

Pseudomonas syringae pv. tomato exploits light signals to optimize virulence and colonization of leaves

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Summary

Light is pervasive in the leaf environment, creating opportunities for both plants and pathogens to cue into light as a signal to regulate plant-microbe interactions. Light enhances plant defences and regulates opening of stomata, an entry point for foliar bacterial pathogens such as *Pseudomonas syringae* pv. tomato DC3000 (*PsPto*). The effect of light perception on gene expression and virulence was investigated in *PsPto*. Light induced genetic reprogramming in *PsPto* that entailed significant changes in stress tolerance and virulence. Blue light-mediated up-regulation of type three secretion system genes and red light-mediated down-regulation of coronatine biosynthesis genes. Cells exposed to white light, blue light or darkness before inoculation were more virulent when inoculated at dawn than dusk probably due to an enhanced entry through open stomata. Exposure to red light repressed coronatine biosynthesis genes which could lead to a reduced stomatal re-opening and *PsPto* entry. Photoreceptor were required for the greater virulence of light-treated and

dark-treated *PsPto* inoculated at dawn as compared to dusk, indicating that these proteins sense the absence of light and contribute to priming of virulence in the dark. These results support a model in which *PsPto* exploits light changes to maximize survival, entry and virulence on plants.

Introduction

Foliar bacteria live as epiphytes. To cause disease, however, all foliar pathogens must enter the leaf tissues. Although wounds can enable pathogen entry, stomata are the primary natural entry point for most pathogens of leaves, with stomatal entry providing access to the network of intercellular spaces known as the apoplast. The probability of bacterial entry into the apoplast is greatly increased when a pathogen can prevent or reduce pathogen-triggered stomatal closure, which is an important component of plant innate immunity (Melotto *et al.*, 2008). This entry also requires active bacterial motility (Panopoulos and Schroth, 1974; Hattermann and Ries, 1989; Antúnez-Lamas *et al.*, 2009; Río-Álvarez *et al.*, 2014). Once in the apoplast, bacterial expression of virulence traits, including the introduction of effector proteins that suppress additional innate immune defences, promotes the eventual induction of disease symptoms. Pathogen survival and growth on leaf surfaces and in the apoplast requires adaptations that confer tolerance to a highly dynamic abiotic environment resulting from continuous changes in temperature, water availability and light intensity (Vorholt, 2012; Yu *et al.*, 2013). Therefore, survival and infection strategies are central elements dictating foliar disease outcomes.

Light and circadian rhythms are integral to the physiology of plants, regulating stomatal movement as well as plant defence (Kangasjarvi *et al.*, 2012; Sharma and Bhatt, 2015). Light has a particularly large role in plant defences that involve salicylic acid signalling, as was illustrated by the severe attenuation of defence gene expression, hypersensitive cell death, systemic acquired resistance and accumulated salicylic acid following inoculation of avirulent *Pseudomonas syringae* into *Arabidopsis* and incubation in the dark (Zeier *et al.*, 2004; Griebel and Zeier, 2008). The circadian clock also

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influences plant defences based, in part, on circadian regulation of defence genes (Covington *et al.*, 2008) and pathogen-triggered immunity, namely defences induced by pathogen-associated molecular patterns (PAMPs) (Bhardwaj *et al.*, 2011). Thus, daytime stomatal opening, which optimizes carbon dioxide uptake when sunlight is available for photosynthesis, may be coordinated with robust PAMP-triggered defences to compensate for the associated vulnerability to pathogen invasion (Bhardwaj *et al.*, 2011; Zhang *et al.*, 2014). Diurnal regulation of plant defences suggests opportunities for pathogens to exploit light signals to counteract or evade these defences. Pathogens have evolved numerous mechanisms for counteracting or evading plant defences, as illustrated by the secretion of compounds by some *P. syringae* and *Xanthomonas campestris* pathovars that interfere with PAMP-induced stomatal closure (Melotto *et al.*, 2008; Gudesblat *et al.*, 2009; Panchal *et al.*, 2016). However, the extent to which pathogens exploit light signals to promote virulence and plant infection is poorly understood. Photosynthetic and non-photosynthetic bacteria are able to sense light and regulate various physiological processes in response to light (Armitage and Hellingwerf, 2003; Purcell and Crosson, 2008; Elias-Arnanz *et al.*, 2011). These responses include protection against phototoxicity-associated stresses (Ziegelhoffer and Donohue, 2009) and lifestyle choices, such as between host- and non-host-associated life stages or between motile single-cells and non-motile multicellular assemblages (Gomelsky and Hoff, 2011). Like plants, bacteria possess diverse photoreceptor proteins that detect visible light, including members of the blue light-sensing light-oxygen-and-voltage (LOV)-domain family, blue-light-sensing-using-FAD (BLUF)-domain family, photoactive-yellow-protein (PYP) family, the cryptochrome/photolyase family, proteorhodopsins and the red light-sensing bacteriophytochrome family. Although the photochemical properties of various photoreceptors of animal and plant pathogens are known (Lamparter *et al.*, 2002; Swartz *et al.*, 2007; Barkovits *et al.*, 2008; Rottwinkel *et al.*, 2010; Barkovits *et al.*, 2011; Ondrusch and Kreft, 2011; Losi *et al.*, 2014; Bonomi *et al.*, 2016; Shah *et al.*, 2016), the role of these photoreceptors in regulating host colonization or infection traits has been examined in only a few plant pathogens. In particular, photoreceptors are known to influence motility in *P. syringae* pv. *syringae* (Pss) B728a (Wu *et al.*, 2013), plasmid conjugation in *Agrobacterium fabrum* (Bai *et al.*, 2016), and motility, adhesion and virulence in *Xanthomonas axonopodis* pv. *citri* (Kraiselburd *et al.*, 2012) and *Xanthomonas campestris* pv. *campestris* (Bonomi *et al.*, 2016). Light quality and intensity change over the day-night cycle. Daytime sunlight contains higher proportion

