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Keeping time on the plant-pathogen arms race: A role for the plant circadian clock in immune response

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Plagiarism declaration

I know the meaning of plagiarism and declare that all of the work in this dissertation, save for that which is properly acknowledged is my own.

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Abstract

Plants and pathogens have been engaged in an evolutionary arms race that has led to both species evolving a complex set of defence mechanisms. Initially, plants perceive invading bacteria by recognising microbe associated molecular patterns (MAMPs). This leads to the MAMP triggered immune response (MTI). Bacteria in turn evolved effector proteins that act to repress MTI leading to virulence. However, plant resistance (R) proteins, can recognise these effectors, activating effector-triggered immunity (ETI). While plants are capable of initiating extreme defence responses such as programmed cell death, they display a carefully regulated defence response. Recently, investigations of defence responses to the oomycete *Hyaloperonospora arabidopsis* revealed a molecular link between a circadian clock component and plant defence. A systems approach revealed that a number of defence genes showed peak expression at dawn and possessed CIRCADIAN CLOCK ASSOCIATED1 (CCA1) binding sites in their promoters. In this study, *Arabidopsis thaliana* (*Arabidopsis*) in the Columbia-0 (Col-0) background showed time-of-day variation in susceptibility to the plant-pathogen *Pseudomonas syringae* DC3000 pathovar tomato (*P. syringae* DC3000) when infected under constant light and temperature conditions. Wild type plants showed least susceptibility at circadian time (CT) 26 and 50, which correspond to 'subjective' morning. Plants were most susceptible when infected at CT42 and CT66, 'subjective' night. Additionally, the arrhythmic *CCA1*-over-expressor (*CCA1*-ox) line and *elf3-1* mutant revealed similar levels of susceptibility at both subjective morning and night infections. Callose deposition is an early MAMP defence response. Measuring callose levels in response to infection with non-pathogenic *P. syringae hrpA* demonstrated that callose production also showed time-of-day variation. Higher callose levels were observed after infection at CT26 compared to CT42 in wild type plants, while *CCA1*-ox showed similar levels after infection, at both time points. This result complemented the earlier observation. Paradoxically, promoter activity of *Pathogenesis related1* (*PR1*) a defence gene, indicated quicker induction at CT42 than at CT26 while promoter activity of *Oxidative Stress Induced 1* (*OXI1*) showed no time-of-day variation in activity. Preliminary gene expression measurements indicated that induction of *WRKY29* showed time-of-day variation with greater induction at CT26 than at CT42 in wild type plants but *WRKY22* did not. Analysis of basal expression levels of a gene encoding a suppressor of R proteins (*SRFR1*) also showed time of day variation with higher levels at CT42. While clock control in plant defence is important, measuring promoter activity of *COLD AND CIRCADIAN REGULATED 2* (*CCR2*), a clock controlled output gene, revealed core circadian clock function is unaffected by pathogen infection. Collectively these results indicate that there are clock co-ordinated defence responses in *Arabidopsis* and that plant defence against *P. syringae* is greatest at dawn.

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Abbreviations

AOS	Active Oxygen Species
Arabidopsis	*Arabidopsis thaliana*
AtRBOHD	Arabidopsis Respiratory burst oxidase homologs
BAK1	BRI1 Associated Receptor Kinase 1
CCA1	Circadian Clock Associated 1
CCA1-ox	Circadian Clock Associated 1-overexpresser
CHE	CCA1 Hiking Expedition
Clk	Clock
CLR	CATTERPILLAR
Col-0	*Arabidopsis thaliana* Columbia-0
CRY	CRYPTOCHROME
CSP	Cold Shock Protein
Cfu	colony forming units
Cyc	Cycle
DBT	Doubletime
Drosophila	*Drosophila melanogaster*
EE	Evening Element
EFR1	EF-Tu Receptor 1
EF-Tu	Elongation Factor Tu 18, 20
elf18	Elongation Factor Tu
elf26	Elongation Factor Tu
EMT	Early MAMP triggered defence
ETI	Effector Triggered Immunity
flg22	flagellin 22
FLS2	Flagellin Insensitive 2
GLS5	Callose synthase 5

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Hpa Emwa1	*Hyaloperonospora arabidopsis Emwa1*
JMJD4	Jumonji Domain containing 4
LHY	Late Elongated Hypocotyl
LRR	Leucine Rich Repeat
MAMP	Microbe Associate Molecular Pattern
ME	Morning Element
MTI	MAMP Triggered Immunity
NK	Natural Killer Cell
OXI1	Oxidative Signal Inducing 1
P. syringae	*Pseudomonas syringae DC3000*
PAD4	Phytoalexin Deficient
PCD	Programmed Cell Death
Per	Period
PGN	Peptidoglycan
PMR4	Callose synthase
PR	Pathogenesis Related
PR1	Pathogenesis Related 1
PRR	Pattern Recognition Receptor
qPCR	quantitative PCR
R	Resistance proteins
RIN4	RPM1 interacting Protein 4
RLK	Receptor Like Kinase
RNAi	RNA interference
ROS	Radical Oxygen Species
SA	Salicylic acid
SAR	Systemic Acquired Resistance
SD	Sprague Dawley

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SID2	Salicylic Induction Deficient 2
SRFR1	Suppressor of RPS4-RLD1
Timeless	Tim
TLR	Toll Like Receptor

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Chapter 1: Literature Review

For millions of years, plants and pathogens have been engaged in an arms race that led plants to evolve a two-tiered innate immune system [1]. Plant immunity evolved to recognise conserved structural features of pathogens using transmembrane pattern recognition receptors (PRRs) [2,3]. These conserved microbe structures include flagellin and peptidoglycan (PGN) and are collectively termed microbe associated molecular patterns (MAMPs) [4]. Upon recognition of these MAMPs, plants initiate MAMP triggered immunity (MTI) [1]. To overcome MTI, pathogenic bacteria evolved effector proteins some of which suppress MTI responses thus enhancing virulence [1]. These effectors often delivered by a type three-secretion system into the plant cell act to manipulate host defences and suppress MTI [1]. The plant immune system recognises some effectors through resistance protein (R proteins) resulting in effector triggered immunity (ETI), an accelerated and amplified MTI response [1]. R proteins are intracellular and recognise effectors either directly or indirectly with more severe responses such as programmed cell death (PCD) [1]. This continuous conflict between plants and pathogens characterised by bacteria acquiring new effectors to enhance virulence and plants evolving new R proteins to detect them is described as the plant-pathogen arms race [1]. This review investigates the *Arabidopsis thaliana* (hereafter *Arabidopsis*) immune system and compares aspects of plant innate immunity with immune systems of mammals and insects. Current literature purports that immune systems of plants, insects and mammals evolved strikingly similar features by convergent evolution [5,6]. Circadian clock regulation of innate immunity is also common to insects, animals and humans. The immune systems of mice, humans and fruit flies demonstrated some degree of circadian regulation [7,8,9], while recently potential clock control of plant defence was proposed [10]. This review summarises similarities in innate immunity, particularly focussing on circadian regulation of innate immunity in plants, animals and insects. Additionally, I report on recent advances implicating the plant circadian clock in the regulation of immune responses and speculate on additional questions these latest discoveries reveal. Finally, the review concludes with a brief introduction to the questions this study sought to answer, and the experimental approaches taken.

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The *Arabidopsis* immune system

The *Arabidopsis-Pseudomonas syringae* DC3000 (*P. syringae* DC3000) interaction has been very useful in developing our understanding of the plant immune system. While the *Arabidopsis'* genome is sequenced thus making it an ideal candidate for molecular studies, *P. syringae* DC3000 is also well

characterised. This two-model system contributed greatly to contemporary understanding of plant immunity.

Plants possess a two-tiered innate immune system consisting of MTI and ETI. Perception of pathogens also results in Systemic Acquired Resistance (SAR) [11]. Using the plant hormone, salicylic acid (SA), plants are able to signal messages away from the site of infection to uninfected cells resulting in activation of defence genes, giving broad spectrum protection to the plant known as SAR [12]. Additionally plants use RNA interference (RNAi) against viruses [13]. However, this review focusses on the MTI and ETI responses in plant-pathogen interactions.

As plants lack mobile defence cells, they rely on innate immunity of individual cells and systemic signals emanating from infected sites [1]. MAMPs are common to most bacteria and because they are essential to bacterial survival and fitness undergo very little change over time [14]. PRRs, which are cell membrane bound receptors on plant cells, recognise these conserved MAMPs leading to MTI [1]. Some bacteria have evolved mechanisms to counter MTI. Bacteria possessing type III secretion systems (TTSS) release effector proteins into the intracellular space of plants, aiming to suppress MTI [1]. In turn, plants evolved R proteins that detect these effectors leading to ETI. Bacteria seek to evade ETI by losing or modifying the effectors in their repertoire, restoring virulence [1]. This zigzag model of plant-pathogen interactions is described as the plant-pathogen arms race [1]. A previously established view of plant innate immunity is that the plant immune system is a two-branched innate system, however more recently experimental evidence summarised in reviews by Tsuda (2010) and Thomma (2011) [15,16], suggest there is considerable overlap between MTI and ETI. The notion that plant innate immunity involving MTI and ETI is a continuum rather than a two-branched system will be discussed in detail later in this review. I will begin by discussing each branch separately.

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MAMP triggered immune response (MTI)

The MTI response is initiated by plant recognition of MAMPs [1,6]. Known MAMPs include flagellin (flg22), elongation factor-EF-Tu (elf18 and elf26), chitin and PGN [1,4,17,18,19]. These MAMPs are recognised by PRRs such as FLAGELLIN INSENSITIVE 2 (FLS2), which are Receptor like kinases (RLK) with an extracellular Leucine Rich Repeat (LRR) domain and an intracellular kinase signalling domain [1,4,17,18,19]. Upon recognition of MAMPs, the surface receptor is internalised by endocytosis and activation of defence results [20]. Upon recognition of flg22 by the FLS2 receptor

there is rapid transcriptional induction of 1100 *Arabidopsis* genes [21]. Another MAMP, elongation factor Tu (EF-Tu), is recognised by the PRR EF-Tu Receptor1 (EFR1) [18] and triggers induction of a similar set of genes to flg22, suggesting that although MAMP receptors are different, the downstream defence pathway activated by their recognition is very similar [22,23]. In addition to the MTI response, a weak ETI response is also initiated as some effectors are weakly recognised [1]. Defence responses include strengthening of cell walls (by lignification), callose deposition, and production of hydrolytic enzymes and expression of Pathogenesis Related (PR) proteins such as phytoalexins [24,25,26]. Additionally hormonal changes occur with levels of ethylene, jasmonic acid and salicylic acid all affected [27,28]. These hormones are crucial for plant defence regulation [27,28]. Callose production is a classical marker of MTI after treatment with MAMPs or non-infectious *P. syringae* [29,30]. Perception of fungal and bacterial pathogens elicits callose synthesis by callose synthase GLS5/PMR4 [31,32,33]. Compared to wild type plants, *pmr4* mutant plants showed 20-fold higher counts of *P. syringae hrcC* which lack the type three-secretion system [33], illustrating the importance of the callose response during immune responses. Callose is secreted by the plant in response to infection, slowing down the rate at which bacteria proliferate. This gives the plant time to mount a stronger defence response while slowing down rate of infection.

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Early molecular events upon MAMP perception:

FLS2 is a membrane spanning plant LRR receptor essential for recognition of flagellin [34,35]. The intracellular domain of FLS2 interacts with a receptor-like kinase (RLK) called BRI1 associated receptor kinase (BAK1) [34,35]. The interaction between FLS2 and BRI1 is vital for activation of downstream responses after recognition of flagellin as downstream signalling is strongly impaired in *bak1* null mutants [34,35]. Plant recognition of MAMPs results in alkalinisation of the intracellular space within 0.5-2.0 min [36,37,38]. Cytoplasmic levels of Ca^{2+} and other charged ions also increase upon perception of MAMPs and play a critical role in the activation of defence responses [39]. A virulence strategy employed by invading bacteria such as *P. syringae* is to sequester Ca^{2+} ions using bacterial exopolysaccharides to chelate free ions [40]. This is an indication of the importance of Ca^{2+} in defence signalling [36].

In addition to a flux in ions such as Ca^{2+}, production of reactive oxygen species (ROS) which occurs as an oxidative burst is also reported [36]. The ROS signal, generated by membrane bound NADPH oxidases such as *Arabidopsis Respiratory burst oxidase homologs* (*AtRbohD*), results in the production of active oxygen species (AOS) such as H_2O_2, which act as a form of defence as they are

harmful to the invading bacteria [41,42]. AOS also activate Oxidative Signal Inducing 1 (OXI1), a serine/ threonine kinase, which is needed for full activation of the mitogen-activated protein kinase (MAPK) pathway and MTI response [43,44]. Perception of MAMPs such as flg22, chitin and PGN activate the MAPK cascade [45,46,47,48,49]. FLS2 simultaneously activates MPK3/6 via MKK4/5, and MPK4 via MEKK1/2, which act as activators and repressors of defence responses, respectively. While MPK3/6 contributes positively to MTI, MPK4 acts to suppress MTI. These contradictory responses to MAMP perception are mediated by a PP2C phosphatase [36,50] and will be discussed in detail later.

One of the effects of activation of MPK3/6 pathway by MAMP perception is the activation of WRKY22/29 transcription factors that are involved in defence gene expression [45]. The ability of plants to respond to multiple biotic and abiotic signals is achieved through complex molecular pathways that are yet to be fully dissected. However, the family of WRKY transcription factors is a key signalling network involved in numerous processes, most notably in biotic and abiotic stress response [51]. The WRKY family of transcription factors get their name from the highly conserved WRKYGQK peptide sequence and a zinc finger motif present in all members [52]. The large WRKY family shows overlapping functionality among its member proteins for example, WRKY22 is homologous to and shows functional redundancy with WRKY29 [53]. WRKY29 was shown to be a key transcriptional activator of defence genes in *Arabidopsis* innate immunity [45]. *WRKY29* induction occurs rapidly, within 30min of MAMP perception whereas defence responses such as *PATHOGENESIS RELATED 1* (*PR1*) production occur much later [45]. *PR1* expression is associated with systemic acquired resistance (SAR) and the hypersensitive response (HR), which is mediated by salicylic acid and salicylic acid associated genes such as *SALICYLIC INDUCTION DEFICIENT2* (*SID2*) and *PHYTOALEXIN DEFICIENT* (*PAD4*) [54]. Initially HR and SAR were thought to be characteristic of ETI responses only but recently, MTI was shown to initiate them too [55]. Thus, while the MTI response shares similar downstream molecular pathways as ETI, ETI is initiated by recognition of effectors which are often strain specific and delivered through the type-three secretion system of bacteria into the plant intracellular space [1].

To evade the MTI response, bacteria evolved effectors that suppress MTI responses with the model bacterium *P. syringae* DC3000 secreting more than 30 effectors [1]. These effectors abrogate MTI responses by targeting plant defence molecules for degradation, for example, the bacterial effector AvrRpt2 targets the *Arabidopsis* RPM1 interacting protein (RIN4) for degradation [56]. RIN4 regulates stomata aperture during pathogen attack thus limiting bacterial invasion [57]. Effectors such as AvrPto which is a kinase inhibitor [58] suppresses auto-phosphorylation of PRRs such as FLS2

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[59], while AvrPtoB possesses an E3 ligase domain [60] and targets FLS2 for degradation. The presence of effectors abrogates MTI responses but effectors themselves are targets for the plant immune response.

Effector Triggered Immune response (ETI)

Recognition of effectors results in avirulence, a rapid and strong defence response and activation of HR and PCD at a much quicker rate than MTI [1]. Through direct gene-for-gene resistance, the 'guard' model and the 'decoy' model, plants detect effector molecules resulting in a stronger and more prolonged defence response compared to MTI [1,61]. The ETI response has a biphasic nature to it, with the initial phase triggered by MAMP recognition, which is supressed by effector molecules [1]. The effector molecules in turn trigger the second phase of plant defence as R proteins recognise them [16,62]. *Arabidopsis* possess approximately 125 R proteins that may recognise effectors secreted by bacteria [63]. Notably, R proteins are race specific and different populations of a single species may have different R proteins [63]. These R proteins, which are intracellular, are important in mediating the effector-triggered response as they detect effectors secreted by bacteria into the cytoplasm [16,36]. This detection can be direct or indirect.

The effectors discussed earlier: AvrPto and AvrPtoB, are capable of suppressing MTI by binding to FLS2 [58,59]. However, resistant *Solanum lycopersicum* (tomato plants) detect the effectors directly using the RLK PTO and the R protein PRF that directly interact to activate ETI [64,65]. The direct interaction of PTO with AvrPto occurs in the presence of PRF, a Nucleotide Binding- Leucine Rich Repeat (NB-LRR) R protein [59]. Interestingly AvrPto only contributes to virulence in the presence of FLS2 suggesting binding of FLS2 and other RLKs are the operative mechanism by which virulence is achieved [59]. In a recent review proposing the decoy model for plant defence, PTO was suggested as a decoy molecule to bacteria expressing AvrPto thus attenuating virulence [66]. PTO is a RLK, similar to FLS2 and competes with FLS2 for AvrPto effectively sequestering the effector before it can bind to FLS2 and other RLKs involved in defence [64]. PTO interacts *in vivo* with PRF and its accumulation is dependent on levels of PRF [64]. This suggests that PTO functions in a dose-dependent manner, outcompeting other RLKs for AvrPto, thus reducing virulence [67]. This is termed the 'competing decoy' model [66].

