage of research and several questions need to be addressed. The circadian clock of individual plant species is modulated in accordance with its surroundings. Thus, it would be interesting to identify the degree of conservation of this cross-talk across a wider range of species from different geographical backgrounds. The identification of a connecting link between defence signals and the circadian clock is a major challenge. It is also important to define how hormonal signalling affects this cross-talk. The combinatorial effect of major plant hormones on the outcome of plant pathogen interaction is difficult to understand. An integrative study of plant growth regulators, the circadian clock and defence signalling could demarcate the role of the above components in the establishment of disease resistance in plants.



**Fig. 2** A model representing circadian clock–defence cross-talk. Pathogen elicitors provide an input to the circadian clock via an unknown intermediate (depicted by a question mark). The circadian clock thus modulates the expression of various defence-related circadian-controlled genes (CCGs) via a putative binding of clock components to the promoter region of CCGs. CCA1/LHY, Circadian Clock Associated 1/Late elongated HYpocotyl; EE, evening element; ETI, effector-triggered immunity; PTI, pathogen-associated molecular pattern (PAMP)-triggered immunity; SAR, systemic acquired resistance; TOC1, Timing Of CAB2 expression 1.

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