and intensity of blue wavelengths than sunset. At twilight, short wavelengths are scattered and red wavelengths more easily reach the surface of the earth (Thorington, 1985; Bedrosian and Nelson, 2017). Therefore the role of different specialized photoreceptors could be related with the bacterial adaptation to the changing environment during plant infection.

Pseudomonas syringae pv. tomato DC3000 (*PsPto*) is the causal agent of bacterial speck of tomato, which is characterized by the development of chlorotic or necrotic symptoms in leaves, stems and fruits. *PsPto* is a hemibiotrophic pathogen that can multiply on leaf surfaces and in the leaf apoplast. After entering the leaf, *PsPto* uses a type three secretion system (T3SS) to inject effector proteins (T3Es) into plant cells. These T3Es have been widely investigated for their ability to subvert plant cell processes; subversion strategies include modulating the host ubiquitin-proteasome system, host cytoskeleton or MAPK cascades to disrupt pathogen recognition and receptor signalling, hormone signalling and vesicle trafficking (Gohre and Robatzek, 2008; Block and Alfano, 2011; Toruño *et al.*, 2016). In addition to these T3Es, *PsPto* produces coronatine, a chlorosis-inducing phytotoxin that functions in part by mimicking a jasmonic acid conjugate and activating a signalling cascade that inhibits salicylic acid accumulation (Zheng *et al.*, 2012; Geng *et al.*, 2016). Coronatine contributes to bacterial growth and lesion induction in plants (Brooks *et al.*, 2004) and, importantly, antagonizes PAMP-induced stomatal closure to promote bacterial entry (Melotto *et al.*, 2006; Panchal *et al.*, 2016).

The *PsPto* genome encodes a single blue light-sensing LOV-domain protein and two red light-sensing bacteriophytochromes. Our previous studies demonstrated that *PsPto* light perception affects motility, attachment to plant leaves and virulence, and these phenotypes require the LOV photoreceptor (Río-Álvarez *et al.*, 2014). Interestingly, light impacted these plant-based pathogen behaviours when the pathogen was exposed to the light signal for only 10 min prior to inoculation onto tomato leaves (Río-Álvarez *et al.*, 2014). This discovery illustrates that light can evoke changes in pathogen traits or behavior that are detectable after the initial light stimulus is gone. Such enduring environmental reprogramming of bacterial behavior is generally a result of changes in global gene-expression patterns.

Here, we explored the interplay among light-mediated impacts on plant host physiology, *PsPto* global gene expression patterns, *PsPto* photoreceptors, and *PsPto* virulence. We carried out microarray hybridization experiments to explore the effect of light perception on *PsPto* at a global level, demonstrating that light induces genetic reprogramming in *PsPto* that

involves large-scale changes in stress tolerance- and virulence-related traits. These changes were accompanied by changes in virulence output that varied depending on the wavelength to which the bacteria were exposed before inoculation and the physiological stage of the plants with respect to the diurnal cycle. We discovered that *PsPto* expresses selected virulence-related traits in the absence of light, and provide evidence that this nocturnal priming optimizes bacterial entry through the stomata at dawn. The behavior of mutants altered in the *PsPto* photoreceptors corroborates the role of light perception during the early stages of infection, likely at the point of bacterial entry. Collectively, these results support a model in which *PsPto* optimizes leaf invasion and subsequent growth in the apoplast by exploiting cues based on the presence, absence and quality of light.