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Another way in which plants detect effector activity is via the 'guard' model. This model is described in detail by Dangl and Jones (2006) [1] and the key tenets are described briefly. The guard model proposes that bacteria secreting effectors that suppress MTI may alter plant proteins, creating 'pathogen-induced modified-self' molecular patterns, which in turn are detected by R proteins leading to ETI. The effector proteins AvrB and AvrRpt2 both interact with RIN4, phosphorylating it and targeting it for degradation, respectively [56,68] effectively modifying the plant molecule. The NB-LRR protein RPM1 detects AvrB activity indirectly, while RPS2 indirectly detects AvrRpt2 activity [68,69]. Thus, RPM1 and RPS2 guard proteins monitor RIN4 and upon modification of RIN4 by effectors, initiate ETI. In the *rpm1rps2* double-null mutant RIN4 is manipulated by bacterial effectors to promote virulence [1]. The effector-triggered immune response also results from direct gene for gene recognition. While ETI is a stronger and more prolonged response, it shares similar molecular pathways to MTI and results in the activation of similar defence responses, hormonal changes and systemic responses. Recent reviews [15,16] excellently show that there is considerable overlap between MTI and ETI. The shared molecular circuitry in *Arabidopsis* suggests that while R proteins are thought to have evolved after PRRs, they evolved to share the same molecular pathways and defence outputs [16].

The two-branched paradigm challenged:

Recent reviews have questioned the basis of the widely held view that the plant immune system possesses two distinct branches of immunity [15,16]. Using data from recent experiments, Thomma *et al.* (2011), illustrated that widely held assumptions about MAMPs, effectors, PRRs and R proteins are all debatable [15]. MAMPs, recognised for their importance in bacterial survival and fitness [70,71], are structural features that are highly conserved across genera, while effectors are specific to single or a few related species [1,15,72]. Apart from their distribution their intrinsic roles are used to differentiate MAMPs and effectors: MAMPs contribute to survival and fitness while effectors specifically contribute to virulence by targeting host defence [15]. Several examples show that it is difficult to separate virulence from fitness and survival [15]. Experiments showed MAMPs such as flagellin and harpin (HrpZ) are essential for virulence [73,74,75,76]. Another MAMP Ax21 released in a cell density dependent manner could be important in quorum sensing and therefore play a role in virulence of bacteria [77]. Perhaps one of the ways MAMPs contribute to survival and fitness is through their role as virulence molecules.

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In addition to MAMPs contributing to virulence, they are also able to suppress MTI, a feature previously assigned to effectors alone [15]. Chitin from *Cladosporium fulvum* (*C. fulvum*) activates MTI after recognition by a LysM PRR protein [78]. Using the zigzag model, one could speculate that the fungal pathogen may produce an effector to suppress the resulting MTI response. Indeed the fungus does produce a molecule, Ecp6 that mimics the host Lys M PRR and is a receptor-like protein (RLP) [79]. Ecp6 attenuates the MTI response to chitin by binding to any free chitin before the plant Lys M receptor can, thus sequestering any free chitin [80]. All strains of *C. fulvum* possess Ecp6 with little sequence variation and conserved Ecp6 orthologs are common in the Fungal Kingdom [79]. Its wide distribution across strains of *C. fulvum* and conserved sequence mean it is comparable to a MAMP [15] yet its ability to suppress MTI qualifies it as an effector. Conversely, MAMPs with a narrow distribution have also been described, with only a narrow range of host plants recognising them, a characteristic often attributed to effectors [15].

PRRs and R proteins show distinct characteristics. PRRs are membrane bound, highly conserved and evolutionarily ancient while R proteins are intracellular and evolved more recently [15]. The discoveries of R proteins that display typical characteristics of PRRs further blur the line between MTI and ETI. The R protein Ve1 found in tomato contributes race specific resistance to race 1 strains of vascular fungal pathogens of the *Verticillium* genus [81]. However, the race 1 resistance affects two distinct species of fungus *V. dahliae* and *V. albo-atrum* [15]. This suggests Ve1 mediated resistance is activated by an elicitor common to different fungi, most likely a MAMP, thus Ve1 shows characteristics of a PRR, not an R protein [15]. Ve1 shows additional PRR characteristics such as activating only a weak MTI response [15] and interacting with BAK1 a crucial component of MTI responses [34,35]. A defining characteristic of PRRs is their capacity to recognise broad structural patterns across species but recently close inspection of PRRs revealed some PRRs have a narrow host range [19,22,82,83]. The PRR EFR1 that recognises Ef-Tu is found in *Arabidopsis* but not in -*Nicotiana benthamiana* (tobacco) [22] while bacterial cold shock protein (CSP) a MAMP, elicits MTI responses in tobacco but not in tomato, rice and *Arabidopsis* [19]. These results show that only a narrow host range recognises widespread MAMPs. Additionally PRRs that are specific for the same MAMPs recognise different MAMP epitopes implying that some PRRs may have evolved recently thus challenging the assumption that PRRs are evolutionarily ancient [15].

MTI is often characterised as a weak defence response while ETI is characterised as a stronger and more prolonged response. Features thought to be unique to ETI such as HR and SAR have recently been shown to occur in MTI [55,84] . In addition to strong MTI responses, weak ETI responses also occur for example RPS4 resistance to AvrRps4 in *Arabidopsis* [85].

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Thomma *et al.* (2011) present an excellent review citing numerous examples all pointing towards the conclusion that a continuum exists between MTI and ETI responses. They conclude that the robustness of MTI or ETI depends on the specific interaction; different molecules activate defence pathways of varying strength, depending on the trigger, the receptor and possibly environmental conditions [15]. Interestingly, they also suggest that plant innate immunity evolved to regulate the strength of defence depending on the type of microbial attack. Ausubel (2005) [6] introduced a similar idea in a review where he suggested that the two-tiered response might have evolved as a mechanism to distinguish between non-pathogenic and pathogenic bacteria. As immune responses are energetically expensive, this 'two hit' approach to defence would allow a moderate defence response to less harmful bacteria and full response would only manifest in the event of infection by pathogenic bacteria. The two-branched model and zigzag model while distinctly separating the defence responses, acknowledged that an overlap might exist. Recent findings support the view that a continuum exists between the MTI and ETI responses and as our understanding of plant immunity grows these two previously separate branches may merge even more.

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Negative regulation of plant defence

Plants are able to distinguish between pathogenic and non-pathogenic bacteria suggesting careful control of defences. This is important as a full immune response is energetically expensive and comes at great cost to the plant often resulting in cell death. Understanding negative regulation of plant immunity will help to explain how plants co-ordinate defence responses to deliver a proportionate response.

Genetic studies revealed a number of negative regulators of various aspects of immunity: SNI1 represses systemic acquired immunity, LSD1, and ACD11 prevent programmed cell death [86]; and SRFR1 regulates levels of NB-LRR R proteins preventing them from over-accumulating [87], thus avoiding autoimmune responses. Other negative regulators include a number of *WRKY* genes, KAPP that negatively regulates FLS2 and PUB22, 23 and 24. The PUBs negatively regulate MTI possibly by binding to PRRs and degrading positive regulators of MTI [88]. Genetic studies revealed that the plant innate immune system is under negative regulation prior to MAMP perception [89]. However, negative regulation not only occurs in the absence of infection but persists even after activation of defence.

Identification of MAMPs leads to activation of both MPK3/6 and MPK4, which positively and negatively regulate MTI, respectively. PP2C phosphatase AP2C1 regulates both pathways possibly to ensure a sensitive and controlled defence response upon MAMP perception [90]. This may allow plants to distinguish pathogens from commensal microbes [6]. Recently, a comprehensive study, which divided plant defence responses into sectors based on positively co-ordinated pathways, identified four sectors: The Early MAMP Triggered (EMT) genes, the Salicylic Acid-associated (SA) genes, the Jasmonic Acid genes (JA) and Ethylene associated genes [54]. Early MAMP triggered proteins include MPK3/6, WRKY29, PMR4 (callose synthase) and AtRbohD NADPH oxidase. The proteins that make up the SA sector include PAD4, SID1, PR1 and NPR1 which are involved in SA signalling thus activating SAR and HR [54]. While both sectors positively contribute to plant defence, this study reported that the EMT sector negatively regulates the SA sector and that this relationship is antagonistic [54]. For example, PMR4, which is part of EMT, negatively regulates SID1 and *pmr4* null mutants show greater SA mediated activity [32]. Collectively these results suggest that PMR4 negatively affects SA mediated responses and *vice versa* [32]. MPK3/6, Jasmonic Acid and Ethylene all showed negative relationships with the SA sector [54].

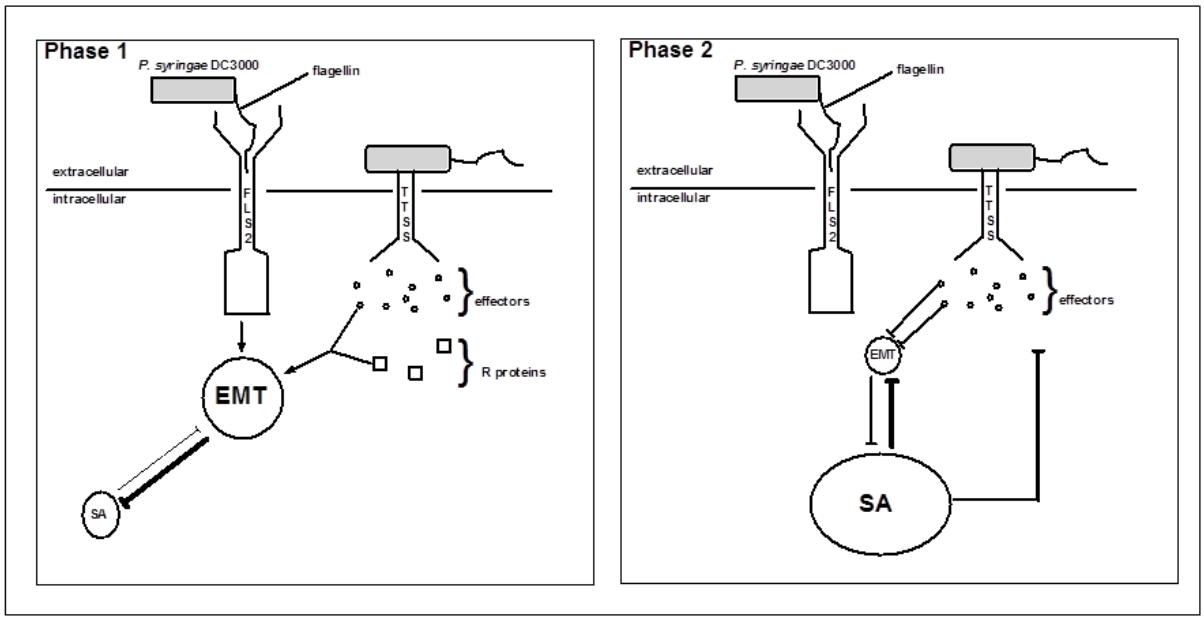

Figure 1. The 'two-hit' model of plant defence: An illustration of the EMT and SA sectors which show an antagonist relationship. Phase 1 is marked by the recognition of MAMPs such as flagellin and effectors by PRRs and R proteins. This activates the Early MAMP Triggered (EMT) sector. A strong EMT response (shown by a larger circle) clears infection while simultaneously suppressing the SA sector (thicker arrow). Importantly, EMT responses are not harmful to the plant. In the event that the bacteria possess effector molecules capable of suppressing EMT, phase 2 is initiated. Suppression of EMT by bacterial effectors inadvertently activates the SA sector as the antagonistic repression of SA by EMT weakens (with a weak EMT shown by thinner arrow). A strong SA sector in phase 2 (larger circle) also represses EMT in phase 2 and initiates more severe defence responses such as programmed cell death. This model for infection shows how plant defence responses are hierarchical, reserving the most severe response for instances when EMT genes cannot clear infection. Additionally, this model shows how repression of EMT by bacteria results in even stronger responses.

These results indicate that activation of one defence pathway results in repression of another, even though collectively they aid in defence against pathogens [54]. This apparently paradoxical phenomenon points to the need for a nuanced look at plant immunity. Mounting evidence suggests that plants employ a carefully moderated defence response to bacteria [15,16,54]. This restrained approach may allow plants to distinguish between non-pathogenic and pathogenic bacteria thus reserving a full defence response only when necessary. Additionally by dividing defence responses into mutually antagonistic sectors, plants evolved an energetically favourable method of clearing infection. The *pmr4* null mutants that are unable to initiate a callose defence response display a compensatory effect by showing greater SA mediated responses [32].

This finding is important as it points to a hierarchical defence response of increasing severity. Early defence responses such as callose production, the production of antimicrobial peptides and production of AOS are not harmful to the plant compared to defence responses activated by SA, which eventually result in programmed cell death. The antagonistic relationship between the EMT sector and the SA sector is important in plant defence responses (Fig 1). When this antagonistic relationship is perturbed as in the case of *pmr4* null mutants lacking callose synthase, a weak EMT response ensues and so the EMT sector weakly represses the SA sector, which results in a stronger SA sector response. In turn, a strong SA sector response represses the EMT sector.

With these observations, a new approach to understanding events in immune response becomes apparent. PRRs and intracellular R proteins recognise pathogenic bacteria, resulting in a strong EMT response. As a result, a strong EMT response has an antagonistic effect on the SA sector thus repressing it. This is phase 1 of infection (Fig 1). In the event that the bacteria possess effectors or MAMPs capable of suppressing EMT, phase 2 is initiated. Suppression of EMT by bacterial effectors inadvertently activates the SA sector as a weak EMT response is only weakly antagonistic to the SA sector (Fig 1). Additionally, a stronger SA sector has an antagonistic effect on the EMT sector, further repressing it. Thus, by overcoming early defence responses virulent bacteria switch on the SA sector, activating stronger defence responses and SAR. The ultimate effect of the SA sector is programmed cell death, which is costly to the plant but necessary to isolate the pathogen at the site of infection. This model offers a new explanation of events in defence responses to pathogenic and non-pathogenic bacteria and could be described as the ‘two-hit’ approach to plant defence, a term coined by Ausubel (2005) [6]. Furthermore, this model explains how plants may keep non-pathogenic and commensal microbes at bay, without having to sacrifice large resources or initiate strong defence responses that

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are detrimental to the plant. Commensal bacteria may trigger EMT as they possess MAMPs but lack effectors or MAMPs that can suppress EMT responses and so a full-blown defence response is not required. Since the EMT sector is not repressed, it has an antagonistic effect on the SA sector preventing extreme defence responses to harmless bacteria. The 'two-hit' model proposed here is largely similar to the zigzag model. However, the zigzag model proposes that the perception of effectors triggers a stronger response called ETI but it does not explain how that may occur. The 'two hit' model shows that suppression of EMT by effectors and possibly MAMPs, is what triggers stronger defence responses, as weakening of EMT has an antagonistic effect on the SA sector (Fig 1). I base the 'two-hit' model on ideas reported by Thomma *et al.* (2011) and experiments by Sato *et al.* (2010) [15,54]. As some MAMPs can suppress EMT genes [40], the 'two-hit' model also explains why MAMPs were reported to trigger HR and PCD [55].

Comparing innate immunity in plants, animals, insects and invertebrates:

Plants, animals, insects and invertebrates such as *C. elegans* possess innate immune systems. Plants and animals share a number of common features in their immune systems for example both recognise MAMPs such as flagellin and harpin using membrane bound PRRs [6]. This observation initiated a debate questioning whether these similarities were due to divergent evolution or convergent evolution. A number of scientists have commented on whether the shared features of innate immunity found across kingdoms are the result of divergent evolution from a common ancestor or the result of convergent evolution [91,92]. A number of reviews as well as mounting experimental evidence indicate that common features of innate immunity arose independently in each kingdom and are therefore best explained by convergent evolution [6,14,37]. Owing to a limited set of molecules and being subject to the same environmental constraints perhaps led plants and animals to independently evolve analogous approaches in dealing with microbe attack [6].

The most obvious parallel between plant and animal innate immunity is the presence of defined receptors for detecting MAMPs. Both plants and animals share similar PRRs which recognise MAMPs such as flagellin and both animal and plant PRRs activate defence signalling via a MAPK signalling pathway [6]. In plants, the LRR protein FLS2 recognises flagellin. The analogous PRR in animals is the toll like receptor 5 (TLR5) [6]. These PRRs share a number of similarities: they are membrane bound receptors with Leucine rich regions and upon recognising flagellin initiate defence responses [6]. However, closer inspection revealed that these PRRs recognise different epitopes of flagellin [93]. Additionally, the LRR domains are very divergent and plant FLS2 lacks an intracellular

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TIR domain and other downstream components present in TLR5 [6]. Additionally, FLS2 has an intracellular kinase domain whereas TLRs recruit IRAK kinase for downstream signalling [6] Flagellin perception in plants results in activation of defence via WRKY transcription factors which are absent in animals and insects [6]. Animals and insects evolved the transcription factors NF-κβ and Dif respectively to activate transcription of antimicrobial peptides [6].

