The Plant Cell, Vol. 21: 2546–2552, September 2009, www.plantcell.org

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Lights, Rhythms, Infection: The Role of Light and the Circadian Clock in Determining the Outcome of Plant–Pathogen Interactions [C]

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The importance of light with respect to the outcome of plant–pathogen interactions is becoming increasingly evident: light affects both the host response and the virulence of some pathogens. The response of plants to environmental signals and stresses is modulated by the circadian clock, and it is apparent that this may include immune responses. Photo and temporal regulation of immune responses may allow plants to anticipate and react more effectively to particular pathogen infections. These aspects of regulation are sometimes overlooked when designing experiments to understand plant–pathogen interactions, complicating the interpretation of the outcomes and the direct comparisons of studies. We review recent key findings in these areas and discuss the implications for experimental design and analyses.

INTRODUCTION

Successful disease development requires that pathogen, susceptible host, and favorable environmental conditions come together at the same time. However, the vulnerability of the susceptible host and the virulence of the pathogen may vary both with developmental stage and time of day, thus affecting the outcome of the interaction. The importance of light with respect to the outcome of plant–pathogen interactions is becoming increasingly apparent; recent reports have shown direct effects of light on the both the defense response in the host and on the virulence of the attacking pathogen (Chandra-Shekara et al., 2006; Griebel and Zeier, 2008; Oberpichler et al., 2008). In addition, there is growing realization that circadian rhythms may play an important role in disease outcomes. Insights into the circadian regulation of mammalian physiology, immunology, and the cell cycle have influenced the way that human disease is managed (Lévi et al., 2007). A new branch of medicine called chronotherapeutics has been developed, based on the administration of various agents at the optimal time for effectiveness or avoidance of unwanted side effects (Smolensky and Peppas, 2007; Baraldo, 2008). Circadian modulation of resistance to *Pseudomonas aeruginosa* and *Staphylococcus aureus* in *Drosophila melanogaster* has been reported, with clock mutants found to display altered survival rates following infection (Shirasu-Hiza et al., 2007; Lee and Edery, 2008). Here, we review reports of experiments involving plant–pathogen interactions in light or dark or at different times of the day, which suggest that plant biologists should consider temporal and light regulation of physiology in understanding plant disease. Sites of potential crosstalk between the clock, light, and immune response pathways are illustrated in Figure 1.

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[C] Some figures in this article are displayed in color online but in black and white in the print edition.

www.plantcell.org/cgi/doi/10.1105/tpc.109.069922

The Role of Light in the Host Defense Response

Light has long been known to be required for a full defense response in plants, as attenuated responses to a range of viral, bacterial, and fungal pathogens have often been observed in the dark (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Chandra-Shekara et al., 2006). While some plant defense responses occur independently of light, such as camalexin biosynthesis and jasmonic acid production (Zeier et al., 2004), light has been shown to play a particularly important role in salicylic acid (SA)–mediated defense responses. SA is a key signaling molecule involved in all three levels of plant innate immunity (Loake and Grant, 2007), namely, pathogen-associated molecular pattern–triggered immunity (mediated by pattern recognition receptors at the plasma membrane), effector-triggered immunity (ETI; activated following the recognition of pathogen effector molecules by plant resistance proteins, commonly leucine-rich repeat–containing intracellular receptors), and systemic acquired resistance (SAR; whereby a localized primary infection results in increased resistance in systemic tissue against secondary pathogen attack). See excellent reviews by Jones and Dangl (2006) and Chisholm et al. (2006) for an overview of plant innate immunity.

The accumulation of both free SA and the glucoside-bound form (SAG) in *Arabidopsis thaliana* following challenge with either *Pseudomonas syringae* pv *tomato* DC3000 *avrRpt2* (*Pst* DC3000 *avrRpt2*) or *P. syringae* pv *maculicola* ES4326 *avrRpm1* (*Psm*