Results and discussion

Light regulates major fitness and virulence genes in PsPto

Previous studies indicate that light influences motility, attachment and virulence in *PsPto*, with white and blue light inhibiting motility but promoting bacterial attachment to leaves (Río-Álvarez *et al.*, 2014). Here we performed global transcriptome analyses to analyse the response of *PsPto* to light. We used custom-designed microarrays (Agilent Technologies) with probes isolated from either *PsPto* cells after a 10 min treatment with 70 $\mu\text{E}/\text{m}^2\text{s}$ white light, or *PsPto* cells exposed to only darkness. The intensity used is close to the illumination level at dusk and dawn on a day with clear sky conditions (Thorington, 1985). As a complement to white light, which contains a profile of distinct wavelengths across the visible spectrum (Supporting Information Fig. S1), we also performed treatments in which *PsPto* cells were exposed to a 10 min treatment with either red or blue light (20 $\mu\text{E}/\text{m}^2\text{s}$). The short durations of the light treatments were selected based on the rapid kinetics of the photocycle for the *PsPto* blue light photoreceptor (nanoseconds) and red light photoreceptor (milliseconds) (Cao *et al.*, 2008; Takala *et al.*, 2014), and to minimize growth and light stress (Ziegelhoffer and Donohue, 2009) during the treatments. In order to discard major pleiotropic effects, we carried out an experiment to evaluate cell viability and metabolic performance after light treatment in a real time mode (Shah and Naseby, 2014). We have analysed the luciferase activity after light treatments on a *PsPto* derivative stably transformed with the *luxCDABE* operon (Choi and Schweizer, 2006) under the control of a constitutive promoter (Supporting Information Table S1). The

luciferase pathway is highly energy dependent and requires the essential cofactors NADPH/FMNH₂ and ATP. Moreover, the expression of the luciferase and accessory proteins and the process of light emission are considered to be a significant burden to the bacterial metabolism (Waidmann *et al.*, 2011). The results showed no differences on luciferase activity between dark and light treated cells (Supporting Information Fig. S2). In addition, growth under the different light conditions has been previously checked and no differences were observed respect to darkness (Río-Álvarez *et al.*, 2014).

Genes were identified as differentially expressed based on a ≥ 2 -fold change between the control and experimental conditions and a false discovery rate (FDR) ≤ 0.01 . The results were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through GEO series accession GSE101783.

We identified 326 genes that were differentially expressed in response to the white light treatment, with 85% up-regulated and 15% down-regulated. The blue light treatment changed the transcript levels of 607 genes, with 64% up-regulated and 36% down-regulated. The red light treatment changed the expression of 341 genes, with 68% down-regulated and only 32% up-regulated. These results indicate a strong and distinct bias of the red light treatment toward gene repression, in contrast to the primary influence of white and blue light toward gene induction. Despite the broad spectrum of the white light, there were more genes shared between the blue- and red-light treatments (161) than between the blue- and white-light treatments (109) or red- and white-light treatments (17) (Fig. 1). Among the differentially expressed genes, only 10 were shared among all three light treatments (Fig. 1A). However, when analysing up or down regulation independently, only four genes are shared among the up-regulated genes in the three treatments and one among the down-regulated genes. Interestingly, almost all the genes whose expression is altered by both white and blue treatments, are up-regulated (97) while most of the genes with an expression level under the control of both red and blue light, are down-regulated (98). An analysis of the functional categories that were overrepresented among the differentially-expressed genes identified nine categories that were overrepresented among genes responding to the white light treatment (Fig. 2, Supporting Information Tables S2 and S3). The flagellar synthesis and motility category was dominated by down-regulated genes; this down-regulation was consistent with our previous findings that white light inhibits *PsPto* swarming motility, a flagellum-dependent movement (Río-Álvarez *et al.*, 2014). Most of the other functional categories overrepresented after white light

treatment are dominated by up-regulated genes and relate to tolerating water limitation and osmotic stress, including polysaccharide synthesis and regulation, compatible solute synthesis, oxidative stress tolerance and quaternary ammonium compound (e.g. betaine and choline) transport. We previously reported the up-regulation of transcripts encoding proteins involved in alginate biosynthesis and regulation by white light and correlated this with increased attachment to leaves (Río-Álvarez *et al.*, 2014). Alginate production is associated with tolerance to water limitation (Chang *et al.*, 2007; Freeman *et al.*, 2013) and *PsPto* biofilm formation (Keith *et al.*, 2003). Similarly, the biosynthesis of two major compatible solutes, N-acetylglutaminylglutamine amide and trehalose, is associated with water stress tolerance by *PsPto* and *Pss* B728a in culture (Freeman *et al.*, 2013), and genes for the biosynthetic enzymes are induced in B728a *in planta* (Yu *et al.*, 2013). Among the genes within the oxidative stress tolerance category, *katB* was particularly highly induced. KatB is the major inducible catalase in *PsPto* that responds to hydrogen peroxide (Guo *et al.*, 2012), and it is required for full virulence of *PsPto* in *Arabidopsis thaliana* (Guo *et al.*, 2012). In fact, the *katB* gene was induced by white and blue light. This induction by light and oxidative stress (Guo *et al.*, 2012), which are both dominant features of aerial leaf habitats, suggests a possible adaptive advantage of genetic co-regulation of *katB* in *PsPto* by these distinct environmental signals. Collectively, these findings suggest a co-association among *PsPto* responses to white light, water limitation and oxidative stress, including responses that enhance stress tolerance and reduce flagellar-dependent motility. This co-association may reflect the co-occurrence of these stresses on aerial leaves over the diurnal cycle of a day, with water limitation, light and reactive oxygen species (ROS)-associated oxidative stress all potentially peaking at mid-day.