Both plants and animals produce an AOS signal upon MAMP perception and is mediated by $gp91^{phox}$-like NADPH oxidase (AtRbohD in plants) common to both [94,95,96]. This observation could support the view that shared components of plant and animal innate immunity suggest a common ancestor. However, $gp91^{phox}$-like NADPH oxidase is also involved in a number of other biological processes in both plants and animals and therefore could have been recruited to pathogen defence independently [6]. Lastly, the best-characterised plant defence molecules are the R proteins (NBS-LRR) which possess a tripartite structure similar to animal CATERPILLAR (CLR) also called Nod proteins [97,98]. While both classes of defence molecules initiate programmed cell death as a defence mechanism, there is no evidence suggesting common signalling components [6]. Collectively, these examples show innate immune systems share similarities but these similarities are likely due to plants and animals converging on similar solutions to similar problems.

The similarities in the innate immune systems of animals, insects and plants suggests a common 'logic' to the overall structure of innate immunity in these kingdoms [6]. Studies on plant immunity revealed novel insights into animal innate immunity and *vice versa* and continue to do so. Advances in mammalian and insect immunity showed the circadian clock contributes to regulation of innate immunity. The plant circadian clock contributes to general fitness and recent experimental evidence linked a component of the plant circadian clock to plant innate immunity. Understanding similarities among the innate immune systems of animals, plants and insects is essential in getting a clearer picture of the inner workings on innate immunity.

The Circadian Clock and Immunity

The circadian clock regulates a number of biochemical and physiological processes in plants, fungi, animals and insects. The Earth rotates on a fixed axis, which results in a 24h day with light and dark cycles of varying length. Over evolutionary time, it is widely thought that organisms independently evolved circadian clocks to regulate various metabolic and physiological processes [99]. Circadian

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clocks confer an evolutionary advantage as they enable organisms to anticipate regular daily events, thus priming various physiological processes to coincide at the most favourable time [99,100,101]. Circadian clocks are characterised by three features: they are regulated by transcriptional-translational feedback loops, can be reset by light and temperature input and show temperature compensation and continue to run under constant conditions [100,102,103,104]. The ability of circadian clocks to reset is essential as it allows organisms to synchronise their internal processes with the external environment, adapt to changing day length and anticipate seasonal change [99]. Temperature compensation is also an important characteristic of the circadian clock. Usually for a 10ºC, increase in temperature there is an associated increase in biochemical rate of activity. If the circadian clock was not temperature compensated, it would run faster at higher temperature and slower at lower temperature [99]. However, the 24h period of the clock is largely unaffected by temperature and this is reflected by circadian clocks retaining near 24h rhythms at varying temperatures, which is important in organisms that cannot regulate their own temperature [102,103]. Circadian clocks are essential for a number of processes and have been demonstrated to play a role in immune responses in flies, mice, humans and plants.

The Circadian Clock of *Drosophila melanogaster* and its role in immunity

The circadian clock of *Drosophila melanogaster* (*Drosophila*) the fruit fly maintains a 24h period by regulating expression of *Clock* (*Clk*), *Cycle* (*Cyc*), *Period* (*Per*) and *Timeless* (*Tim*) [99]. Using at least two interconnected transcriptional-translational feedback loops, *Drosophila* is able to retain rhythmic expression of a number of output pathways [105]. PER and TIM associate together, interacting with the CLK-CYC complex and block it from transcribing their expression forming a negative feedback loop [106,107]. This leads to down regulation of *Per* and *Tim* transcription and activation of *Clk* transcription [107,108,109,110]. Transcription of *Per* and *Tim* begins around mid-day with RNA levels peaking in the early evening and protein levels peaking at midnight [110]. While PER is phosphorylated by a kinase DOUBLETIME (DBT), which targets it for degradation, TIM is degraded in a CRYPTOCHROME-dependent manner by light [111,112]. At night, TIM binds to PER and this prevents DBT from phosphorylating PER, thus PER avoids degradation [111,113,114]. When TIM and PER associate they are able to localise to the nucleus [114,115,116]. Nuclear localised TIM-PER undergo phosphorylation events, which eventually mark the complex for degradation [110,117]. After degradation of TIM-PER repression of CLK-CYC ceases resulting in activation of *Per* and *Tim* transcription (the positive feedback loop) and repression of *Clk* transcription [118]. These interconnected feedback loops form the core rhythms of the circadian clock in *Drosophila*.

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Lee and Edery (2008) showed circadian regulation of *Drosophila* immune response to *Pseudomonas aeruginosa* and *Staphylococcus aureus*. To investigate clock control of immune responses in *Drosophila*, infections were performed at various time points under light-dark cycles and in free running conditions of constant dark [119]. Circadian rhythms persist under constant light or dark and constant temperatures, known as free-running conditions. Flies showed greater susceptibility to the bacterial pathogens after daytime infections compared to infections at night. The time-of-day variation to infection was also observed under constant dark conditions suggesting circadian clock regulation of defence responses [119]. To test this, experiments using clock mutant flies: Per^{01}, Clk^{Jrk}, Cyc^{01} and Tim^{01}, showed that loss of clock components resulted in loss of time-of-day variation in susceptibility to infection by *P. aeruginosa* [119]. Interestingly, the clock mutants behaved differently to infection, some showing enhanced defence responses while others were more susceptible to infection. This suggests loss of different components of the clock affected immune responses uniquely [119]. The Per^{01} mutant showed greater susceptibility to infection while Clk^{Jrk} was less susceptible than the wild type [119]. Per^{01} mutants also showed greater susceptibility than wild type flies when infected with *Streptococcus pneumoniae* and *Listeria monocytogenes* [7].

In contrast the clock mutant Clk^{Jrk} showed lower bacterial counts than Per^{01} with bacterial levels comparable to wild type flies that were infected at night [119]. Collectively these observations implicate clock control of defence genes leading to the *Drosophila* immune system being better primed for defence at night than during the day. Microarray data revealed that basal levels of a key regulator of defence called *imd* showed time-of-day variation in expression with peak expression at night [120]confirmed by quantitative PCR [119]. Additionally, both PGRP-SA a PRR essential for detecting peptidoglycan and *drc* a gene encoding antibacterial peptides accumulate to greater levels at night than during the day [119]. Interestingly the Per^{01} mutant accumulated greater amounts of *drc* than Clk^{Jrk} despite Per^{01} showing greater susceptibility [119]. This contradictory observation indicates an instance where incorrect or over-expressed immune genes can have deleterious effects. Overexpression of defence genes perhaps abrogates other defence responses while also being physiologically expensive [119,121,122,123]. This in turn leads to diminished overall fitness and greater susceptibility to infection. Lee and Edery (2008) illustrated the role of circadian rhythms and clock genes in immunity. Their work suggests PER may play a protective role in innate immunity by regulating expression of defence genes.

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Circadian regulation of immune responses in mammals

Mammalian clock components share remarkable sequence similarity with those of *Drosophila* and similarly the clock functions by transcriptional-translational feedback loops. The positive arm of the clock involves CLOCK:BMAL1 associating to regulate expression of *Per 1*,*2* and *3*, *Cry 1* and *2* and *Bmal1* while PER 1 and 2 and CRY1 and 2 constitute the negative arm of the clock [124]. CRY1 and CRY2 interact with PER1 and PER2, enabling nuclear translocation where they repress CLOCK activity [124]. Like in *Drosophila* PERs 1, 2 and 3 are phosphorylated repeatedly, eventually marking them for degradation [99].

Natural killer (NK) cells are an integral component of mammalian innate immunity [70]. NK cell activity displayed circadian rhythms when investigated under free running constant dark conditions [8]. NK cells produce cytolytic factors and cytokines in response to infection and these molecules play a vital role in NK activity [8]. Expression of the cytolytic factors: granzyme B and perforin, as well as cytokines: IFN-γ and TNF-α, all showed rhythmic expression in NK cells of Sprague Dawley (SD) rats, peaking in expression at night [8]. Additionally, RNA- mediated knockdown of *Per2* resulted in reduced levels of granzyme B and perforin levels [8].

NK cells are essential for detection of infected and malignant cells [70]. NK cells destroy infected cells by calcium-dependent release of cytolytic granules and activation of death receptors in target cells by TNF-related ligands [8]. When NK cell activity in SD rats was investigated for time-of-day variation, maximum activity was reported at night [125]. The collective results show NK cell activity in SD rats is under clock control and peaks at night. SD rats are nocturnal and are most active at night. It is possible that NK cell activity increases at night when exposure to pathogens and other environmental insults would be greatest [8]. That loss of *Per2* resulted in reduced levels of granzyme B and perforin suggests *Per2* positively contributes to production of cytokines and cytolytic factors.

A well-characterised output of the circadian clock of animals is the sleep-wake cycle [126]. An excellent review documents how infections in animals and humans leads to altered sleep patterns, an extreme example is sleeping sickness (*Trypanosomiasis*) caused by a tick-borne pathogen [9,126]. The link between altered sleep patterns after infection by pathogens suggests that the human immune system may also show circadian regulation. Studies revealed that levels of lymphocytes and monocytes reach maximal levels around midnight while NK cells reach peak levels in the afternoon,

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decreasing in number at midnight [127,128]. These daily fluctuations are under circadian control, showing a link between sleep and disease response [9]. The effect of sleep on immune responses is beyond the scope of this review but an excellent treatise discussing it can be found here [9]. In summary, the circadian clock regulates immune function of fruit flies, rats and humans and probably other animals.

The Plant Circadian Clock and its role in Immunity

Plants are sessile organisms and unlike animals lack mobile defence cells, rather evolving to have innate immunity in each cell [1]. Similarly, each cell possesses the molecular circuitry that makes up the circadian clock [100]. The synchronised expression of circadian clock components in each cell gives the plant a collective rhythm [129]. In plants, the circadian clock is essential for a number of physiological and biochemical processes including photosynthesis, flowering and seed germination. As in animals and insects, the plant circadian clock is temperature compensated, can be reset by light input and possesses multiple transcription-translational feedback loops [100]. Plant circadian rhythms have a 24h period and persist under free-running conditions [100]. While the circadian clocks of animals, plants and insects share a number of similarities, the actual components of the plant clock are not conserved across the kingdoms and the presence of similar clock circuitry may be a result of convergent evolution [130,131].

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While the basics of clock regulation in plants are now known, understanding components of the plant circadian clock is still the subject of active research. Additionally, identifying output pathways regulated by the circadian clock is also researched extensively. Using the model plant, *Arabidopsis*, molecular biologists have managed to dissect the molecular pathways that drive the positive and negative loops of the plant circadian clock [131]. Studying plant circadian rhythms and its clock components are useful not just for the advancement of understanding plant circadian rhythms but also for advancing our understanding of the circadian clocks of animals and insects. Plants, animals and insects share a common architecture among their clocks. Therefore, findings in one system may have implications in another.

The interrelated nature of clock components can be seen in the following example [130]. A histone demethylase jumonji domain containing 5 (JMJD5) which is found in plants and humans [130]. In both *Arabidopsis* and humans, knock down mutants lacking *JMJD5* have a fast-running circadian oscillation phenotype compared to wild type. Intriguingly, the *Arabidopsis* and human JMJD5

ortholog retain enough similarity to rescue the mutant phenotype in reciprocal systems [130]. The human cell line U2OS-B6 has a fast-running oscillation and as it lacks human *JMJD5* [130]. To test if *Arabidopsis AtJMJD5* could rescue the human phenotype, an *AtJMJD5* + U2OS-B6 cell line was created that stably expressed the *Arabidopsis* gene [130]. This cell line was transfected with human short interfering (si) RNA *JMJD5* and the period length measured showing a significantly longer period [130]. However, this was only a partial rescue as further experiments showed a modest decrease in period length in the presence of human siRNA coding *JMJD5* compared to scrambled siRNA [130]. Jones *et al.* (2010) also tested whether the human *JMJD5* ortholog was functional *in planta*, showing that under the control of the *Arabidopsis* promoter human *JMJD5* indeed can restore a longer period.

Like animals and insects, the plant circadian clock has multiple, interlocking feedback loops. The core loop that was initially identified comprises two Myb transcription factors- CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY)- and a pseudo-response regulator called TIMING OF CAB EXPRESSION 1 (TOC1) [131,132]. CCA1 and LHY act together in another loop as positive regulators of PRR7 and PRR9 and this pair in turn, indirectly repress *CCA1* and *LHY* expression [131]. In the core loop, CCA1 and LHY bind together to repress *TOC1* expression and by doing so form the negative arm of the circadian clock [132]. On the other hand, TOC1 positively regulates *CCA1* and *LHY* [132]. Interestingly TOC1 lacks DNA binding motifs and does not directly regulate CCA1 and LHY. Recently, CCA1 Hiking Expedition (CHE) a TCP (TB1, CYC, PCFs) Myb-like transcription factor that binds TOC1 was identified and could aid in understanding the positive limb of the circadian clock [133]. Additionally CHE binds to the *CCA1* promoter repressing *CCA1* expression. Therefore, TOC1 may bind to free CHE, sequestering this repressor of *CCA1* enabling transcription of *CCA1* [131]. This aspect of the core loop is still the subject of current research as our understanding of the positive arm of the plant circadian clock is still incomplete [131]. CCA1 and LHY form homo and heterodimers driving transcriptional processes in various output pathways [134,135] such as expression of the evening phased *AtGRP7* also called *COLD AND CIRCADIAN REGULATED 2* (*CCR2*) [136].

As plants are sessile, the circadian clock is crucial for fitness as it enables plants to anticipate regular biotic and abiotic changes, thus priming appropriate metabolic processes to occur at optimal times [100]. A number of plant physiological and biochemical processes are optimal when their internal clock matches the periodicity of the external environment [100]. A short period mutant *toc1-2* when grown under 28h day conditions (14h light and 14h dark) showed reduced fitness with stunted growth and decreased chlorophyll levels, while a long period mutant *ztl-27* grown under 20h days (10h light

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and 10h dark) also showed similarly stunted features and overall reduced fitness [101]. These observations indicate the importance of matching the internal clock with the external environment.

Microarray experiments suggest that approximately one-third of expressed *Arabidopsis* genes are under circadian regulation [137]. Analysis of promoter regions of expressed genes also suggests 36% of *Arabidopsis* genes are under clock control [138]. These data suggest the *Arabidopsis* circadian clock regulates a wide range of physiological processes that occur at various times during the light-dark cycle. The genes encoding clock proteins are classified as morning and evening-phased genes depending on when their peak expression occurs. These clock proteins can bind to the promoter region of downstream genes, activating or repressing gene expression at various times of day [100]. The dawn-phased proteins, CCA1 and LHY, bind the Evening Element (EE) found in the *TOC1* gene, resulting in repression of *TOC1* [139,140,141,142]. Dusk-phased genes display enrichment of the EE in their promoters, while dawn-phased genes show overrepresentation of the morning element (ME) [139,141,142]. In addition to ME and EE, PBX regulates expression of night-phased genes and GATA (G-box), is important for expression of late-morning and afternoon-phased genes [139,141,142,143]. Through these various upstream promoter elements, plants may regulate multiple physiological processes to occur at appropriate times during the day.

Plant circadian regulation through complex feedback loops integrates multiple signals thus switching on output pathways at the most advantageous time of day [100]. In addition to generating rhythmic expression of biological pathways, the circadian clock also regulates a number of responses through a mechanism called gating [144]. Gating of gene expression by the circadian clock is a phenomenon where application of a particular stimulus elicits a response only at a specific time in the 24h day [100]. Therefore, a stimulus of equal intensity applied at different times will elicit a response of varying amplitude depending on when the stimulus is applied. An example of circadian gating is shade avoidance in plants [144]. Plants are able to perceive shade in the form of a change in the equilibrium between far-red and red light, through phytochromes [145] with a low ratio of red light to far-red light triggering shade avoidance in plants. Investigating shade avoidance mechanisms in *Arabidopsis* seedlings revealed that 4-day-old seedlings switched on shade avoidance mechanisms such as hypocotyl elongation, in a time-of-day dependent manner [145]. In controlled experiments, plants were transferred from 12h light-dark cycle to constant low red to far-red light mimicking shading but shade avoidance mechanisms were only activated at subjective dusk [144]. Through a gating mechanism, shade avoidance pathways were only activated at dusk even though the stimulus necessary to activate shade avoidance was present throughout the day [144]. Thus, when the circadian clock gates gene expression, the stimulus alone is insufficient to elicit a full response: rather the

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stimulus applied at the correct time activates a full response. Gating by the circadian clock perhaps enables plants to regulate physiological processes to occur at the most optimal time of day, ensuring that costly metabolic processes are initiated only at the most opportune moment.

Microarray studies implicate circadian clock regulation of plant responses to biotic stress, suggesting some circadian clock control of plant defence responses to pathogens [137], this idea was clearly articulated by Roden and Ingle (2009) [10]. A number of defence genes have shown circadian regulation and recently a molecular link between the circadian clock and immune responses was established [146].