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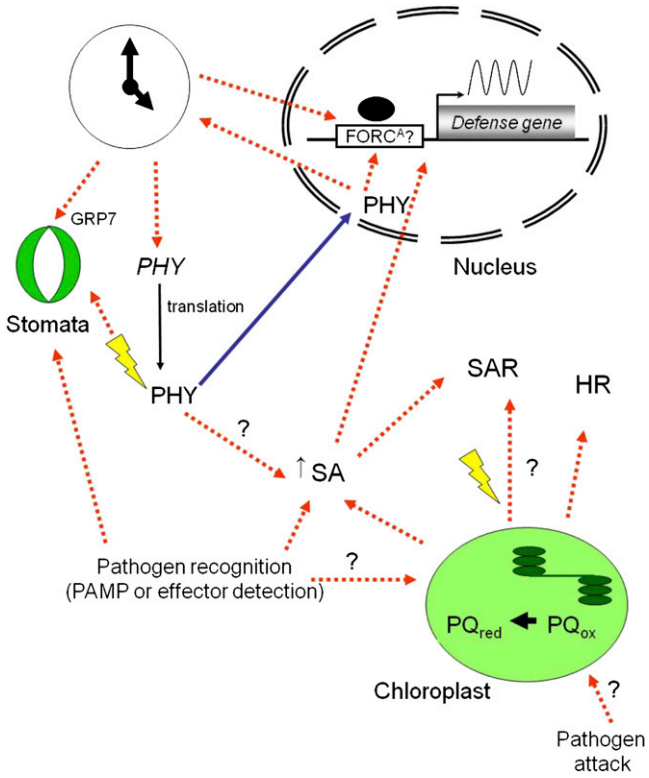

Figure 1. Interplay between Light, Defense, and Circadian Signaling.

Stomatal opening is regulated by light and the circadian clock but is also modulated following pathogen detection. Entrainment of the circadian clock by light is mediated by phytochromes (PHY) and cryptochromes that translocate to the nucleus in response to light where they regulate gene expression. Expression of several defense genes has been shown to be modulated by both the circadian clock and PHY activity. Additionally, regulatory elements in promoters have been recently identified that are responsive to both light and pathogen-derived signals (Evrard et al., 2009). Pathogen detection leads to an increase in SA and downstream changes in defense gene expression. Both the synthesis and downstream perception of SA require light and may be modulated by PHY activity (Genoud et al., 2002). Systemic acquired resistance (SAR) is associated with an increase in SA content but can occur independently of SA under high light conditions (Zeier et al., 2004), possibly via a chloroplast-derived signal. In the plastid, reduction of the PQ pool is a consequence of excess light but might also occur due to downregulation of photosynthesis following pathogen attack or detection, resulting in the activation of SA-dependent signaling pathways. Both functional chloroplasts and light are required for the HR in incompatible interactions with avirulent pathogens. Signaling events are indicated by dashed lines (red) and translocations by solid lines (blue); light is indicated by a jagged arrow, and the question mark indicates potential signaling pathways. For the sake of clarity, many known signaling components, such as reactive oxygen species, have been omitted. [See online article for color version of this figure.]

ES4326 *avrRpm1*) is light dependent (Genoud et al., 2002; Zeier et al., 2004; Griebel and Zeier, 2008). However, following infection of the *Arabidopsis* ecotype Dijon-17 with turnip crinkle virus, accumulation of free SA (but not SAG) was observed in the dark (Chandra-Shekara et al., 2006). Thus, this response may be to some extent pathogen specific. In all plant–pathogen interactions, however, light apparently is required for the activation of downstream SA-mediated defense responses. In particular, light is required for the hypersensitive response (HR), a form of localized programmed cell death at the site of infection, activated during ETI. Plants grown in the dark show greatly reduced lesion formation in response to incompatible bacterial and viral pathogens (Mateo et al., 2004; Zeier et al., 2004; Chandra-Shekara et al., 2006; Griebel and Zeier, 2008). SAR has also been shown to be light dependent. Plants inoculated with the avirulent strain *Psm* E4326 *avrRpm1* display greatly increased resistance to subsequent infection with virulent strain *Psm* E4326. However, when the primary infection was performed in the dark, the establishment of SAR was totally abolished in *Arabidopsis* (Zeier et al., 2004), which may be a consequence of a reduced immune response to the primary infection.