We found that the functional categories that were over-represented among the differentially-expressed genes responding to blue light were distinct from those responding to white light (Fig. 2, Supporting Information Tables S2 and S3). A large number of genes encoding proteins comprising the T3SS, including T3Es, were up-regulated. The T3SS and its associated T3Es are major determinants of *PsPto* pathogenesis (Lindeberg *et al.*, 2012), and these include effectors that induce an aqueous environment in the apoplast that promotes *PsPto* growth (Xin *et al.*, 2016). Moreover, some *P. syringae* T3Es, at least in B728a, contribute to epiphytic fitness (Lee *et al.*, 2012). The induction of these genes was surprisingly as experiments were conducted on KB medium which has been described as an hrp-inhibiting medium (Lam *et al.*, 2014). To corroborate the inducing activity of

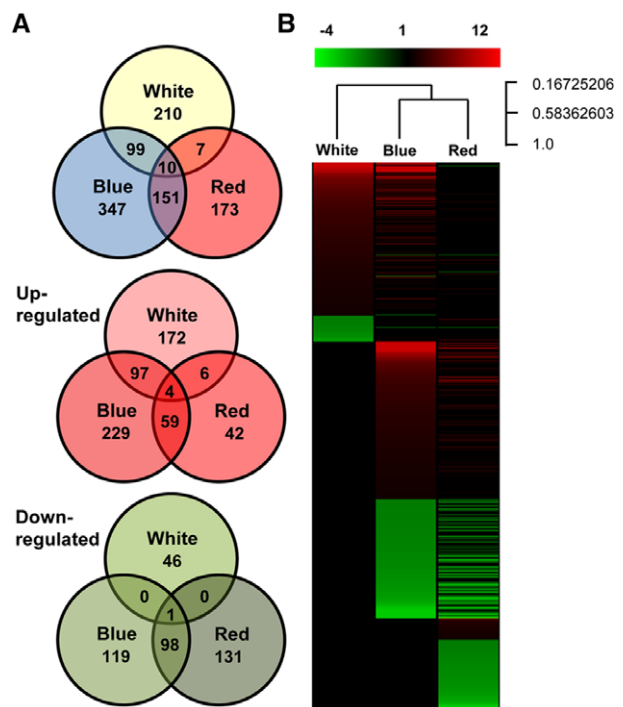
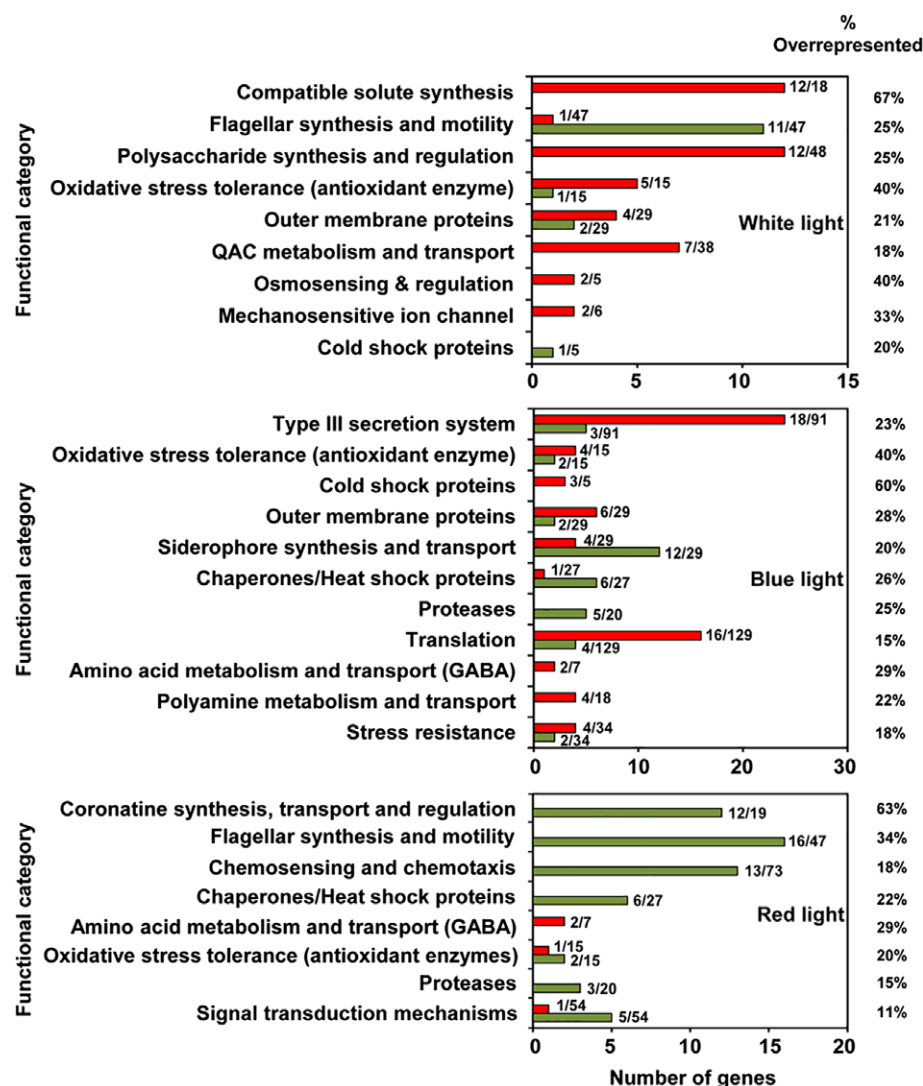