Seasonal fluctuation in disease in plants is a well-understood phenomenon. Success of plant pathogens in infection is dependent on both host susceptibility and favourable environmental conditions. An example is the release of spores by *Phytophthora ramorum* and *Fusarium circinatum*, which occurs in cool and humid conditions [90,147]. The temporal variation in biotic stress also extends to bacteria on plant leaves showing diurnal variation in abundance [148]. These observations indicate that favourable conditions for infection vary and possibly plants have evolved mechanisms to anticipate them. Indeed a number of plant defence genes display rhythmic expression under the control of the circadian clock [149,150,151,152]. Additionally the defence gene *DEA1* in tomato shows rhythmic expression in long days but is constitutively expressed in short days, suggesting seasonal influence on defence gene expression [10,149]. While these defence genes display circadian rhythms, it would be interesting to investigate whether their induction is regulated by circadian clock gating.

A well-characterised output under clock control is the opening and closing of stomata [153], pores found on the abaxial side of plant leaves. These pores are important for gaseous exchange but are also exploited by bacteria as a portal of entry into the plant leaf. Novel work has revealed that stomata may present a barrier to pathogen entry [154]. Experiments showed that stomata close upon perception of MAMPs [154] while certain bacteria secrete a molecule called coronatine that can re-open closed stomata [154]. Further experiments showed that RIN4 (a target of a number of bacterial virulence effectors) regulates stomata opening and closing during pathogen attack [57]. Regulation of stomata is under the circadian clock and it would be interesting to investigate whether stomata sensitivity to MAMPs and coronatine varies at different times of day as plants attempt to balance the requirements of photosynthesis and pathogen resistance. The clock controlled AtGRP7 sometimes called CCR2

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also plays a role in stomatal regulation [155] and is under the control of the circadian clock showing peak expression in the evening [136]. CCR2 is also a target of bacteria with the virulence effector HopU1 affecting the ability of CCR2's to bind to RNA [156]. As CCR2 has been shown to regulate a number of biotic and abiotic stress responses [136], this result suggests that bacteria have evolved mechanisms to target circadian clock output pathways that contribute to defence responses.

Recently, seminal work by Wang and colleagues (2011) [146] established a molecular link between plant defence and a component of the circadian clock called CCA1. Using a systems approach, they identified novel R genes and MAMP genes as they sought to understand how these sets of genes are activated in response to biotic stress [146]. Inadvertently they noticed enrichment of CCA1 and EE binding motifs in the promoter regions of these genes. The R gene *RPP4* involved in plant immune response displayed a circadian rhythm and possessed CCA1 binding elements in its promoter. Wang *et al.* 2011 showed a number of defence genes were potentially under clock control. Using the null mutants' *ztl4* and *cca1* they showed that plant defence responses are compromised in clock mutants. Additionally the over-expressing *CCA1* showed enhanced defence responses to infection. Furthermore, they showed that under alternating light-dark conditions over expression of *CCA1* contributed positively to defence against *Hyaloperonospora arabidopsis Emwa1* (*Hpa Emwa1*). Since a number of defence genes possessed a CCA1 binding domain, Wang and colleagues investigated whether *CCA1* expression increased upon infection [146]. Promoter activity of *CCA1* was measured by using the reporter gene luciferase fused to the *CCA1* promoter. Interestingly, *CCA1* expression showed rapid induction and became arrhythmic after infection with *Hpa* [146]. This suggests a *CCA1* mediated defence pathway but further work is required to establish this molecular pathway [157].

Wang and colleagues showed that plants anticipated dawn infection, which corresponded with the release of spores by *Hpa* suggesting that the circadian clock regulated defence responses to occur when perceived threat was greatest. Conversely, greater pathogen success was reported after infection at dusk. This time-of-day variation was absent in the *cca1* mutant [146]. These results show time-of-day variation in the wild type and not in the mutant and they mirror studies performed on *Drosophila* discussed earlier [119]. However, these experiments should be carried out under free-running conditions to investigate whether there is circadian clock regulation [10,119,146,157]. Nonetheless, their novel results provided a link between plant defence responses and the circadian clock showing that absence of a functional circadian clock compromises plant defence.

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A systems approach as used by Wang *et al.* (2011) [146] is most likely to yield mechanistic insights into the *Arabidopsis* circadian clock network and its plant innate immune network. Additionally network studies similar to those employed by Sato *et al.* (2010) [54] will also be useful in understanding the relationship between the plant circadian clock and its immune responses.

Concluding remarks

Innate immunity and circadian clock regulation arose independently in insects, plants and animals. These complex systems share similarities across species that perhaps arose because of convergent evolution. The two systems of innate immunity and circadian clock regulation are both the subject of constant research and more recently, the link between the two is gaining greater attention. As our understanding of plant innate immunity grows, a clearer picture emerges which shows that plants attack pathogens with a carefully coordinated defence response. While previous research focussed on innate immunity simply as an on and off system, recent advances indicate that the plant innate immune system is more subtle than that [54]. Implicating the circadian clock in plant defence responses lends weight to the view that plant immunity is carefully coordinated and that plant defence responses may follow a diurnal rhythm showing peak defence when pathogens are most anticipated. The plant defence response is an energetically costly process that affects plant physiology and metabolism irreversibly if fully activated. Therefore, plants perhaps have evolved mechanisms to strike a balance between ensuring protection against pathogens while avoiding the detrimental effects of full-blown immunity. One of the mechanisms could be regulation by the circadian clock and this needs further research.

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Research question

The hypothesis that was tested was that defence responses in *Arabidopsis* are regulated by the circadian clock. This work tested the hypothesis by investigating the outcomes of infection by virulent *P. syringae* DC3000 under constant light and temperature conditions. Infections were carried out every four hours over two circadian cycles so that if differences in susceptibility were detected, they could be attributed to endogenous factors. Additionally the arrhythmic clock mutant *elf3-1* and the *CCA1*-overexpressing line were infected with *P. syringae* DC3000. These plant lines are arrhythmic under constant light conditions, thus enabling one to distinguish whether the circadian clock regulates time-of-day variation in response to infection. In addition to using bacterial counts as a measure of plant defences, callose production in response to infection of wild type *Arabidopsis* Col-0 and *CCA1*-

ox with *P. syringae* DC3000 *hrpA* was also measured at subjective day and night. This was used as a measure of the strength of the MTI response. To investigate circadian regulation of specific R protein triggered defence responses wild type, *elf3-1* and *CCA1*-ox were infected with avirulent strains of *P. syringae* DC3000 avrB and *P. syringae* DC3000 avrRpt2. Promoter activities of *OXI1* and *PR1* after infection with *P. syringae* DC3000 at different times of day were investigated using luciferase reporter constructs and transcript levels of *WRKY22* and *WRKY29* were measured in an attempt to understand the basis for differential responses depending on time-of-day of challenge.

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Chapter 2: Materials and Methods

2.1 Plant Materials

All plant lines used for pathogen assays were in the Columbia background (Col-0). Wild type *Arabidopsis thaliana* were from the Arabidopsis Biological Resource Centre and Seed Stock Centre (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm) while the *CCA1*-ox seeds were a kind gift from Dr Alex Webb at Plant Sciences, Cambridge, England. Seeds with *elf3-1 CAB*::LUC were obtained from Dr Frank Harmon, University of California, Berkeley. This *elf3-1*line was used as it was originally made by introgressing the *CAB2::LUC* reporter from wild type C24 into Columbia. This was then backcrossed seven times and while there may be a small amount of C24 around the reporter, the majority of the genome is derived from Columbia (personal communication, Dr. Bryan Thines, UC Berkeley). To measure the promoter activity of *PR1 Arabidopsis* Col-0 *PR1*::LUC constructs from [158] were used. Promoter activity of *OXI1* was measured using *OXI1*::LUC in the Col-0 background. *Arabidopsis* in the Wassilewskija (WS) background was used for *CCR2*::LUC promoter activity measurements and were kindly donated by Dr. Seth Davis (Max Planck University Institute for Plant Breeding Research, Cologne, Germany). *OXI1*::LUC plants were a contribution from Dr Robert Ingle. The *elf3-1* null mutant and *CCA1*-ox lack a functional clock meaning they are arrhythmic under free-running conditions of constant light and temperature. Many other clock mutants retain some degree of rhythmicity albeit with altered period. Using *CCA1*-ox and *elf3-1* meant that the role of the clock in regulating defence responses could be investigated, rather than a particular clock component.

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2.2 Plant growth conditions

All seeds were sterilised for 5 min in 2% (v/v) sodium hypochlorite and 0.025% (v/v) Tween 20 solution. Seeds were washed three times in sterile distilled water and placed on Murashige and Skoog (MS; Highveld biological PTY, Ltd., Lyndhurst, South Africa) medium, pH 5.8 solidified with 0.8 % (w/v) agar. All seeds were stratified at 4ºC in the dark for two to three days after which, they were placed in a growth room with long day photoperiod (16h light, 8h dark) with cool white light (60-100µmol $m^{-2}s^{-1}$) at 22ºC and 55% relative humidity, except for *CCR2*::LUC plants which were grown under 12h light dark cycles. The seedlings grew on MS agar for seven days and thereafter were transferred to Imidocloprid-treated ('Gaucho', Bayer, Paarl, South Africa) soil which was composed of peat (Jiffy Products, International AS, Norway) and vermiculite in a 1:1 (v/v) ratio. Plants were grown for three to four weeks in plant growth rooms using earlier mentioned conditions. Plants were

moved, within the light period, from long days (LD), to constant light (LL) at least 24 hours prior to pathogen treatment. An explanation of circadian time is shown in Fig 2.

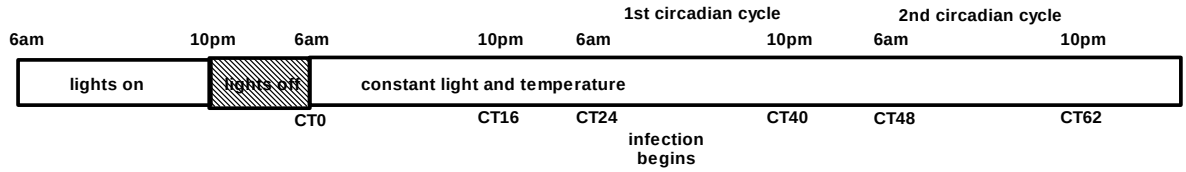

Figure 2. An illustration explaining circadian time and free-running conditions. Plants were grown for 4 weeks in alternating light-dark cycles of 16h and 8h respectively. After that, they were transferred to constant light and temperature conditions. Once in constant light, time is measured as circadian time (CT). Plants were infected at CT26 and then every four hours thereafter, i.e. CT30, CT34 and so on up to CT70 specifically for the pathogen assays. For gene expression work and callose deposition assays infections were at CT26 and CT42. These times correspond to subjective morning and evening respectively.

2.3 Bacterial growth conditions

All bacteria were grown in King's Broth [159] supplemented with the required antibiotics (Table1). Bacteria were cultured at 30ºC for 12 to 14 hours, corresponding to middle to late log-phase and prepared for inoculation as recommended in [160]. *P. syringae* DC3000 expresses avirulence B (avrB), an effector protein that is recognised by the R protein RPM1. *P. syringae* DC3000 expresses avirulence Rpt2, an effector protein recognised by the R protein RPS2. *P. syringae hrpA* lacks a TTSS and is therefore unable to deliver effectors.

Table 1. The various strains of *P. syringae* used and the required antibiotics

Pseudomonas syringae DC3000	Rifampicin 50µgmL^{-1}
Pseudomonas syringae DC3000 avrB	Rifampicin 50µgmL^{-1} Tetracycline 15 µgmL^{-1}
Pseudomonas syringae DC3000 avrRpt2	Rifampicin 50µgmL^{-1} Kanamycin 50µgmL^{-1}
Pseudomonas syringae DC3000 *hrpA*	Rifampicin 50µgmL^{-1} Kanamycin 50µgmL^{-1}

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2.4 Pressure infiltrating *Arabidopsis* plants for pathogen assays

Plants were inoculated by pressure infiltration using a 1mL needleless syringe to inject the abaxial side of the leaf as described in [160]. Bacteria were pressure infiltrated into three leaves per plant with three biological replicates. Mock treated plants were infected with 10mM $MgCl_2$. After infection, the infected leaves were harvested to measure bacterial growth. This was done at 4 and 48h after infection. To measure bacterial growth, leaf discs were homogenised in 1mL of 10mM $MgCl_2$ and appropriate dilutions plated onto KB agar supplemented with the necessary antibiotics. Petri dishes were incubated in the dark for two days at 30ºC. All infiltrations were under constant light with the plants remaining in constant light for the remainder of the experiment. Counts measured as colony forming units per cm^2 of leaf.

2.5 Callose deposition assays

Callose levels were compared in wild type *Arabidopsis* Col-0 (hereafter referred to as Col-0) and the overexpressing line, *CCA1*-ox at CT26 and CT42. Four-week-old plants were infected with *P. syringae* DC3000 *hrpA* mutant lacking type three-secretion system and callose levels were measured 14h after infection. Plants were infected with a bacterial suspension at an OD of 0.2 which corresponds to 1×10^8 $cfuml^{-1}$ as recommended by [161]. Using a 1 mL needleless syringe, bacteria were pressure inoculated through the abaxial side of the plant leaf surface [161]. After 14 hours, the infected leaves were detached and subjected to staining by aniline blue [161]. Infections were under constant light and temperature conditions at two time points, CT26 and CT42. Mock-treated plants were pressure inoculated with 10mM $MgCl_2$ and also subjected to staining by aniline blue [161]. After staining, leaves were fixed on glass slides and sealed with nail polish. Images were collected using a fluorescent inverted microscope (Nikon TMD-EF DIAPHOT-TMD, Tokyo, Japan) of callose deposition and these were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, California).

2.6 Measuring gene expression

2.6.1 Gene expression using luciferase

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Promoter activity of *Pathogenesis Related1::LUCIFERASE* (*PR1::LUC*), *Oxidative Inducing Signal 1 ::LUC* (*OXI1::LUC*) and *Cold and Circadian Regulated 2::LUC* (*CCR2::LUC*) was measured. Bioluminescence was measured in four-week-old plants inoculated with *P. syringae* DC3000 or mock treated with 10mM $MgCl_2$, using a Xenogen Lumina IVIS (Caliper Life Sciences, MA, USA) with thermoelectrically cooled, low-light-detecting charge-coupled device (CCD) camera, in a temperature-controlled dark box. Plants were sprayed with 5 mM luciferin (Biosynth AG, Switzerland) 24 h and 12 h before the start of experiment, and were subsequently sprayed every 8 h with 1mM luciferin during the experiment. Bioluminescence was captured for 5 min, every 2 h in the Lumina IVIS. In order to calculate levels of bioluminescence, Living Image v4.1 software (Caliper Life Sciences, MA, USA) was used. Total bioluminescence counts were divided by the area to yield counts per cm^2. Only infected leaves were measured and luciferase levels were measured in four biological replicates per time point. Plants were grown in 16h light and 8h dark cycles and transferred to constant conditions 24h prior to infection. Bioluminescence measurements were carried out 24h after infection at CT26 and CT42.

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2.6.2 Quantitative PCR

2.6.2.1 RNA extraction and cDNA synthesis

Col-0 and *CCA1*-ox plants were grown for four weeks in 16h light/ 8h dark cycles, and were transferred to constant light at least 24 h before infection. Three plant leaves per plant of Col-0 and *CCA1*-ox were inoculated at CT26 and CT42 using a 1 mL needleless syringe with either *P. syringae* DC3000 or 10mM $MgCl_2$. Inoculated leaves were harvested in liquid nitrogen 4h after infection and stored at -80ºC. RNA was extracted as described by Smart and Roden [162]. Quantity and integrity was tested by spectrophotometric readings (Nanodrop ND11) and separation on a 1.2% agarose gel by electrophoresis, respectively. For cDNA synthesis RNA was reverse transcribed using ImpromII reverse transcription system (Promega, Madison WI, USA), following the manufacturer's protocol except half reaction-volumes were used and random hexamer primers (Promega) were added. Two technical repeats for cDNA synthesis were carried out to account for variation in efficiency of cDNA synthesis. The pooled cDNA was used for quantitative-Polymerase Chain Reaction (qPCR) experiments. Three biological replicates were used for each treatment.

2.6.2.2 qPCR using standard curve method

Real Time PCR was carried out using a Rotor Gene 6000 machine (Corbett Life Science Pty. Ltd., Sydney, Australia). Transcript levels of *WRKY22*, *WRKY29* and *SRFR1* were measured in Col-0 and

CCA1-ox infected at CT26 and CT42. Basal expression was determined for *SRFR1* at CT26 and CT42 while basal and induced transcript levels were measured for *WRKY22* and *WRKY29* at CT26 and CT42. The Dynamo flash SYBR Green qPCR kit (Finnzymes, Keilaranta, Finland) manufacturer's protocol was followed except half reaction volumes were used. A total reaction volume of 10µl per reaction consisted of 1µl of cDNA template, 0.5 µl each of forward and reverse primers, 5µl of SYBR green mix and 3µl of sterile H_2O. The housekeeping gene was *UBIQUITIN10* (*UBIQ10*) and the genes investigated were *WRKY22*, *WRKY29* and *SRFR1*. *UBIQ10* was chosen as the housekeeping gene as it is expressed constitutively and is unaffected by the circadian clock [163]. Primers, annealing conditions and R^2 values are listed in Table 2. The R^2 values are above 0.995 for the housekeeping gene and *WRKY22* and *WRKY29*. *SRFR1* has a R^2 value of 0.988 (Table 1.). R^2 values of 0.995 indicate a linear standard curve. To determine copy number the standard curve method was used [164,165]. A standard curve was made, ranging from 1×10^1 copy numbers/µL to 1×10^6 copy numbers/µL using purified PCR product of known concentration and base pair length for each gene investigated including the housekeeping gene. The R^2 values shown in Table 2 indicate excellent correlation coefficients for the standard curves. Using Rotor Gene 6000 software, copy number for the housekeeping gene was determined from the standard curve. Similarly, copy number for the genes of interest was also determined. To calculate relative gene expression the copy number of the gene of interest was divided by the copy number of the housekeeping gene.