The molecular mechanisms linking light perception and the plant immune response have been a focus of recent research. Initially, a link between phytochrome-mediated signaling and the defense response was proposed, as *phyA phyB* double mutants were reported to display a reduced HR and increased susceptibility to the avirulent strain *Pst* DC3000 *avrRpt2* (Genoud et al., 2002). However, subsequent studies failed to find an analogous role for phytochromes in ETI against turnip crinkle virus or avirulent *Psm* E4326 *avrRpm1* (Chandra-Shekara et al., 2006; Griebel and Zeier, 2008), although *phyA phyB* double mutants were compromised in the establishment of SAR (Griebel and Zeier, 2008). The other known plant photoreceptors, cryptochromes and phototrophins, do not appear to play any role in plant immunity (Griebel and Zeier, 2008). However, the observation by Genoud et al. (2002) that functional chloroplasts are required for the HR suggested the existence an alternative signaling pathway linking light and plant immunity related to the redox status of the chloroplast.

While the majority of studies on plant signaling pathways are performed in controlled environments where single stimuli are manipulated, in the natural environment, plants face multiple environmental challenges at the same time. An increase in light intensity leads to excess excitation energy (EEE), that is, energy in excess of that required for photosynthetic activity (Bechtold et al., 2005). Similarly, any environmental stress that impacts the rate of photosynthesis, such as drought or pathogen infection, can also result in EEE. Plant responses to EEE have a number of striking parallels with the plant immune response, including the production of reactive oxygen species, SA-mediated signaling regulated through EDS1 and PAD4, the formation of lesions via programmed cell death, and the expression of a number of common genes, such as *GST6* and *PR2* (Bechtold et al., 2005; Mateo et al., 2006; Mühlenbock et al., 2008). Importantly, plants acclimated to high light displayed increased resistance against

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virulent *P. syringae* DC3000 both in tissues exposed directly to excess light and in systemic leaves, analogous to the establishment of SAR (Bechtold et al., 2005; Mühlenbock et al., 2008).

A key component of acclimation to EEE appears to be the redox status of the plastoquinone (PQ) pool. Treatments that result in increased reduction of the PQ pool, such as light enriched at λ 680 nm or the electron transport inhibitor 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinine, lead to increased programmed cell death, H_2O_2 production, and accumulation of ethylene in *Arabidopsis* (Mühlenbock et al., 2008). These treatments also resulted in increased resistance to virulent *Pst* DC300 infection, mimicking the effect of EEE from exposure of plants to high light. By contrast, plants treated with DCMU (which results in oxidation of the PQ pool) prior to exposure to high light displayed the same susceptibility to *Pst* DC3000 as control plants grown under low-light conditions. These results indicate that the redox poise of the PQ pool appears to play a central role in plant responses to both light and pathogen attack. At the level of gene expression, comparatively little is known about the integration of light and pathogen-induced signaling. However, a hexameric motif known as FORC^A has recently been identified in the promoters of genes coexpressed in response to light treatments and fungal pathogens that may play a role in this process (Evrard et al., 2009). FORC^A is a light-responsive element whose activity is modulated by the length of the light period; transgenic plants grown under constant light display higher FORC^A-mediated reporter gene expression than those grown under a 14-h-light/10-h-dark photoperiod or in constant darkness. The activity of this light-responsive element is also modulated by exposure to pathogens. Infection with a virulent *Hyaloperonospora parasitica* isolate Noco2 (or treatment with exogenous SA) led to repression of FORC^A-mediated expression under a 14-h-light/10- h-dark photoperiod or in constant darkness, whereas increased reporter gene activity was observed under constant light (Evrard et al., 2009). Thus the FORC^A motif may serve as a point of integration between light and pathogen signaling pathways.

Light as a Determinant of Virulence in Pathogens

In addition to direct effects on the plant immune response, it is becoming apparent that light can also act as a determinant of virulence in plant pathogens. While it has long been known that motility affects the virulence of plant pathogens, a recent study by Oberpichler et al. (2008) has provided evidence linking light perception and virulence via the control of cell motility. Analysis of the *Agrobacterium tumefaciens* C58 proteome revealed that the flaA and flaB flagellin subunits are significantly upregulated in dark-grown bacteria. Bacteria cultured in the light had a reduced number of flagella (one or two compared with three to five in the dark) and exhibited reduced mobility in colony assays. Importantly, virulence was also directly affected by light; reduced root attachment was observed in tomato (*Solanum lycopersicum*) and smaller tumor formation in cucumber (*Cucumis sativus*) in the presence of light compared with darkness (Oberpichler et al., 2008). Two putative phytochromes and one cryptochrome have been identified in the *A. tumefaciens* genome (Goodner et al., 2001), but knockout mutants showed normal light-dependent regulation of Fla protein levels (Oberpichler et al., 2008), suggesting that an as yet unidentified photoreceptor may be involved in this process.