Fig. 1. *P. syringae* pv. tomato DC3000 (*PsPto*) exhibits distinct changes in gene expression in response to distinct wavelengths of light. **A.** Venn diagram showing overlap among the genes that were differentially expressed in response to a 10 min exposure to white, blue or red light as compared to maintained in the dark. **B.** Hierarchical clustering of these differentially-expressed genes, where red indicates higher expression of the gene in the light than the dark (i.e. up-regulation) and green indicates down-regulation. Bar indicates fold-change in expression.

blue light we performed an expression analysis of *hrpR* and *avrPto* genes, as representatives, in hrp-inducing medium. Results (Supporting Information Fig.S3) confirmed the induction observed in the microarray data and point to a prevalent function of blue light as an inducer of the expression. Coronatine is a phytotoxin produced by *PsPto* that acts both prior to and during lesion development (Melotto and Kunkel, 2013). Coronatine production together with the T3SS effectors are considered the main components of *PsPto* virulence and, as the genes responsible for these functions have been observed to be coregulated (Sreedharan *et al.*, 2006), blue light upregulation of the Type III secretion genes suggest that coronatine synthesis might also be upregulated by blue light. The *corR* gene, which encodes the main regulator of *cor* gene expression, was in fact induced 1.70-fold by blue light (FDR = 0.014), although this was not declared to be differentially expressed because it fell below our twofold threshold for differential expression. The light treatment used in our analysis is very short and we cannot discard that at longer sample recovering time we could significantly detect also the expression of *cor* genes. In a time course frame it seems that blue light has an earlier

Fig. 2. Light induces gene-expression reprogramming in *PsPto*, with both overlap and specificity in the functions affected by distinct wavelengths. The data shown are the functional categories that are overrepresented among the differentially-expressed genes responding to a 10 min treatment with white, blue or red light (fold change ≥ 2 , FDR ≤ 0.01). Only functional categories that are significantly overrepresented are shown ($p \leq 0.05$). Red and green indicate the number of up-regulated and down-regulated genes, respectively, within each functional category. The percentage of overrepresented indicates the percentage of observed genes out of the total number of genes annotated in each functional category.



impact on T3SS gene regulation than in *cor* gene regulation compared with darkness.

The functional category containing genes involved in γ -amino butyric acid (GABA) metabolism and transport was also significantly overrepresented among blue light-responsive genes, and like the T3SS genes, these genes primarily exhibited up-regulation. These genes include *gabP*, which encodes a permease for GABA uptake, and *gabD-2*, which encodes an enzyme implicated in GABA catabolism. GABA is the most abundant amino acid in the tomato apoplast and increases in abundance under abiotic and biotic stress conditions (Park *et al.*, 2010). GABA can serve as a nitrogen source for *PsPto* (Park *et al.*, 2010) and as a signal that represses the virulence genes *hrpL* and *avrPto* and induces the *Arabidopsis* defence gene PR1 (Park *et al.*, 2010; McCraw *et al.*, 2016). The GABA uptake and catabolism genes were up-regulated in *Pss* B728a during growth *in planta* (Yu *et al.*, 2013), supporting the prediction that GABA uptake and

catabolism may prevent GABA-mediated virulence repression (Yu *et al.*, 2013; Park *et al.*, 2010). The blue light-mediated activation of cell genes involved in coping with GABA in a broth media in which this compound is not present, strongly indicates that *P. syringae* has evolved mechanisms of using light signals to enhance pathogenesis and epiphytic fitness which actually correlates with a successful infection process.

Like the overrepresented functional categories responding to white light, those responding to blue light also include categories related to stress responses (Fig. 2, Supporting Information Tables S2 and S3). The oxidative stress tolerance and cold shock protein categories were overrepresented in response to white and blue light. However, the chaperones/heat shock proteins, polyamine metabolism and transport and stress resistance categories were overrepresented specifically in response to blue light. It is noteworthy that except for the functional category of chaperone/heat

shock proteins, the expression of genes belonging to these functional categories is up-regulated. This may reflect the protective response against the particularly oxidative stress known to be induced by blue light (Liang *et al.*, 2013). After a blue light treatment, up-regulation of bacterial genes coding for chaperones/heat shock proteins would be an expected consequence of the high energy status associated to blue light. However, we have used LED lights in our analysis to discard the associated heat response.