Relative transcript abundance = gene of interest (copy number) ÷ housekeeping gene (copy number)

Table 2. Primers used, annealing conditions and R^2 values.

Primer	Primer sequence	Annealing temperature	R^2 value
UBIQ10 f	5' TTGTCGATGGTGTCGGAGCTT 3' [166]	Anneal at 60ºC	0.997
UBIQ10r	5'TAAAAACTTTCTCTCAATTCTCTCT 3' [166]		
WRKY22f	5'CGACAAAGTAATGCCGTCTCC 3' [167]	Anneal at 55ºC	0.996
WRKY22r	5'CGTTTCTGGTTCTGTGGCTTT 3' [167]		
WRKY29f	5'-ATCCAACGGATCAAGAGCTG-3' [168]	Anneal at 60ºC	0.996
WRKY29r	5'-GCGTCCGACAACAGATTCTC-3' [168]		

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SRFR1f	5' CTGG`ATATGCCTCACTAGAA3' [169]	Anneal at 60ºC	0.988
SRFR1r	5' CACTGGGTCACAAGGCTCTG 3' [169]		

2.7 Online Bioinformatics tools

To obtain accession numbers for genes examined in this study the online *Arabidopsis* resource was used http://www.arabidopsis.org/. To analyse promoter regions of plant genes the online tool found at this link http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl (*Arabidopsis thaliana* expression network analysis) (ATHENA) was used.

2.8 Statistical analysis

To determine whether time of infection and/or plant genotype affect bacterial titre and callose deposition general linear model (GLM) analysis was performed using Statistica v10.1 followed by the Bonferroni post-hoc test. To analyse bioluminescence data for *PR1* and *OXI1* expression Two Way Repeated Measures ANOVA was used. Student's t-test was used to analyse qRT-PCR data. A p-value of less than 0.05 was considered significant.

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Chapter 3: Results

3.1 Investigating the MAMP-triggered immune response for circadian control

3.1.1 Investigating the effect of time-of-day on *Arabidopsis* response to *P. syringae* DC3000:

To investigate the potential circadian regulation of plant defence responses, 4-week-old Col-0 plants were infected with the plant pathogen *P. syringae* DC3000 at 4h intervals under constant light and temperature conditions. As infections were carried out in free running conditions, the time at which plants were infected was recorded as circadian time (CT), i.e., the plant's subjective or internal time, shown on the x-axis (Fig 3.1). Subjective evening is indicated by the shaded regions in the figure (Fig 3.1).

Bacterial titres measured 48h after infection indicate that Col-0 plants were least susceptible to infection at CT26 and CT54, while greatest success of infection was recorded at CT42 and CT66. Generally, the trend suggested infections during subjective day led to a better defence response than infections during the subjective night, with lower bacterial counts recorded for the former (Fig 3.1). This time-of-day difference in susceptibility to infection is observed through two circadian cycles. The peaks and nadirs in susceptibility to infection under free-running conditions indicated a possible circadian clock driven influence on plant responses to *P.* syringae DC3000. To investigate this possibility, defence responses of Col-0 plants were compared with defence responses in clock deficient plants under free running conditions.

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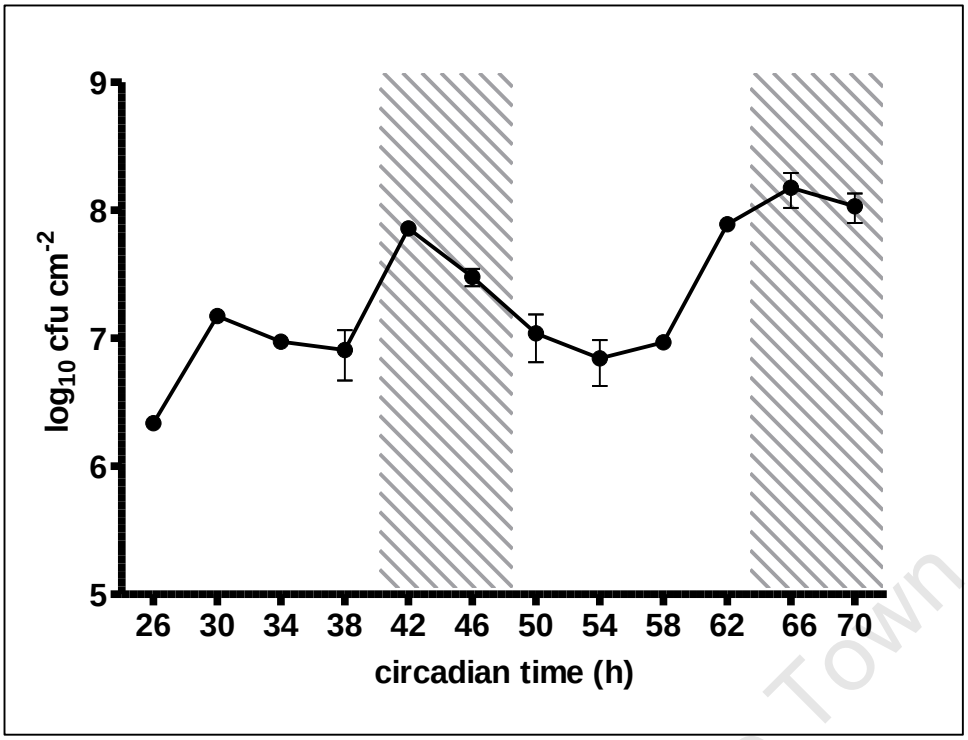

Figure 3.1 *Arabidopsis thaliana* (Col-0) shows variable susceptibility to *P. syringae* DC3000 under free-running conditions of constant light. Bacterial growth in leaves was measured 48h after infection under constant light conditions at each of the 12 time points. The success of growth was used as a measure of virulence (measured as colony forming units per cm^2 cfu cm^2). The error bars indicate standard error of mean for three measurements from three biological replicates. A similar pattern in the variation in susceptibility was observed in three independent experiments. (Absence of error bars implies little variation).

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3.1.2 Investigating *elf3-1* and *CCA1*-ox for time-of-day variation in susceptibility to *P. syringae* DC3000:

The circadian clock null mutant, *elf3-1* and *CCA1*-overexpressing line *CCA1*-ox, were investigated for time-of-day variation in susceptibility to infection by *P. syringae* DC3000 (Fig 3.2). Under constant light conditions *CCA1*-ox has been shown to have decreased fitness, arrhythmic circadian regulation and lower photosynthesis [101], while in constant light, the null mutant *elf3-1* was reported to be arrhythmic [170]. Recently researchers showed that the *cca1* null mutant displayed no time-of-day variation in susceptibility to *Hyaloperonospora arabidopsis* (*Hpa*) [146] when infected under light/dark cycles. In addition, *CCA1*-ox showed enhanced disease resistance to *Hpa* [146] compared to Col-0 after infections in the morning.

In this study, plants were infected under free-running conditions of constant light. Col-0, *elf3-1* and *CCA1*-ox were infected at CT26 and CT42 as these time points corresponded to least susceptible and

most susceptible in Col-0, respectively (Fig 3.1). Bacterial titres at 4hpi indicated that equal numbers of bacteria were inoculated across time points and genotypes (Fig 3.2). Plant susceptibility to *P. syringae* DC3000 was measured after 48h as in Fig 3.1. Once again, Col-0 showed time-of-day variation to pathogen infection with lower titres recorded at CT26 than at CT42 (Fig 3.2). Interestingly, the arrhythmic plants displayed equal susceptibility to infection, regardless of when the infection was carried out. Furthermore, these arrhythmic plants showed greater susceptibility to infection than Col-0 plants at CT26 with bacterial titres comparable to Col-0 at CT42. To test whether these differences were statistically significant, a general linear model (GLM) analysis comparing the effects of genotype and time was used followed by Bonferroni's post-hoc test grouping bacterial counts based on homogeneity. Similar counts were assigned the same letter indicating no significant difference in mean value (Fig 3.2).

The statistical analysis revealed that Col-0 plants showed a time-of-day variation in susceptibility to infection with bacterial levels significantly lower at CT26 compared to CT42 ($p<0.05$). However, this time-of-day variation was absent in the arrhythmic plants, *elf3-1* and *CCA1*-ox (Fig 3.2). Collectively these results indicated that Col-0 showed the least susceptibility to infection at CT26 and displayed a time-of-day variation in defence response while the arrhythmic plants, *elf3-1* and *CCA1*-ox showed no time-of-day variation to infection and higher bacterial counts comparable to Col-0 at CT42. This further demonstrated a clock controlled time-of-day variation in defence responses of Col-0 but not of the arrhythmic plants.

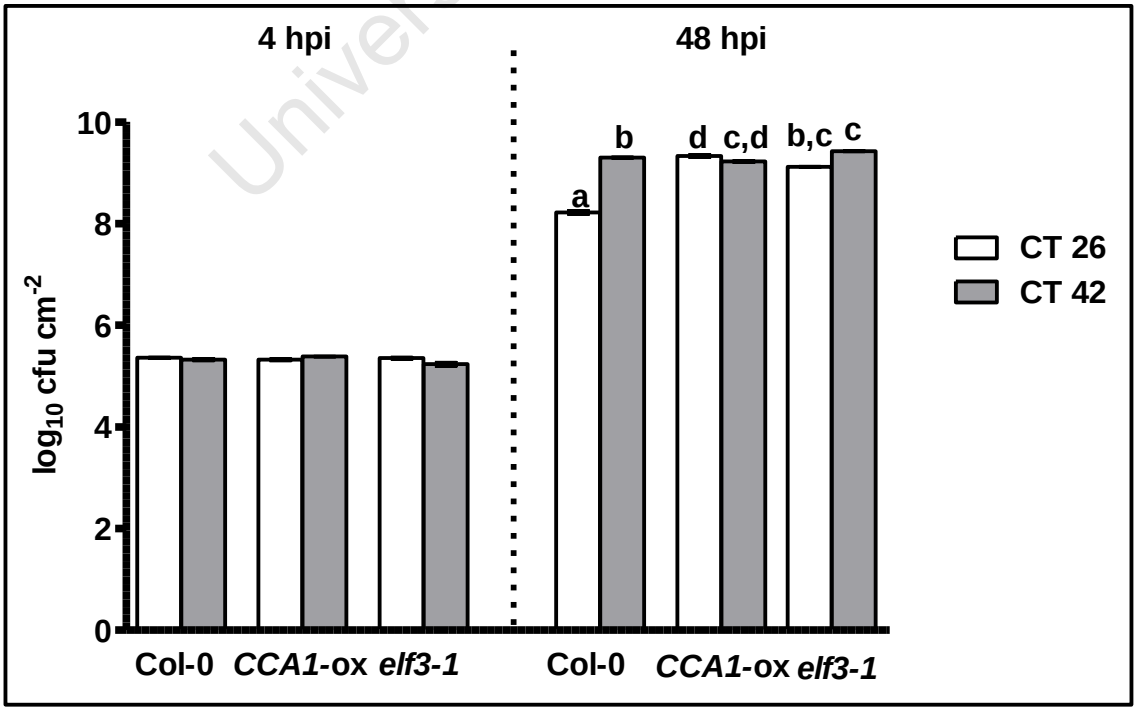

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Figure 3.2 Arrhythmic plants show no variation in susceptibility with respect to time of infection under free-running conditions of constant light. The wild type, *elf3-1* null mutant and *CCA1*-ox were infected with *P. syringae* DC3000 at CT26 and CT42. Bacterial growth at 48h after infection shows variation in susceptibility with time of infection in Col-0 but not in *CCA1-ox* and *elf3-1.* To test whether time of infection and genotype were significant, a general linear model was used followed by Bonferroni's post hoc test ($p<0.05$). Dissimilar letters above the bars represent statistically significant differences in mean bacterial counts. Each measurement is the average of three biological repeats and error bars indicate standard error of mean (Note small error indicated little variation).

3.1.3 Investigating time-of-day variation in *CCA1*-ox in the second cycle:

To confirm observations made in Fig 3.2 that Col-0 showed a time-of-day variation in susceptibility but not *CCA1*-ox, Col-0 and *CCA1*-ox were infected in the second cycle at CT50 and CT66 that correspond to subjective morning and subjective evening infections respectively (Fig 3.3). Bacterial titres were measured at 4h post infection (hpi) and 48hpi. The 4hpi counts show equal numbers of bacteria inoculated at both time points and across plant genotypes. At 48h after infection, bacterial counts are lower for Col-0 at CT50 than at CT66 while *CCA1*-ox shows levels comparable to Col-0 at CT66 for both infection times. To test whether Col-0 levels were significantly lower at CT26, a two-tailed Student's t-test was carried out, giving a p-value of 0.037. This indicates that Col-0 is significantly lower at CT20. Additionally a p-value of 0.2852 after a two-tailed Student's t-test showed that mean counts in *CCA1*-ox at CT50 and CT66 are not significantly different.

These infections revealed that time-of-day variation to *P. syringae* DC3000 occurs in Col-0 plants while arrhythmic plants show equal susceptibility to infection. Furthermore, plants lacking a functional clock also show a greater susceptibility to infection than Col-0 at CT50, with levels comparable to Col-0 at CT66 (Fig 3.3).

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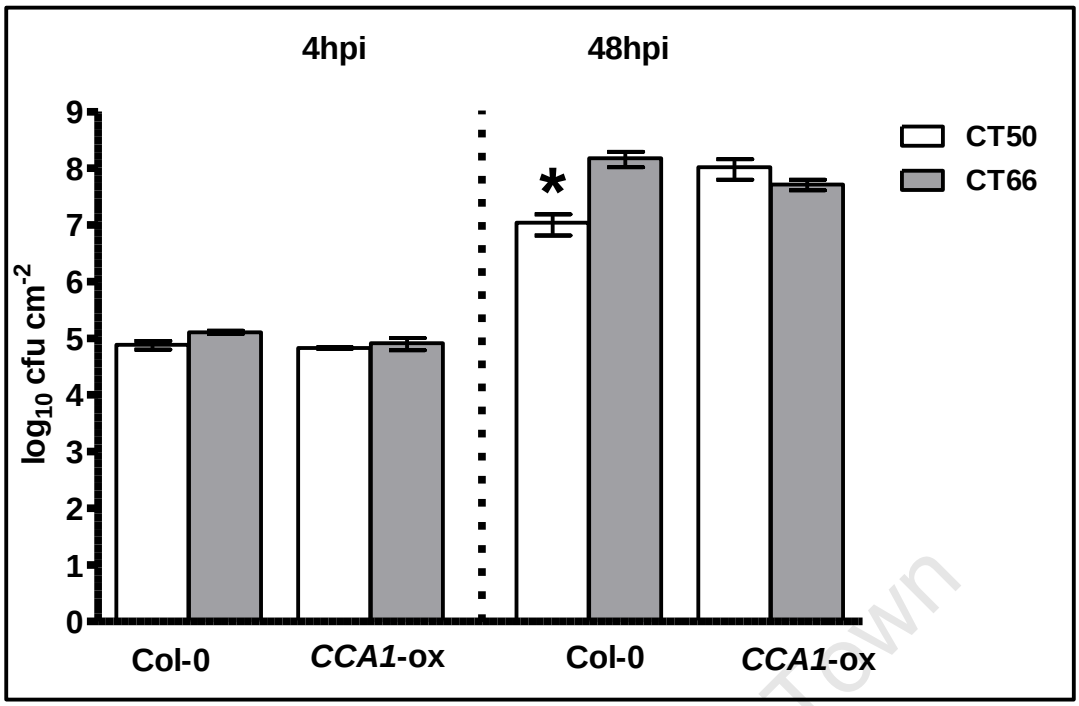

Figure 3.3 Second cycle infections show time-of-day variation in Col-0 but not in *CCA1ox*. Levels of bacteria 48h after infections in Col-0 are lowest at CT50 while at CT66 are comparable to *CCA1*-ox mutant. Error bars represent standard error of mean from three biological replicates. A two tailed Student's t-test showed Col-0 at CT50 was significantly lower than at CT66 (shown by asterisk, $p<0.05$). No significant differences were reported in *CCA1*-ox.

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3.2 Investigating the ETI response of *Arabidopsis* for time-of-day variation under free running conditions:

3.2.1 Infecting wild type and clock mutants with *P. syringae* DC3000 avrB and avrRpt2 at CT26 and CT42:

Next, the effect of time-of-day variation on ETI was investigated using two avirulent strains: *P. syringae* DC3000 avrB and *P. syringae* DC3000 avrRpt2. These strains possess effectors that are recognised by the plants cognate R proteins thus initiating ETI.