In plants, flavin binding LOV (light, oxygen, or voltage) domains have long been known to act as light sensory modules, best characterized in the blue light–sensing phototrophin receptors (Huala et al., 1997). Swartz et al. (2007) recently demonstrated that four prokaryotes, *Brucella melitensis*, *Brucella abortus*, *Erythrobacter litoralis*, and *P. syringae*, possess histidine kinases containing LOV domains that bind a flavin mononucleotide chromophore. Light-induced absorption changes in affinity-purified proteins were indicative of cysteinyl-flavin adduct chemistry in response to blue light (analogous to that observed in the plant phototropins), and increased kinase activity of the LOV-HK proteins was observed in response to light. In the mammalian pathogen *B. abortus*, light perception was found to increase virulence, with increased infection of macrophages observed in response to light. A functional role of the LOV-HK in this process was demonstrated as a knockout mutant did not display this response (Swartz et al., 2007). Whether the *P. syringae* LOV-HK plays an analogous role in virulence remains to be tested.

Developmental Stage of the Host

Seasonal variation in incidence and severity of plant and animal infections and disease is well known. Based on epidemiological studies, vaccination programs are performed on seasonal bases to reduce the incidence of human influenza and other diseases. The incidence and spatial distribution of canker disease in oak trees has been attributed to the need for synchronicity of pathogen activity and seasonal host development (Dodd et al., 2008). The peak of sporulation by *Phytophtora ramorum* and *Fusarium circinatum*, the casual agents of sudden oak death canker disease and pine pitch canker, respectively, is when the climate is cool and humid (Schweigkofler et al., 2004; Dodd et al., 2008). Dodd et al. (2008) demonstrated that the development of sudden oak death canker disease required coincidence of pathogen sporulation and activity of the host cambial tissue, and they found a strong correlation between date of largest lesion size and timing of spring bud burst. This implies that not only do particular environmental conditions favor pathogen activity and infection but that there are specific times in development when the host is more susceptible to successful infection and disease development than others.

The host developmental stage, specifically vegetative growth versus the transition to flowering, has been implicated in differential disease symptom development in *Arabidopsis* infected with cauliflower mosaic virus (CaMV) (Cecchini et al., 2002). These authors had previously noted that *Arabidopsis* plants infected with CaMV under short days displayed much more severe symptoms than under long days (Cecchini et al., 1998)

and therefore investigated the link between the transition to flowering, which is responsive to photoperiod and disease response. *Arabidopsis* is a facultative long-day plant, which means that long-day photoperiods accelerate flowering. They tested plants with mutations at the *FCA* and *GIGANTEA* (*GI*) loci, which delay flowering under long days (Koornneef et al., 1998; Fowler et al., 1999; Park et al., 1999). FCA is a nuclear RNA binding protein involved in the autonomous flowering pathway (Macknight et al. 1997), and in RNA-mediated chromatin silencing at a number of loci (Bäurle et al., 2007). GI, a nuclear protein with no functional similarity to other proteins, plays a role in the plant circadian clock, probably in entrainment to environmental signals and in photoperiodic flowering (Fowler et al., 1999; Locke et al., 2005; Gould et al., 2006). Wild-type plants that were infected with CaMV under long days accumulated higher viral loads than those under short days, yet symptoms were more severe under short days than long days (Cecchini et al., 2002). The *fca-1* mutant displayed severe symptoms under both long and short days, even though viral load was estimated to be higher in longer days than short, as in the wild type (Cecchini et al., 2002). This result indicated that the developmental stage of the plant affected the outcome of the viral interaction rather than solely the duration of the light or dark phase. However, the authors tested a suite of *gi* alleles, all of which were late flowering in long days; all accumulated virus to similar levels but displayed differences in their symptom severity loosely related to the level of *gi* expression in each. Whereas *gi-4*, which has a single point mutation and high levels of *gi* expression (Fowler et al., 1999), displayed mild symptoms under long days and severe symptoms under long days, *gi-11*, a T-DNA insertion allele in which *gi* expression is not detectable (Fowler et al., 1999), displayed only mild symptoms under both long and short days (Cecchini et al., 2002). This decoupling of symptom development and virus accumulation seems to implicate GI in symptom response and may be unrelated to its role in photoperiodic flowering, but perhaps more to its role in the entrainment and function of the circadian clock (Park et al., 1999; Mizoguchi et al., 2005; Gould et al., 2006). It also highlights the importance of distinguishing between successful infection (i.e., pathogen numbers or load) and disease symptom development when analyzing the outcome of plant–pathogen interactions.