Blue light treatment is also accompanied by an up-regulation of genes related to translation. The enrichment of this functional category could be associated with an active protective response mediated by the induction of specific protein synthesis. Actually in *P. aeruginosa* it has been previously described how a protective response against the stress induced by an antimicrobial agent involves the up-regulation of translation leading to the increased synthesis of membrane-related proteins and virulence proteins (Nde *et al.*, 2008). Not only in bacteria but also in plants it has been described a dynamic light regulation of translation (Juntawong and Bailey-Serres, 2012) showing that light has an up-regulatory effect over translation as compared to darkness conditions.

Among the functional categories containing genes that were differentially expressed by the red light treatment, eight functional categories were overrepresented (Fig. 2, Supporting Information Tables S2 and S3). Notably, most of the genes contributing to coronatine synthesis, transport and regulation were down-regulated. Coronatine suppresses stomatal defence and thus facilitates pathogen entry into the apoplast (Melotto and Kunkel, 2013). Once *PsPto* is in the apoplast, coronatine promotes growth, at least in part, by modulating plant defence responses and this growth contributes to eventual disease symptoms (Melotto and Kunkel, 2013). Red light-mediated down-regulation of almost all of the *PsPto* coronatine biosynthetic genes (63% of the genes in this category) suggests that *PsPto* perceives red light as a signal to remain in a non-invasive stage.

Another functional category that was overrepresented among differentially expressed genes was flagellar synthesis and motility (Fig. 2). Among the genes in this category, 34% were down-regulated in *PsPto*, which is consistent with a report of red light-mediated repression of swarming motility in *Pss* B728a (Wu *et al.*, 2013). Invasion of the plant apoplast requires active movement (Río-Álvarez *et al.*, 2014), including movement toward open stomata (Melotto *et al.*, 2006), and potentially even chemotaxis given that *PsPto* do not move towards open stomata when the stomata are closed (Melotto *et al.*, 2006). The chemosensing and chemotaxis category was also

overrepresented among genes expressed after the red light treatment, and like the flagellar genes, this reflected exclusively down-regulation of genes. Together, these findings suggest that red light negatively regulates *PsPto* movement, including possible movement toward stomata, and thus could reduce its probability of invasion.

Although red and blue light similarly affect genes in the GABA metabolism and transport category (Fig. 2, Supporting Information Tables S2 and S3), in contrast to blue light, red light represses genes in several overrepresented functional categories related to virulence and stress tolerance. In particular, within the oxidative stress tolerance category, red light down-regulates *katB*, a gene known to contribute the *PsPto* virulence (Guo *et al.*, 2012). Red light also down-regulates many genes in the chaperones/heat-shock proteins, proteases and signal transduction mechanisms categories, with the latter generally involved in responding to environmental signals (Lavín *et al.*, 2007).

Collectively, our results indicate that light influences the expression of many genes involved in *PsPto* fitness and plant infection. Based on global transcriptome data, many of the *PsPto* traits regulated by light *in vitro* are similar to the *Pss* B728a traits differentially expressed during growth in epiphytic sites, such as the down-regulation of flagellar motility, or during growth in the apoplast, such as up-regulation of GABA transport and catabolism (Yu *et al.*, 2013). Moreover, genes involved in responding to environmental stresses were strongly influenced by light and by growth *in planta* in these *P. syringae* strains. These results therefore support a role for light as a prevalent signal during the *P. syringae* life-cycle and as a regulator of the pathogenic behavior of this bacterium.

The light conditions at the time of inoculation significantly affect virulence outcomes

We postulate that *PsPto* uses light cues to ensure stomatal entry at a time favourable for multiplication in the apoplast. To test this hypothesis, we performed an experiment in which *PsPto* cells were treated for 10 min with white, red or blue light, or were maintained in the darkness, and then were inoculated into tomato plants at either a simulated dawn or simulated dusk. Inoculation at a simulated, or subjective, dawn involved inoculation at the end of the night cycle and then plant transfer to the light. Inoculation at a subjective dusk involved inoculation at the end of the day cycle followed by plant transfer to the dark. Plants were inoculated by dipping in the inoculum to enable natural bacterial entry into the apoplast through stomata. At 6 days post-inoculation, *PsPto* populations were higher in plants infected at subjective dawn

than at subjective dusk when the *PsPto* cells had been pre-treated with white light, blue light or no light (Fig. 3A). These leaves also exhibited more severe symptoms (Fig. 3B). The *PsPto* cells treated with red light established populations similar to the other light treatments when inoculated at subjective dusk, but did not establish larger populations or more severe symptoms when infected at subjective dawn (Fig. 3A, B).