P. syringae DC3000 avrB initiates ETI if the infected plant possesses the cognate resistance (R) protein RPM1 [171]. Absence of RPM1 results in successful infection with avrB enhancing virulence [171]. Col-0, *CCA1*-ox and *elf3-1* were infected with *P. syringae* DC3000 avrB at CT26 and CT42 and bacterial counts carried out 4h and 48h after infection (Fig 4i). Titres at 4h showed equal numbers of bacteria infected across time points and plant lines (Fig 4i). Bacterial counts at 48hpi indicated levels of bacteria were lowest in Col-0 at CT26, with slightly higher counts at CT42 (Fig 4i). Both *CCA1*-ox and *elf3-1* showed higher bacterial titres at CT26 than CT42 with *elf3-1* showing greater susceptibility to infection than Col-0 at both time points (Fig 4i). To examine if these differences were statistically significant GLM analysis and Bonferroni post-hoc analysis were performed. These tests suggested the observed differences in Fig 4i are not statistically significant with $p>0.05$.

P. syringae DC3000 *avrRpt2* activates ETI if the plant possesses the cognate R protein RPS2 [171]. If the plant does not have the R protein, avrRpt2 enhances infection. To investigate the effect of time-of-day, Col-0, *CCA1*-ox and *elf3-1* were infected with *P. syringae* DC3000 avrRpt2 (Fig 4ii). Pathogen assays at 4hpi show equal bacterial titres across genotypes and plant lines (Fig 4ii). After 48h, the lowest counts were noted at CT26 in Col-0, with slightly higher counts at CT42 in Col-0. Bacterial levels in *elf3-1* at both CT26 and CT42 were comparable to Col-0 at CT42 while *CCA1*-ox was the most susceptible with levels higher than both Col-0 and *elf3-1* (Fig 4ii). To investigate whether these differences were statistically significant GLM analysis followed by Bonferroni post hoc analysis were carried out. Interestingly GLM analysis suggested the effect of genotype alone on bacterial counts was significant ($p=0.025$) and Bonferroni post-hoc test identified Col-0 as behaving differently from *CCA1*-ox but similar to *elf3-1* ($p= 0.0395$). However, when investigating the effect of genotype and time Bonferroni post-hoc test showed no significant differences, which suggests no effect of the clock on ETI response.

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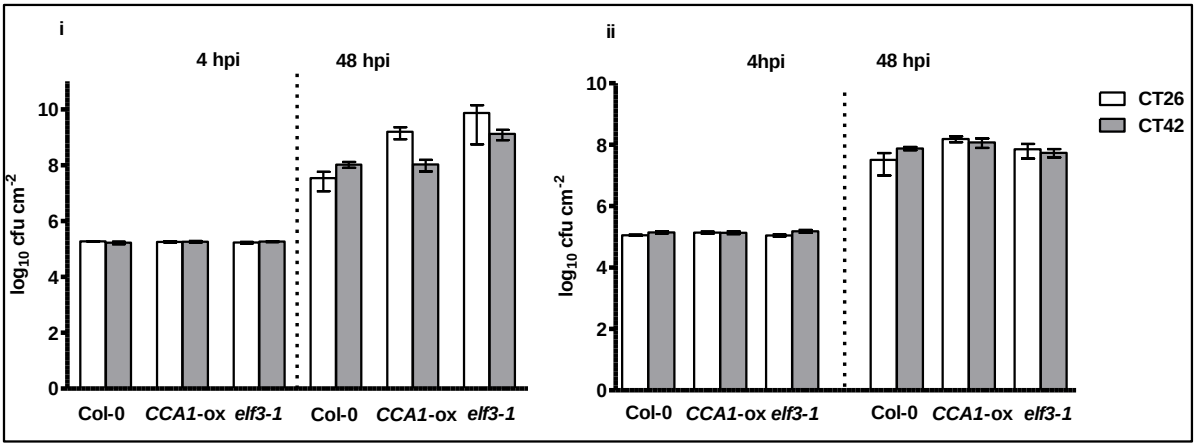

Figure 4 Bacterial counts in Col-0, *CCA1*-ox and *elf3-1* after 4 and 48h when infected with (i) *P. syringae* DC3000 *avrB* and (ii) *P. syringae* DC3000 *avrRpt2* at CT26 and CT42. Error bars represent standard error of mean for three biological replicates. Experiment performed only once.

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3.3 Measuring callose levels of *Arabidopsis* in response to *P. syringae hrpA* at CT26 and CT42:

Fig 3.1 shows time-of-day variation in Col-0 plants to infection with *P. syringae* DC3000 suggesting the circadian clock perhaps modulates MTI. One of the early responses to flg22 and non-pathogenic *P. syringae* is the production of callose regulated by callose synthase (GSL5/PMR4) [54]. *In silico* analysis of *PMR4* (At4g03550) using the online bioinformatics tool (ATHENA) found at this link- http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl, showed *GSL5/PMR4* to have a CCA1 element in the promoter region (-405 to -398bp upstream of +1). The presence of circadian clock binding motifs in *PMR4* and the earlier result reported in Fig 3.1 indicated that the circadian clock may modulate callose production in a time-of-day manner. To investigate this, Col-0 and *CCA1*-ox were infected with non-pathogenic *P. syringae* DC3000 *hrpA* at CT26 and CT42 under free running conditions (Fig 5.1 and 5.2). As *P. syringae hrpA* lacks a functional type III secretion system, it is unable to deliver effectors, and so is non-pathogenic [172]. Furthermore, detection of MAMPs from *P. syringae hrpA* initiates a strong MTI response. Callose levels were measured 14h after infection at either CT26 or CT42 (Fig 5.1 and 5.2). The highest callose levels were recorded at CT26 in Col-0 while much lower callose levels were detected in Col-0 at CT42 (Fig 5.1) Callose levels of *CCA1*-ox were greatly reduced at both time points with levels comparable to those of Col-0 at CT42 (Fig 5.1). These results mirror observations made in Fig 3.1 and Fig 3.2.

To test if the observations in Fig 5.1 were significantly different, GLM analysis testing the effect of genotype and time of infection and Bonferroni post-hoc analysis were performed. GLM analysis showed both time and genotype ($p=0.00002$ and $p=0.02$ respectively) to be significant when factored

independently. Furthermore interaction between genotype and time was highly significant (p=0.0006). Callose levels with similar counts were grouped together using the Bonferroni post-hoc analysis. Callose counts showing significantly different levels ($p<0.05$) are represented with different letters (Fig 5.1). Notably, callose deposition showed time-of-day variation in Col-0 but not in *CCA1*-ox.

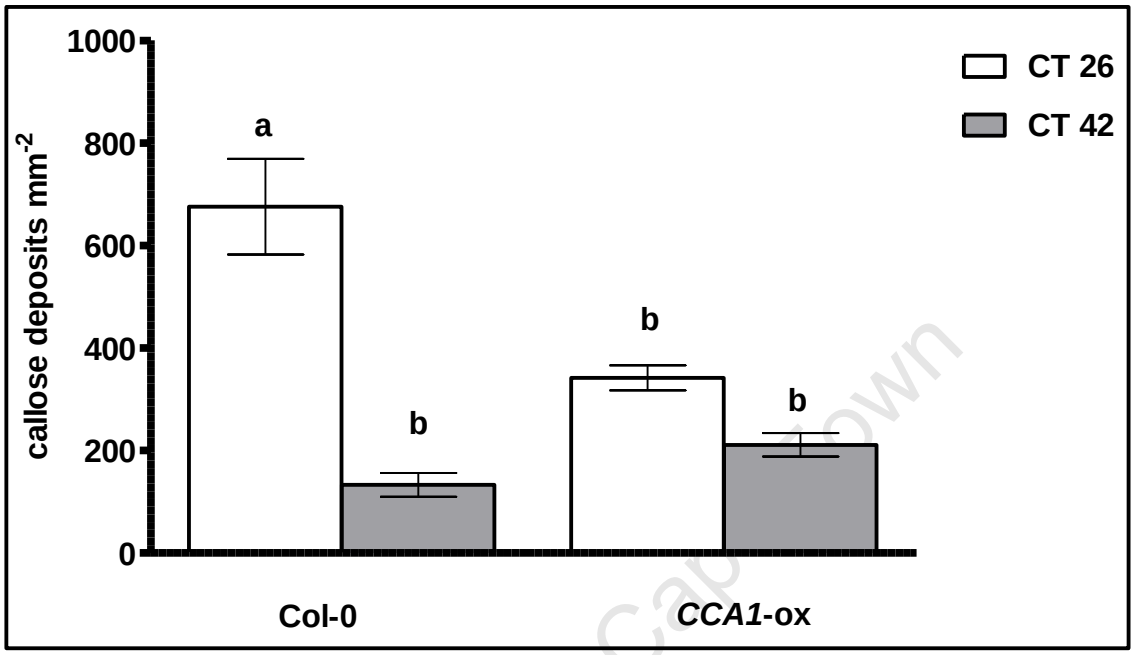

Figure 5.1 Callose levels measured at CT26 and CT42 in Col-0 and *CCA1-ox*. Levels of callose measured 14h after infection with *P. syringae hrpA* show higher production of callose at CT26 in Col-0 than at CT42. Production of callose is abrogated in *CCA1*-ox with levels comparable to Col-0 at CT42. Error bars are standard error of mean from six biological replicates. Letters above each bar represent statistically significant levels of callose (GLM analysis, Bonferroni's post-hoc test $p<0.05$). Experiments were performed twice with similar results.

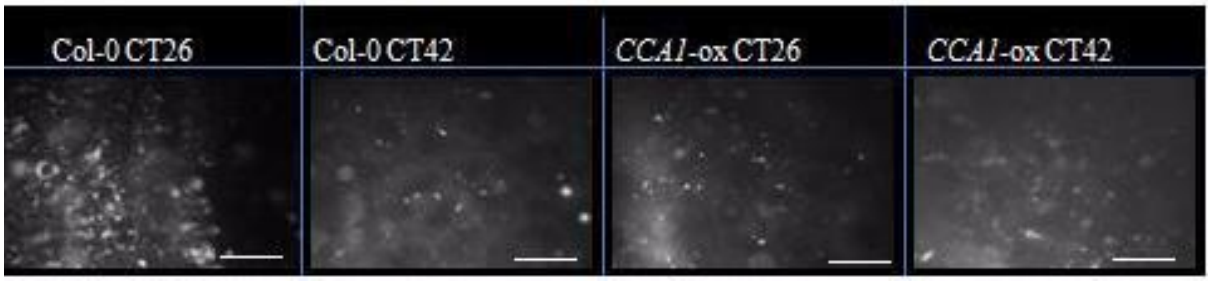

Figure 5.2 Fluorescent microscope images showing callose deposits. Callose deposits appear as white specks when they fluoresce. For each plant, two leaves were infected and stained with aniline blue 14h later. Several regions of the infected leaves were photographed and quantified. (Scale bar = 0.2mm).

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3.4 Promoter activity and expression of genes involved in the MTI response to *P. syringae* DC3000 infection:

Given that the circadian clock plays a role in plant defence [146], and these experiments suggested MTI could be modulated by the clock, investigating genes involved in MTI was essential. Upon recognition of MAMPs, one of the immediate results is the production of active oxygen species (AOS) such as H_2O_2 [44]. This AOS burst activates Oxidative Signal Inducing 1 (OXI1) which is important for full MTI response to *P. syringae* [43]. OXI1 is also necessary for the full activation of the mitogen activated protein kinases, MPK3 and MPK6 [44]. In turn, the MAP kinase cascade involving MPK3 and MPK6 is known to be up regulated in response to MAMPs, activating *WRKY22* and *WRKY29* which are important downstream components of the MAPK cascade [45]. The *WRKY* genes are a plant-specific group of transcription factors and are important in regulating defence gene expression upon infection [45,167,173]. One of the outputs of this pathway is the expression of *PATHOGENESIS RELATED 1* (*PR1*). Importantly though *PR1* is not an early response gene, occurring much later in the defence response [45] and is expressed in response to accumulation of SA [28]. *PR1* is an important defence signal in response to biotrophic pathogens such as *P. syringae* and is important in SAR [174,175]. Here promoter activity of *OXI1* and *PR1* was investigated in response to infection with *P. syringae* DC3000. Additionally, mRNA induction of *WRKY22* and *WRKY29* genes were measured 4h after infection.

3.4.1 Promoter activity of *OXI1* following infection

Wild type plants with the *OXI* promoter fused to a luciferase construct were infected with *P. syringae* DC3000. *OXI1* promoter activity was measured as bioluminescence in response to infection. An upstream analysis of the promoter region of *OXI1* indicated the presence of two putative CCA1 binding elements (-580 to -573 and -949 to -942 from start). Plants were infected at CT26 and CT42 and levels of bioluminescence measured from 24h after infection (Fig 6.1). Bioluminescence levels and peak expression levels for both infection time points were comparable with no significant differences reported after a Two-Way Repeated Measures (RM) ANOVA.

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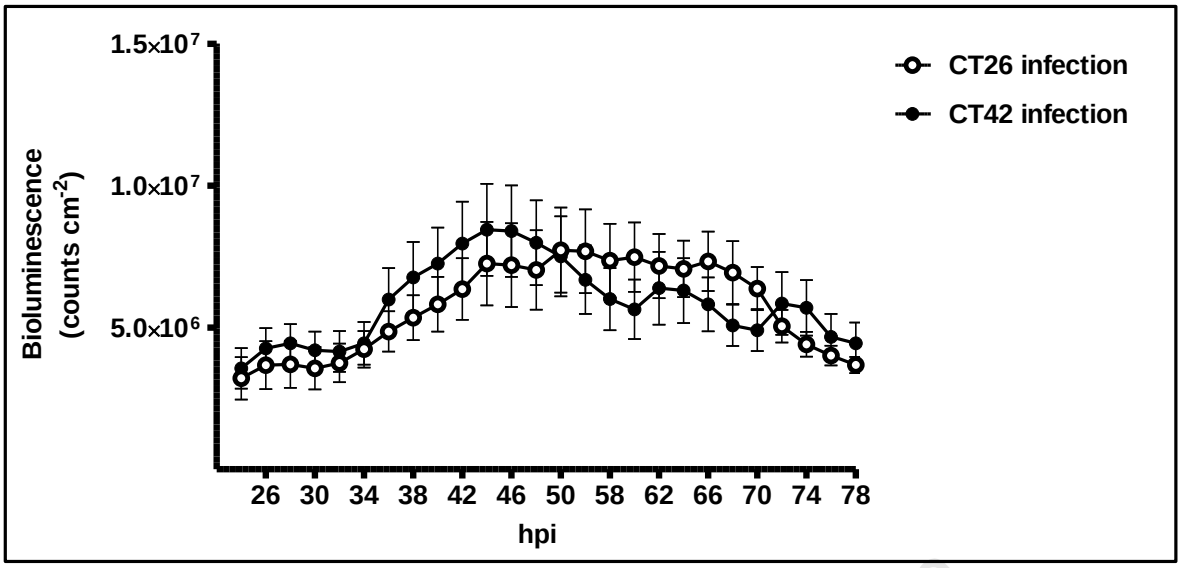

Figure 6.1 *OXI1* expression measured at CT26 and CT42 from 24h after infection with *P. syringae* DC3000. Error bars represent standard error of mean when four biological replicates were infected per time point. A 2 way RM ANOVA showed no significant differences in *OXI1* expression profiles at CT26 and CT42 ($p<0.05$). A similar result was observed in an independent experiment.

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3.4.2 Promoter activity of *PR1* following infection

PR1 promoter activity was also investigated using a luciferase reporter construct. Wild-type *PR1::LUC* plants were infected with *P. syringae* DC3000 under free-running conditions at CT26 and CT42 and bioluminescence was measured from 24h after infection (Fig 6.2).

There is quicker induction of *PR1* following infection at CT42 than at CT26 based on promoter activity of *PR1* measured as bioluminescence (Fig 6.2). A Two Way RM ANOVA confirms this difference in induction of *PR1* as statistically significant ($p<0.001$). Levels of *PR1* are higher at CT42 than at CT26 from 40h after infection, remaining higher until 56h after infection where levels begin to overlap.

PR1 expression occurs much later and is associated with SAR in uninfected leaves, HR, and PCD in infected leaves. While callose experiments (Fig 5.1 and 5.2) indicated a stronger response during subjective morning, complementing earlier observations (Fig 3.1 and 3.2) paradoxically, *PR1* levels were higher after infection at subjective night. We next investigated *WRKY22* and *WRKY29* which encode transcription factors important in activating early defence response genes and are themselves rapidly induced [45].