Circadian Variation in Immunity

Circadian variation in immune function and disease susceptibility has been noted in animals and best characterized experimentally in fruit flies (*D. melanogaster*). In microarray studies, genes involved in innate immunity, such as those involved in recognition and phagocytosis, antimicrobial peptides, chitinase-like molecules, etc., were found to peak in expression at particular phases of the day and were circadian regulated in constant darkness (McDonald and Rosbash, 2001; Ueda et al., 2002). Many of the genes identified in the study by McDonald and Rosbash (2001) were indirectly regulated by the central oscillator component, CLOCK.

Further investigations of the significance of the circadian regulation of innate immunity in *Drosophila* were published recently. Shirasu-Hiza et al. (2007) infected wild-type and circadian clock–defective per^{01} and tim^{01} mutant flies with two Gram-positive pathogens, *Streptococcus pneumoniae* and *Listeria monocytogenes*. The tim^{01} and per^{01} mutants were very sensitive to infection and died significantly faster than wild-type flies. The significance of the *Tim* locus in the immune response was confirmed by a reduced death rate when the tim^{01} mutant was partially rescued with one copy of wild-type *Tim* (Shirasu-Hiza et al., 2007). Lee and Edery (2008) tested whether the endogenous circadian system modulates the susceptibility to infection infected flies at different times of day in both diurnal light dark cycles and in constant darkness. These authors infected wild-type (*yw*) and circadian clock–defective (Clk^{Jrk}, cyc^{01}, per^{01} and tim^{01}) flies with *P. aeruginosa* PA-14-isogenic strain (Gram negative) and *S. aureus* (Gram positive) bacteria at different times of the day and monitored survival rates. They observed that the wild-type flies showed almost identical diurnal and circadian rhythms in survival rates over a broad range of bacterial doses, with peaks of survival in the middle of the night/subjective night. The circadian clock–defective mutant flies showed no rhythms in survival rates, with all except per^{01} mutants having higher survival rates than the wild type (Lee and Edery, 2008). When the authors tried to correlate their observed survival rhythms with induction kinetics of immune-related gene expression at the times of peak and trough of survival rates, only PGRP-SA (peptidoglycan recognition protein-SA; a microbial receptor or scavenger) and drc (drosocin; an antimicrobial peptide) showed any differences in profile as a function of time of day (Lee and Edery, 2008).

Is There Circadian Regulation of Immunity in Plants?

Experiments have demonstrated that having a functional circadian oscillator with the same period as the Earth's rotation gives plants an adaptive advantage and increases fitness (Dodd et al., 2005). Does this anticipation by the plant of regular abiotic environmental changes extend to anticipation of changes in biotic challenges? Certainly pathogen-inducible genes have been identified that have diurnal and/or circadian rhythms of expression (Molina et al., 1997; Wang et al., 2001; Sauerbrunn and Schlaich, 2004; Weyman et al., 2006). The functional significance of this is not yet clear, as it was also recently demonstrated that under defined environmental conditions, 89% of *Arabidopsis* transcripts are expressed rhythmically, being regulated by the circadian clock or directly by environmental changes in light or temperature (Michael et al., 2008). From their microarray studies of *Drosophila* gene expression, both McDonald and Rosbash (2001) and Ueda et al. (2002) noted that genes that were coregulated were found in clusters on chromosomes and considered economy in gene transcription as a possible factor in coordinated expression patterns. Is there an adaptive, functional reason for rhythmic transcription of these genes involved in

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defense, or is it just a result of general regulation of the genome or proximity to regulatory elements?