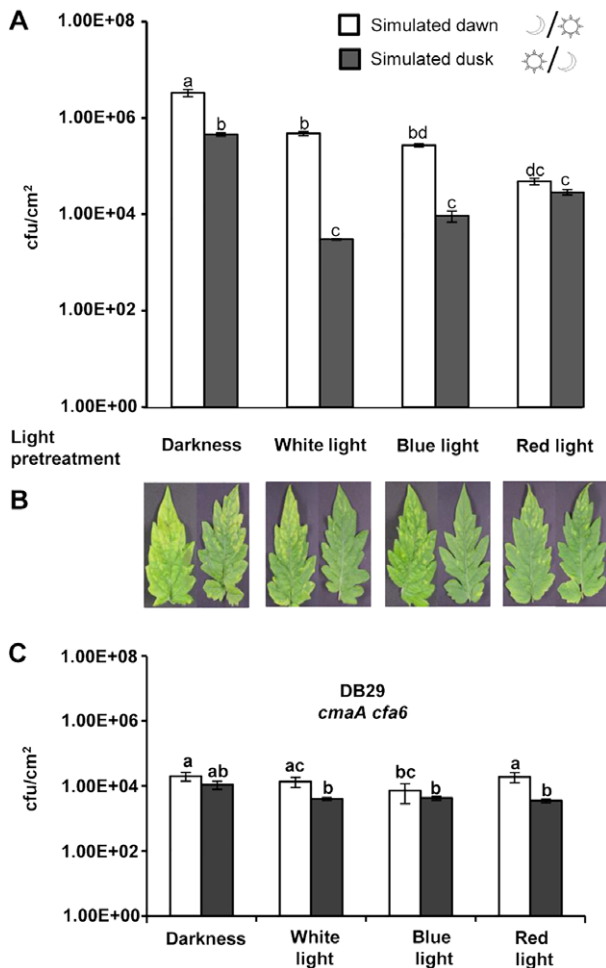


Fig. 3. *PsPto* virulence is affected by both pathogen exposure to light prior to inoculation and the time in the diurnal cycle at which the plants are infected. **A.** Virulence of *PsPto* wild-type, as assessed based on bacterial population sizes in tomato leaves at 6 days post-inoculation (dpi) after immersion inoculation in *PsPto* suspensions (10^8 cfu/ml) that had been pretreated for 10 min either with white light, blue light, red light or maintained in the dark. Plants were infected at subjective dawn, which was at the end of the night cycle with immediate transfer to the light (white bars), or at the subjective dusk, which was at the end of the day cycle with immediate transfer to the dark (grey bars). Data represent the means and standard errors of three replicates, with different letters indicating significant differences among treatments [$p < 0.05$, Fisher's least significant difference (LSD) test]. **B.** Disease symptoms on leaves representative of the results from each treatment. **C.** Virulence of DB29 mutant strain. Different letters indicating significant differences among treatments ($X^2 w = 27.72$, $p < 10^{-3}$ GzLM analysis followed by LSD test).

Stomata are the main entry points for *PsPto* into the plant apoplast, and this entry is a critical step for *PsPto* pathogenesis. Plants regulate stomatal aperture based on environmental conditions and circadian control, with most species opening stomata as morning begins, maintaining open stomata through midday, and closing stomata long before dusk. Some C3 plants may open stomata pre-dawn (Webb, 2003; Hotta *et al.*, 2007). Importantly, plants close stomata after the detection of PAMPs, even under bright light, and this closure helps restrict pathogen invasion (Zeng and He, 2010). Coronatine counteracts PAMP-closed stomata (Melotto *et al.*, 2006), and as has been recently published, coronatine is also able to open dark-closed stomata in *Arabidopsis* leaves (Panchal *et al.*, 2016). In this context, the relative *PsPto* population sizes shown in Fig. 3 may primarily reflect differences in stomatal entry. In particular, when plants are infected at subjective dawn, it is tempting to speculate that coronatine production and motility of dark-treated cells may have enabled them to maximally exploit stomatal entry, thereby allowing them to establish large populations; when infected at subjective dusk, the dark-induced stomatal closure may have attenuated this entry (Fig. 3A). The reduced populations of the light-treated cells following infection at subjective dawn may therefore reflect reduced motility (white light) and/or greatly reduced coronatine production (red light), with the greater reductions in populations in the plants infected at subjective dusk than subjective dawn, which commensurate with these bacterial phenotypes in combination with dark-induced stomatal closure (Fig. 3A). Noteworthy, the induction of the expression of certain T3SS components triggered by the blue light treatment is not enough to improve virulence, which reflects the involvement of the T3SS in later virulence stages, once the bacteria is in the apoplast, perceive several signals from the host, and display an effective cell contact.

As it has been mentioned above, *PsPto* PAMP exposure induces stomata closure. The similarity in the population sizes established by the red-light-treated cells at subjective dawn and subjective dusk could be explained by a deficiency of these strains in coronatine-mediated stomatal re-opening at subjective dawn. In accordance with our results, *A. thaliana* Col-0 plants were observed to be more resistant when spray-infected with *PsPto* at night than at dawn (Korneli *et al.*, 2014; Zhang *et al.*, 2014).

In order to get more insights into the biological relevance of light effects on coronatine mediated entry, we evaluated the virulence of a coronatine deficient mutant after different light treatments. Our results showed that, in contrast to the wild-type strain (Fig. 3A, B), the *cor* mutant established similarly low populations irrespective of the light pretreatment (Fig. 3C). These results suggest

that *cor* mutant may show a reduced entry into stomata due to its inability to reopen stomata both at dawn, when stomata were closed by PAMP induction, and dusk when stomata were naturally closed.