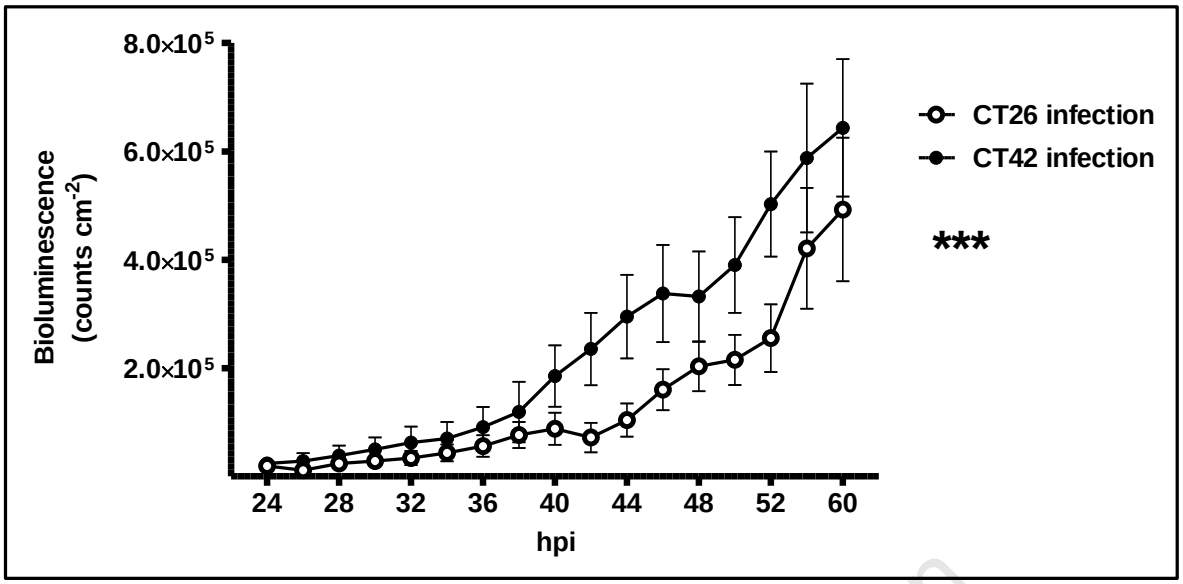

Figure 6.2 shows *PR1* expression measured as bioluminescence at CT26 and CT42. *PR1::LUC* Col-0 plants infected with *P. syringae* DC3000 at CT26 and CT42 are shown with open and closed circles respectively. Error bars are for four biological replicates and signify standard error of mean. A similar result was observed in an independent experiment. The asterisks indicate a significant difference in induction between at CT42 and CT26 ($p<0.001$, 2 Way RM ANOVA).

3.4.3 Measuring transcript levels of *WRKY* transcription factors

WRKY22 and *WRKY29* are downstream of the MAPK pathway and are important in early defence responses to infection [45]. Activation of *WRKY22/29* results in activation of defence genes. Col-0 and *CCA1*-ox plants were infected at CT26 and CT42 with either 10mM $MgCl_2$ or *P. syringae* DC3000, (mock and treated, respectively in Fig 6.3). Expression levels of *WRKY22* (Fig 6.3i) and *WRKY29* (Fig 6.3ii) were measured 4h after inoculation in mock and treated plants.

Expression of *WRKY22* was induced in both genotypes at both time points (Fig 6.3i). The mock treated plants show un-induced levels of *WRKY22* which corresponds to basal levels. These basal levels are similar across both genotypes and time points. While induction of *WRKY22* appears variable, the large error bars suggest this difference is not significant (Fig 6.3i). A Student's t-test confirmed that no significant differences could be observed in induced and basal levels of *WRKY22* across both genotypes at CT26 and CT42 (Fig 6.3i).

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WRKY29 possesses both CCA1 and EE binding motifs in its promoter region. A CCA1 motif1 Binding Site in Chlorophyll A/B Binding protein 1 (CCA1 motif1 BS CAB1) is also reported within the CCA1 motif. The position of the CCA1 motif is from -151 to -144 base pairs upstream of the start site while the CCA1 motif1 BS in CAB1 is from -152 to -143 base pairs from the start site. The evening element is located -314 bases from the start site.

Basal levels of *WRKY29* were similar across genotypes and time points. Though levels of *WRKY29* appear higher in mock-infected *CCA1*-ox at CT26 compared to CT42, the large error associated with this measurement meant no significant differences in variation were observed. Additionally, induced *WRKY29* levels in *CCA1*-ox were similar at both CT26 and CT42. A Student's t-test comparing induced levels of *WRKY29* in *CCA1*-ox showed no significant differences between the two time points (p= 0.2663) (Fig 6.3ii).However, *WRKY29* levels in Col-0 showed higher levels in plants infected at CT26 than at CT42 (Fig 6.3ii). A two-tailed Student's t-test revealed a statistically significant difference (p=0.0105) suggesting significantly higher levels of *WRKY29* are induced at CT26 than at CT42. As basal levels of *WRKY29* in Col-0 are similar, this is potentially a very interesting observation; but these findings are based on one experiment only and therefore must be interpreted with caution.

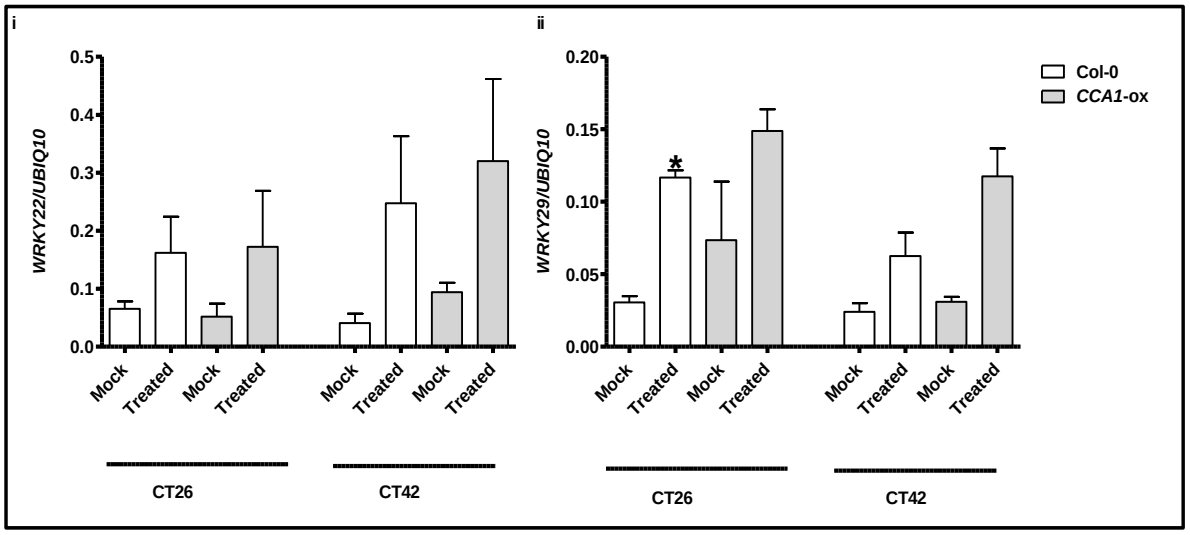

Figure 6.3 Gene expression levels of (i) *WRKY22* and (ii) *WRKY29*. Mock and treated plants were inoculated with 10mM $MgCl_2$ and *P. syringae* DC3000 respectively. Shaded bars represent *CCA1*-ox while un-shaded bars represent Col-0. The plants were infected at CT26 and CT42, tissue harvested 4h later and transcript levels measured by qPCR. Error bars represent standard error of mean for three biological replicates. A 2 tailed Student's t-test comparing induced levels of *WRKY29* show levels are higher at CT26 compared to CT42 ($p<0.05$) as shown by the asterisk. One experiment only.

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3.4.4 *SRFR1* levels at CT26 and CT42

Wang *et al*. (2011) recently showed that plants regulate a number of defence genes to anticipate dawn infection [146]. The authors showed that defence genes are pulse expressed at dawn when likelihood of infection is greatest and that in the event of infection, prolonged expression of these defence genes results in disease resistance and sometimes PCD [146]. Using an avirulent oomycete, *Hpa Emwa1*, they show that plant defence genes time their expression to peak when *Hpa* releases its spores [146]. Furthermore, they showed the circadian clock modulates expression of the defence gene *RPP4*, with its expression peaking at dawn [146].

In a separate study, a *srfr1* null mutant showed overexpression of *RPP4* and enhanced disease resistance [87]. This study revealed *SUPPRESSOR OF RPS4-RLD1* (*SRFR1*) as a negative regulator of NB-LRR proteins, preventing over-accumulation of R proteins thus preventing autoimmunity [87,176,177]. These independent observations led me to predict that *SRFR1* expression could be antagonistic to *RPP4*. *RPP4* has a circadian rhythm with peaks and nadirs at dawn and dusk respectively. I investigated whether *SRFR1* would follow a similar but antagonistic rhythm with peaks at night and nadirs during the day.

Basal levels of *SRFR1* were measured during subjective day and subjective night (CT26 and CT42 respectively) (Fig 6.4). Normalised expression of *SRFR1* shows higher levels of *SRFR1* at CT42 compared to CT26. A two tailed Student's t-test confirmed that these observations are statistically significant ($p=0.007$). However, replicate experiments are required, using a larger sample size to verify observations made in this preliminary experiment.

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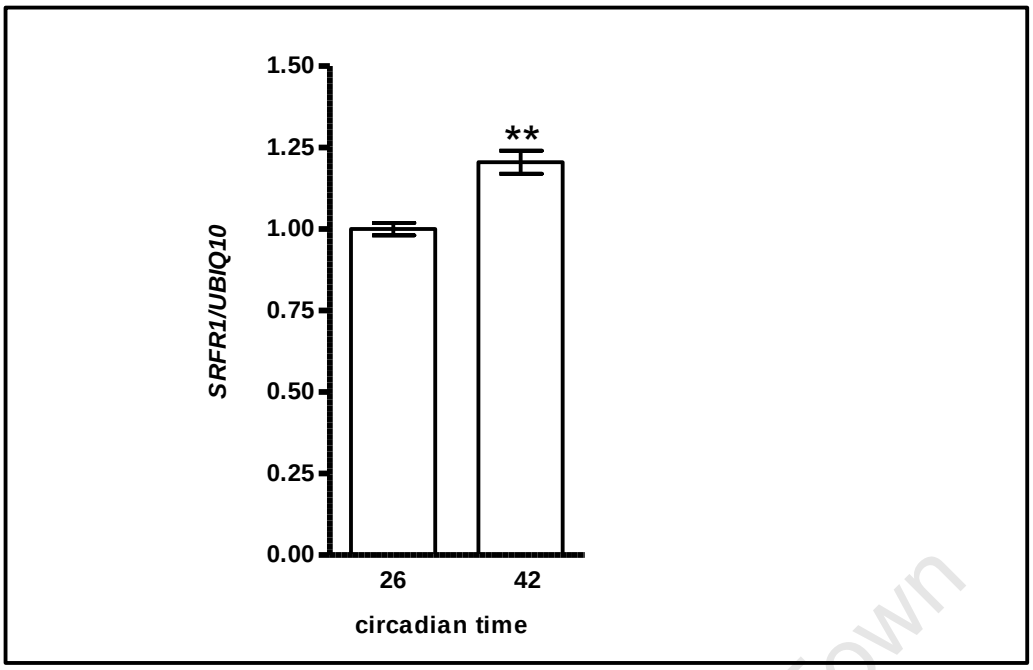

Figure 6.4 Basal levels of *SRFR* in Col-0 at CT26 and CT42. *SRFR1* transcript levels were measured from three biological replicates. Expression of *SRFR1* was normalised using Col-0 CT26 levels. A Two-tailed Student's t-test comparing basal levels of *SRFR1* at CT26 compared to CT42 showed levels of *SRFR1* are higher at CT42 (p=0.007, represented by double asterisks). One experiment only.

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3.4.5 Investigating circadian clock function during infection

AtGRP7 also known as *COLD AND CIRCADIAN REGULATED* (*CCR2*) is an evening-phased gene [178] under the control of the circadian clock [166,179,180,181]. Wang *et al.* (2011) used *CCA1* and *LHY* promoter-luciferase reporter constructs, to measure promoter activity in free-running constant light conditions after infection with *Hpa* Emwa1 [146]. Infection resulted in significant induction and arrhythmic expression of *CCA1* while similar measurements of *LHY* indicated that it was unaffected [146]. As *CCA1* is an important component of the circadian clock, this could lead to disruption of the circadian clock or outputs of the clock because of infection. To investigate this, *CCR2* promoter activity was measured before and during infection. *CCR2::LUC* showed a circadian rhythm, with levels peaking in the evening and this rhythmic expression was unaffected after infection (Fig 6.5).

The results indicate core clock function is not disrupted by infection with *P. syringae* (Fig 6.5) complementing work by Wang *et al*. (2011) showing overall clock function is not disrupted after infection of *Arabidopsis* with *Hpa* under free running conditions [146].

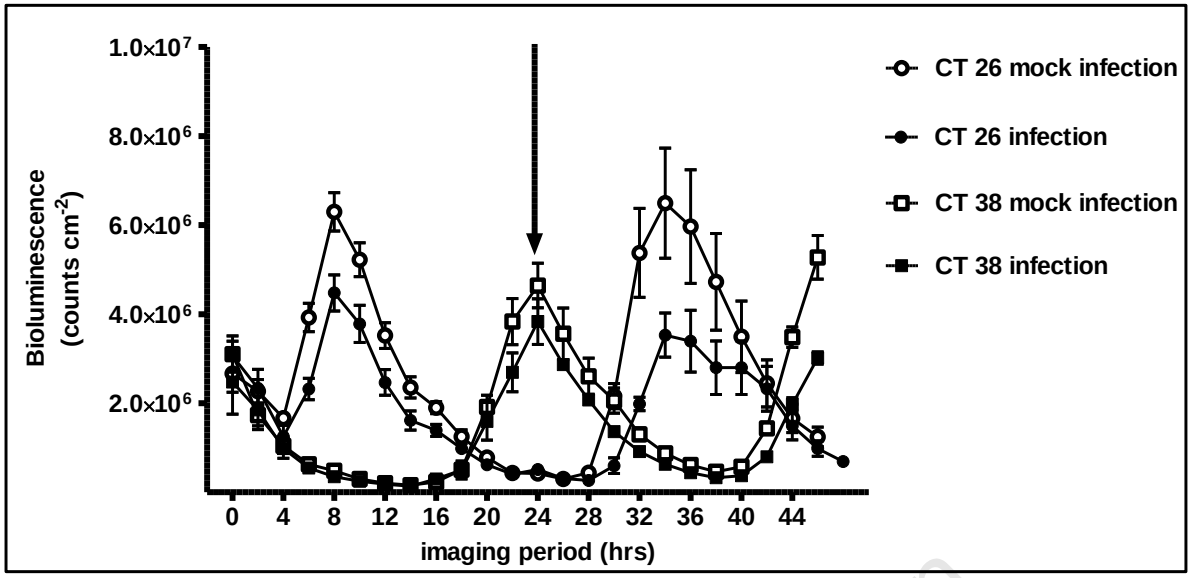

Figure 6.5 Expression profiles of *CCR2::LUC* before and after inoculation with mock (10mM $MgCl_2$) or infected (*P. syringae* DC3000) under free running conditions (LL). Inoculation was at 24h, indicated by arrow. Error bars are standard error of mean for four biological replicates.

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Chapter 4 Discussion

The results reported here indicate that Col-0 plants display time-of-day variation in susceptibility to *P. syringae* DC3000 (Fig 3.1). While previous reports have documented time-of-day variation to plant-pathogens they attributed it to the role of light [182] and the circadian clock [146]. Both these observations were made under light/ dark cycles but to implicate one or the other it was necessary to carry out the infections under free-running conditions [10,157]. The findings in this report indicate that success of infection varies with time-of-day with lowest infection at subjective morning (CT26 and CT50) and greatest infection at subjective night (CT42 and CT66) (Fig 3.1). Success of infection was used as a measure of strength of plant defence response, suggesting that plant defence is better primed for defence at CT26/50 than at CT42/66 (Fig 3.1). Plants activate MTI upon infection by *P. syringae* DC3000 and the reported results suggest that the circadian clock modulates MTI. A stronger MTI response is observed after infection during subjective day than subjective night. While the time-of-day differences in susceptibility in Fig 3.1 are similar to those made under light/dark cycles [146] the data in Fig 3.1 is obtained from infections performed under free-running conditions. The persistence of time-of-day variation in strength of defence responses under free-running conditions firmly implicates control of MTI by the circadian clock.

Infecting the clock mutant *elf3-1* and *CCA1*-ox revealed them to have diminished defence responses and to be equally susceptible to infection in the morning and evening, unlike Col-0 plants (Fig 3.2 and Fig 3.3). This result complements similar findings using a *cca1* null mutant after infection under light-dark cycles with *Hpa* [146]. Contrastingly, *CCA1*-ox was shown to have enhanced defence responses to *Hpa* when infected under light dark cycles [146]. Findings reported here indicate that *CCA1*-ox showed diminished levels of defence with bacterial titres higher than Col-0 at CT26 but comparable to Col-0 at CT42 (Fig 3.2). Therefore, this result contradicted previous work by Wang and colleagues (2011) but there were notable experimental differences between the two studies. They used an oomycete pathogen and performed infections under alternating light-dark cycles while experiments in this report used a bacterial pathogen and were performed under free-running conditions of constant light. One could speculate that under light/dark cycles where there is some degree of driven rhythmicity [183], the over-expression of *CCA1* confers an advantage of heightened disease resistance while under free-running conditions this advantage is lost. Previous work reported that under alternating light dark cycles a number of physiological processes retain rhythmicity in the *CCA1*-ox mutant but are lost upon transfer to free running conditions [183].