So far, the expression of these rhythmically expressed pathogen/defense-related genes has also been found to be inducible by pathogens, signaling molecules, and abiotic stresses (Molina et al., 1997; Wang et al., 2001; Sauerbrunn and Schlaich, 2004; Weyman et al., 2006), but the effect of infections at different times of day on the induction of gene expression or the pattern of expression in circadian clock–defective mutants has not yet been investigated. The *DEA1* gene of tomato, which is inducible upon *Phythophthora infestans* infection, was found to be rhythmically expressed under long days but constitutively expressed under short days (Weyman et al., 2006). As pathogens display seasonality in their infections, it seems reasonable to ask if this observation is significant in terms of plant defenses.

Glycine-rich RNA binding protein 7 (GRP7) is modulated by the circadian clock and in response to various environmental stresses, including cold (Heintzen et al., 1997; Kim et al., 2008). Interestingly, this protein is highly expressed in guard cells, regulates stomatal opening and closing in response to stress (Kim et al., 2008), and was recently shown to be an ADP-ribosylation target for a type III *Pst* DC3000 effector, *hopU1* (Fu et al., 2007). *Arabidopsis* plants deficient in GRP7 due to insertional mutagenesis were more susceptible to *Pst* DC3000 infection that wild-type plants (Fu et al., 2007). The RNA binding on GRP7 is thought to be impaired due to *hopU1* ADP-ribosylation of Arg residues in the RRM domain. GRP7 is thought to play a role in mRNA export from the nucleus under stress conditions (Kim et al., 2008), and ADP-ribosylation of GRP7 may affect the expression of plant defense transcripts (Fu et al., 2007).

The stomata are thought to act as a barrier to bacterial invasion, closing on detection of pathogen-associated molecular patterns and thus playing a role in innate immunity (Melotto et al., 2006). In order to overcome this plant defense, some pathogens that gain entry via stomata have developed mechanisms to reverse the closure. Some *P. syringae* strains secrete the phytotoxin coronatine (Mino et al., 1987; Melotto et al., 2006), while *Xanthomonas campestris* pv *campestris* uses an as yet uncharacterized DSF cell–cell signal-regulated virulence factor (Gudesblat et al., 2009) to cause stomatal opening. As well as being responsive to light, abiotic and biotic stresses, and certain chemical messengers, the stomata of C3 and C4 plants open prior to dawn and begin closing prior to dusk with a circadian rhythm. Each guard cell is thought to have its own circadian oscillator, as rhythms persist even in mature cells that are symplastically isolated, as well as within detached epidermal cells. There is also evidence for circadian gating of stomatal responses to light and dark as well as to chemical messengers indole acetic acid, abscisic acid, and the fugal toxin fusicoccin. The circadian gating makes the guard cells more or less responsive to these signals at particular times of the day (Hotta et al., 2007). The circadian sensitivity of stomatal responses to coronatine has not been investigated but also is likely to vary over the circadian day. During the night, the entry of pathogens is lower due to stomata being closed and probably less sensitive to the effects of coronatine. The bacterial counts of *P. syringae* pv *syringae* (which does not synthesize coronatine) on bean leaves in the field were highest during the day, peaking around midday (Hirano and Upper, 1991). This correlates with the time of maximal stomatal opening in C3 and C4 plants (Hotta et al., 2007) and may partially explain why plant defenses are apparently higher during the light period than at night (Griebel and Zeier, 2008).

Although Griebel and Zeier (2008) attributed the increased plant defenses during the day solely to the presence of light and not due to any circadian rhythm, their experiments used pressure inoculation of *P. syringae* into the leaves, thus bypassing stomatal defense responses. Given the evidence of circadian-regulated pathogen/defense-related genes and stomatal responses, it may be relevant to compare the results obtained in similar experiments when the pathogen is sprayed onto the plants rather than infiltrated into leaves to rule out the role of the circadian clock in modulating defense responses. It is also not obvious why infection of *Arabidopsis* with *Pst* DC3000 *avrRpt2* at dawn caused higher levels of SA to accumulate than inoculation in the middle of the light period (Griebel and Zeier, 2008) as both infections were performed in light of the same intensity and followed by extensive light periods: 9 and 5 h, respectively. When inoculations were performed at two different times under constant light, it appeared that the duration of light preceding the infection also affected SA accumulation (Griebel and Zeier, 2008). Infection of circadian clock or light signaling mutants at different times in constant conditions may provide insights into the mechanism underlying these different responses. Consideration of time of infection and light conditions during studies will allow comparison of results obtained in different laboratories and lead to better understanding of plant resistance toward pathogens, especially when extrapolated to natural or field conditions.

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