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One of the classic MAMP triggered responses (MTI) is the production of callose. *In silico* analysis, revealed *GSL5*/*PMR4* encoding a callose synthase possesses a CCA1 binding motif (found -405 to -398 from +1) in its promoter region. These promoter motifs are small and can occur by chance thus their presence alone is not sufficient to attribute regulation by a certain component. Nonetheless, the presence of these motifs warranted further investigations into whether callose production may be regulated by the circadian clock. Callose levels were measured 14h after infection at CT26 and CT42 under free running conditions in Col-0 and *CCA1*-ox (Fig 5.1 and Fig 5.2). Our results indicate that callose production is seven times greater at CT26 than at CT42 in Col-0. Additionally *CCA1*-ox displayed abrogated levels of callose, comparable to Col-0 at CT42 and showed no significant time-of-day variation (Fig 5.1). These results complemented observations in Fig 3.2 where Col-0 showed time-of-day variation in susceptibility to infection while *CCA1*-ox did not. Collectively these findings indicated that the circadian clock modulates the MTI response and that callose response, a classical marker of MTI varied in strength depending on when the plant was infected. This time-of-day variation of the MTI response and callose production in response to infection by an equal quantity of bacteria at different times suggests that these defence responses may be gated by the circadian clock.

PR1 expression is mediated by the salicylic acid signalling pathway and is part of the systemic response as well as the MTI and ETI responses [84]. Using a luciferase reporter construct, *PR1* promoter activity was measured (Fig 6.2). Paradoxically, *PR1* showed quicker induction after infection at subjective night (CT42) compared to subjective morning (CT26). This result contradicted other results that had indicated a strong defence response during the day. A recent study by Sato and colleagues (2010) provided an intriguing explanation of events. They showed that callose synthase and the salicylic signalling sector mutually inhibited each other [54]. By dividing plant defence genes into sectors based on positive regulatory relationships they identify an early MAMP triggered (EMT) sector of which callose synthase is a member and salicylic acid sector [54]. They showed that these two sectors are mutually antagonistic and were capable of inhibiting each other thus when the EMT sector was fully activated it negatively regulated the salicylic acid sector and *vice versa* [54]. My results indicated a strong EMT response at CT26 and a weak EMT response at CT42 as measured by callose production (Fig 5.1). Based on the observations made by Sato and colleagues, one could speculate that in this study the strong EMT observed at CT26 in Col-0 would mutually inhibit the SA sector thus delaying induction of *PR1*. Furthermore, the weak EMT response observed at CT42 in Col-0 (Fig 5.2) would mean the associated antagonistic effect on the SA sector is weaker at CT42 thus *PR1* accumulated quicker at CT42. These *PR1* data support this hypothesis. Additionally, another early response gene, *WRKY29*, showed greater induction at CT26 than at CT42 (Fig 6.3) lending further support to the proposed idea. Collectively, one could surmise that possibly the time-of-day variation in *PR1* was a downstream effect of varying strength of early defence response genes.

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Transcript levels of WRKY22 and *WRKY29* were also investigated as they are both induced in response to pathogens [45,167,173]. Preliminary data reported here suggested that the circadian clock may gate the strength of *WRKY29* response to bacteria (Fig 6.3ii). A Student's two-tailed t-test showed that *WRKY29* levels were greater at CT26 than at CT42 in infected plants. Mock treated plants showed similar *WRKY29* basal levels at both CT26 and CT42 in Col-0. This indicated that the amplitude of response in Col-0 was greater at CT26 than at CT42. This data, though promising is acknowledged as preliminary data. Furthermore, the biological contribution of such small differences though statistically significant must be verified. Importantly, levels of *WRKY29* in infected *CCA1*-ox were comparable to those of infected Col-0 levels at CT26 (Fig 6.3ii) and showed no time-of-day variation. The absence of time-of-day variation in levels of *WRKY29* in *CCA1*-ox complements other findings in this study (Fig 3.2, Fig 3.3 and Fig 5.1) however; transcript levels are similar to wild type at CT26, which does not explain the higher susceptibility to infection of *CCA1*-ox (Fig 3.2). Thus, while expression of *WRKY29* showed time-of-day variation in Col-0 and not in *CCA1*-ox, expression levels in the arrhythmic mutant were comparable to wild type levels. This weakens attempts to draw significant biological conclusions but warrants further investigations.

In addition, *WRKY22*, which is functionally redundant to *WRKY29*, behaved similarly across time points and genotypes, showing similar basal levels and similar amplitude of induction (Fig 6.3 ii). Collectively then, the *WRKY* dataset suggests that *WRKY* genes investigated here are equally inducible at either night or day in both Col-0 and CCA1-ox and therefore they are unlikely to explain the time-of-day variation observed in previous results. While levels of *WRKY29* in Col-0 plants showed statistically significant differences at different times, this result is based on three biological replicates and should be repeated in independent experiments to verify robustness and reproducibility of this observation. The *WRKY* family of transcription factors positively and negatively regulate defence responses therefore other members of this family could be ideal candidates for further investigation.

Recent results point to plant defences peaking at dawn, showing a 'pulse' in expression of defence genes [146]. One such gene displaying pulse expression at dawn is *RPPR4* [146], a defence gene involved in plant immune responses to infection. *RPP4* is also negatively regulated by *SRFR1* [87]. The observation that *RPP4* displayed pulse expression at dawn and that it is also negatively regulated led to the possibility that its negative regulator may show an antagonistic expression profile. Data reported here indicated that *SRFR1* is lower during the 'subjective' day than 'subjective night' (Fig 6.4). This result suggests that negative regulators of defence may themselves experience down regulation at dawn, thus facilitating induction of defence genes. Suppressors may follow an

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antagonistic circadian rhythm, showing decreased levels at dawn and increased levels at night. While results reported here (Fig 6.4) supported this claim, the difference between levels of *SRFR1* at CT26 and CT42 was less than 25%. Whether such a difference in transcript levels is biologically significant requires further investigation. Additionally this observation was made based on one experiment and therefore must be replicated in larger numbers to ensure that this observation is both robust and replicable. Perhaps the more likely explanation has to account for both down-regulation of suppressor genes and up-regulation of defence genes that results in the pulse expression observed at dawn.

Most of the findings reported here indicate that the circadian clock may modulate MTI. Wang and colleagues showed that the circadian clock also regulated genes encoding R proteins [146]. To investigate whether defence responses showed time-of-day variation with specific R protein and effector protein interaction, Col-0, *CCA1*-ox and *elf3-1* plants were infected at CT26 and CT42. Infections were carried out with *P. syringae* DC3000 avrB and avrRpt2 (Fig 3.3i and ii).

Infections with *P. syringae* DC3000 avrB showed no significant time-of-day variation (Fig 3.3i). Statistical tests indicated that no significant differences could be observed across various infection times and genotypes. While these results indicated that defence responses to *P. syringae* DC3000 avrB showed no time-of-day variation, *P. syringae* DC3000 avrRpt2 showed some differences. Statistical analysis (GLM and Bonferroni post-hoc test) indicated that Col-0 behaved significantly differently from *CCA1*-ox but similar to *elf3-1*. From this data, one could surmise that certain R proteins involved in recognition of avrRpt2 may be affected in *CCA1*-ox plants affecting their ability to recognise and initiate defence. Alternatively, the variation between Col-0 and *CCA1*-ox may hint at a role for CCA1 in plant defence responses, drawing from findings by Wang *et al.* 2011. In contrast, time-of-day and the circadian clock might not affect the recognition mechanisms involved in activation of defence responses to avrB. These observations are mere speculations as the data available are based only on one experiment and further work is necessary to draw significant conclusions. As, Wang *et al.* 2011 reported a number of R defence genes to be under control of CCA1 further experiments should target those R protein interactions using cognate avirulent strains.

An early event upon MAMP perception is production of active oxygen species such as H_2O_2. The production of H_2O_2 results in the induction of *OXI1* which encodes a serine/threonine kinase [43,44]. OXI1 kinase is activated by H_2O_2 and is essential for full activation of the mitogen-activated protein kinases MPK3 and MPK6 [44]. *In silico* analysis revealed that *OXI1* has a CCA1 binding motif in its

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promoter region, which suggested possibility for circadian regulation in the absence of pathogens. Promoter activity of *OXI1* showed no variation in expression profiles between infections at CT26 and CT42 (Fig 6.1) however *OXI1* promoter activity was measured 24h after infection (Fig 6.1). As *OXI1* induction occurs after an AOS burst, which occurs within 30min of perception of MAMPs, measuring early *OXI1* expression may give a better indication as to whether induction varies with time-of-day.

Lastly, this report showed that *CCR2* expression is unaffected after infection with *P. syringae* DC3000 (Fig 6.5). *CCR2* is an evening phased gene regulated by CCA1 and LHY [181] and has been implicated in regulation of stomata [155] and plant defence [156]. Global transcript profiling of an over-expresser of *CCR2* showed enrichment of abiotic and biotic stress response genes including *PR1* [136]. Notably a point mutation in the RNA binding domain of CCR2 diminished any such enrichments showing that the regulation involves the RNA-binding activity of CCR2 [136]. The role of CCR2 in defence is underscored by the observation that it is a target of the bacterial effector HopU1 [156]. HopU1 enhances virulence by ribosylating the RNA binding domain of CCR2 interfering with its ability to bind RNA [156]. The observation that *CCR2*-ox showed enrichment of *PR1* and that *CCR2* expression peaked in the evening (Fig 6.5) may offer an alternate explanation as to why *PR1* showed quicker induction after subjective night infections (Fig 6.2).

Wang *et al*. (2011) demonstrated that *CCA1* expression was rapidly induced and became arrhythmic after infection with *Hpa* [146]. They measured both *CCA1* and *LHY* expression and unlike *CCA1*, *LHY* retained rhythmic expression and was unaffected by pathogen attack [146]. Moreover, *cca1* null mutant plants showed attenuated defences while *lhy* mutants did not [146]. Collectively these results suggest that CCA1 plays a role in a defence output pathway regulated by the clock rather than via the central clock mechanism [184]. As LHY and CCA1 are functionally redundant, Wang and colleagues predicted that overall clock function was unaffected even though *CCA1* becomes arrhythmic [146]. The findings reported here show that *CCR2* retains rhythmic expression after infection with *P. syringae* DC3000 under free-running conditions. As *CCR2* expression is under the control of CCA1 and LHY this would suggest two possible explanations. The first is that functional redundancy of *CCA1* and *LHY* prevents the *CCR2* pathway from losing rhythmic expression after infection. Secondly, induction of *CCA1* may be unique to *Hpa* Emwa1 infection only. Therefore, *CCA1* levels should be measured in response to infection by *P. syringae* DC3000.

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The results presented here as well as those reported by other researchers [146,150,151] demonstrated that the circadian clock modulates plant defence responses to *P. syringae* DC3000 with peak defence occurring in the morning and greatest susceptibility to infection at night. The plant innate immune system and the plant circadian clock are complex aspects of plant physiology and independently are the subject of constant investigation. Recent findings including data reported here show an interface of cross talk exists between the plant circadian clock and its defence responses. Why this is so is a crucial question. Wang *et al.* (2011) offer a possible answer. The circadian clock in plants enables them to anticipate regular diurnal events, priming physiological and biochemical processes to occur at the most advantageous time. Wang and colleagues (2011) showed that defence responses were 'pulse' expressed at dawn. Using *Hpa* because its infection stages were clearly defined [185] they showed that 'pulse' expression at dawn coincided to when *Hpa* released its spores. They concluded that the lifecycles for biotrophic pathogens are intimately linked with their host metabolism, which is controlled by the circadian clock.

P. syringae DC3000 is also a biotrophic pathogen and showed time-of-day variation in its ability to infect *Arabidopsis* plants (Fig 3.1). While numerous researchers have documented various aspects of *P. syringae* DC3000, a clear understanding of the stages of infection has not been completely developed. A possible model of infection could be that plants open and close their stomata with a circadian rhythm, opening stomata at dawn and closing them at night. Stomata are important for gaseous exchange, transpiration and photosynthesis and are a natural portal of entry that bacteria use. This would suggest the ideal time for bacteria to infect plants would be when stomata are fully open, at dawn, and conversely plants anticipate this and so up regulate defence genes to minimise infection. Results reported in Fig 3.1 were after pressure infiltration, thereby bypassing plant stomata altogether and perturbing defence responses at both subjective day and subjective night. A stronger defence response was observed by lower counts at subjective day and a weaker defence response at subjective night (Fig 3.1). This result hints at the possibility that at night when stomata are shut, the anticipated risk of pathogen entry is less and so defence responses are not primed for a response. By pressure infiltrating the bacteria into the plant leaf, the stomata barrier was by-passed and greater infection resulted.

This simple model ignores two important facts though: the ability of stomata to close upon perception of bacteria [154] and that some bacteria synthesise and secrete coronatine enabling them to re-open closed stomata [154]. A close look at the lifecycle of *P. syringae* proved insightful in understanding how it infects plants.

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Studying the life cycle of *P. syringae* indicates that it perhaps infects with greatest success during the early morning period. *P. syringae* occurs in the phyllosphere of plants and microscopy work shows that it is found in aggregates of 100 cells or more on leaf surfaces [186]. Leaf surfaces are nutrient poor areas, akin to a desert with oases of nutrients [187,188,189] and bacteria aggregates have been shown to cluster at the nutrient rich sites. Swarming is a key mechanism used by individual cells to rapidly migrate across the leaf surface in search of these nutrient rich oases [190]. Interestingly, when aggregates form at nutrient rich sites one of the early effects is repression of motility by quorum sensing [186,190,191]. It is advantageous for the small colony to stay at the nutrient rich 'oasis' rather than move away from it into the desert surroundings [186]. The plant leaf surface is a hostile area and in addition to scarce nutrients, strong sunlight and UV irradiation occurs along with exposure to free radicals such as H_2O_2 an effect of photorespiration [148,188,189]. One of the mechanisms bacteria have evolved to deal with this is stress avoidance [189]. UV and sunlight irradiation causes DNA damage possibly favouring bacterial replication at night [192]. Bacterial aggregates at nutrient rich sites replicate to populations reaching 10^8 cells/g of leaf [186]. It has been reported that larger bacterial colonies show greater motility than smaller colonies [191] suggesting as colony size increases a second quorum is reached activating virulence genes such as coronatine production and restoring motility [191].

Thus, if aggregates of 100 cells or more cluster at nutrient rich sites replicating mainly at night, the cell population will be at late log phase, approaching stationary phase at dawn, when virulence genes are switched on by quorum sensing leading to restored motility and production of phytotoxins such as coronatine [191,193]. At dawn plant stomata open under circadian regulation [153] coinciding with peak bacterial population and greatest risk of pathogen entry. Quite possibly, in addition to anticipating *Hpa* spores, plants also anticipate bacterial invasion, thus expressing a pulse in defence gene expression at dawn. These observations could help support the model proposed here, however it will be necessary to carry out elaborate field experiments attempting to mimic natural infection. Identifying mutations in plants with constitutively open stomata would give an indication of whether there is a direct link between up regulated defence and open stomata. The molecular underpinnings of this model could be discovered by measuring transcript changes of defence related genes in response to infection at subjective night and day using microarrays.

One of the early responses to pathogens is the alkalinisation of the apoplast and an influx of Ca^{2+} [36]. This results in activation of calmodulin [194] and a number of other genes in the Ca^{2+} mediated defence pathway. Bacteria actively repress this pathway using exopolysaccharides that actively sequester any free Ca^{2+} [40]. Interestingly cellular levels of Ca^{2+} display robust circadian rhythms

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with peaks and nadirs during the day and night respectively [195]. As infections were performed at subjective day (peak Ca^{2+}) and subjective night (low Ca^{2+}) one could speculate that bacteria are unable to suppress the Ca^{2+} mediated defence pathway during the day but could possible suppress Ca^{2+} when infiltrated at night as levels could be low enough allowing bacteria to sequester any Ca^{2+}. To investigate this, Ca^{2+} levels could be measured *in vivo* using the bioluminescent Ca^{2+}-reporter aqueorin, during infection at both subjective dawn and dusk under free-running conditions.

This study showed promoter activity of *CCR2* is unaffected by infection, with rhythmic expression persisting after infection under free-running conditions (Fig 6.5). However, previous reports show that the HopU1, a bacterial effector, actively targets CCR2 protein [156] resulting in attenuated defence responses. This result suggests further research should also investigate the effects of infection on protein levels of clock output pathways. As the tools for genomic and proteomic studies become accessible and affordable, a clearer picture will emerge illustrating the role of the plant circadian clock in defence responses.

Concluding remarks

There is a growing body of work illustrating that the two complex and vital networks of circadian clock regulation and defence signalling are interconnected. This report highlights the complexity and nuances presented by the plant innate immune system and offers new evidence linking clock control and defence signalling. Plants display a time-of-day variation in susceptibility to the biotrophic pathogen *P. syringae* reported here and *Hpa Emwa1* [146]. This time-of-day variation is lost in arrhythmic *CCA1*-ox and *elf3-1* indicating that the circadian clock drives time-of-day variation. From data reported here, one can surmise that early defence responses perhaps play a key role in defence signalling and maybe gated by the circadian clock, giving a strong response during the day when perceived threat of infection is greater. Callose production, a classic marker of early defence showed time-of-day variation supporting this claim. Mounting evidence, some of which was reported here, shows that plants seek to find a balance between a full immune response and one that is sufficient to clear infection at minimal cost to the plant. In addition to the elegant mechanisms that were discussed earlier, data presented here suggest that circadian clock control may also play a crucial part in striking a balance in defence responses thus avoiding wastefulness. In conclusion, this thesis contributes significantly to studies understanding the link between the plant circadian clock and defence responses.

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