Pathogen exposure to darkness primes virulence

Exposing cells to darkness prior to inoculation under subjective dawn resembles natural conditions for infection by epiphytic *P. syringae* cells. Our finding of maximal virulence with dark-treated cells inoculated at subjective dawn (Fig. 3) suggests a priming effect of night during the infection process. To investigate whether gene expression indicative of motility and coronatine production phenotypes were associated with dark-primed virulence, we evaluated the impacts of the light treatments on the expression of key genes for these phenotypes using qRT-PCR with the RNA isolated for the microarray analysis. We observed that the expression of *fliC*, the gene encoding flagellin, was higher in dark-treated bacteria than in white-light-treated bacteria, as in our previous study (Río-Álvarez *et al.*, 2014); it was also higher than in blue- and red-light-treated cells, consistent with attenuated motility contributing to the reduced virulence of light-treated cells in plants exposed to subjective dawn (Fig. 3). Recognition of flagellin by the FLS2 pattern recognition receptor (PRR) activates plant immune responses during the early stages of infection (Zipfel *et al.*, 2004), suggesting that low levels of flagellin could help *PsPto* evade FLS2-mediated immunity. However, this prediction is contradicted by our findings that dark-treated *P. syringae* cells exhibit strong *fliC* expression (Fig. 4), motility (Wu *et al.*, 2013; Río-Álvarez *et al.*, 2014) and virulence (Fig. 3), whereas light-treated *P. syringae* cells exhibit lower *fliC* expression (Fig. 4), little or no motility (Wu *et al.*, 2013; Río-Álvarez *et al.*, 2014) and virulence (Fig. 3). A previous report shows that a flagellin deficiency was similarly correlated with low virulence, negating the potential benefit from the evasion of FLS2-triggered immunity (Pfeilmeier *et al.*, 2016).

We also examined the expression of genes involved in coronatine biosynthesis. The coronatine biosynthetic genes *cmaA* and *cfa5*, which encode coronamic acid synthetase and coronafacic acid synthetase, respectively, were strongly repressed by red light (Fig. 4), as was highlighted in the functional category analysis above (Fig. 2). They were also repressed by white and blue light, a finding that was not highlighted by the more general functional category analysis, but one that reflects a general role of dark in activating, or light of various wavelengths repressing, coronatine biosynthesis genes. This observation is consistent with a recent report showing that *PsPto* coronatine production at night opens dark-

closed stomata and promotes infection (Panchal *et al.*, 2016).

We previously documented impacts of light on *PsPto* adhesion to the leaf surface (Río-Álvarez *et al.*, 2014); therefore, we also analysed the expression of *algD*, which encodes a GDP mannose dehydrogenase that is critical to the synthesis of the exopolysaccharide alginate. *algD* expression was significantly up-regulated by white light (Fig. 4). Microarray data also revealed that the expression of other alginate biosynthesis related genes was up-regulated after the red and blue light treatments. A transcriptome study with *Pss* B728a showed significant up-regulation of *algD* and most of the alginate biosynthesis genes in response to blue, red, far-red and white light as compared to in the dark (Janssen and Beattie, personal communication), supporting increased alginate production as a *P. syringae* response to light.

PsPto photoreceptor mutants exhibit impaired virulence and an inability to prime virulence in the dark

To investigate the effect of light perception on *PsPto* infection, we evaluated the virulence of mutants inactivated in each of the *PsPto* photosensory proteins. We had previously constructed a mutant insertionally inactivated in the blue light-sensing LOV protein (Río-Álvarez *et al.*, 2014), and here we constructed mutants that were inactivated in each of the two red/far-red light-sensing bacteriophytochromes in *PsPto*. *In vitro* growth of the mutant strains was checked showing no differences with respect to the wild-type (data not shown). We examined the virulence of all three of these mutants following exposure to a 10 min light treatment or constant darkness with subsequent inoculation onto tomato plants, as described above, and again assessed virulence based on the population sizes after 6 days of light/dark cycles. When tomato plants were inoculated at subjective dawn and dusk with strains that had been maintained in the dark, the *PsPto* photoreceptor mutants were less virulent than the wild-type strain (Fig. 5A). The observation that they showed reduced virulence when inoculated at subjective dawn and subjective dusk indicates that the photoreceptor-mediated benefit was not a function of the 12 h of light following inoculation at subjective dawn. We also observed that the mutants showed reduced virulence when pre-exposed to dark conditions (Fig. 5A) but not white or blue light conditions (Fig. 5B, C) following inoculation at subjective dusk, which indicates that the photoreceptor-mediated benefit was not realized during the 6 days of day/night growth, as this light/dark cycle was common to all treatments. Thus, the inability of the photoreceptor mutants to attain the full virulence advantage associated with the dark pre-treatment indicates that

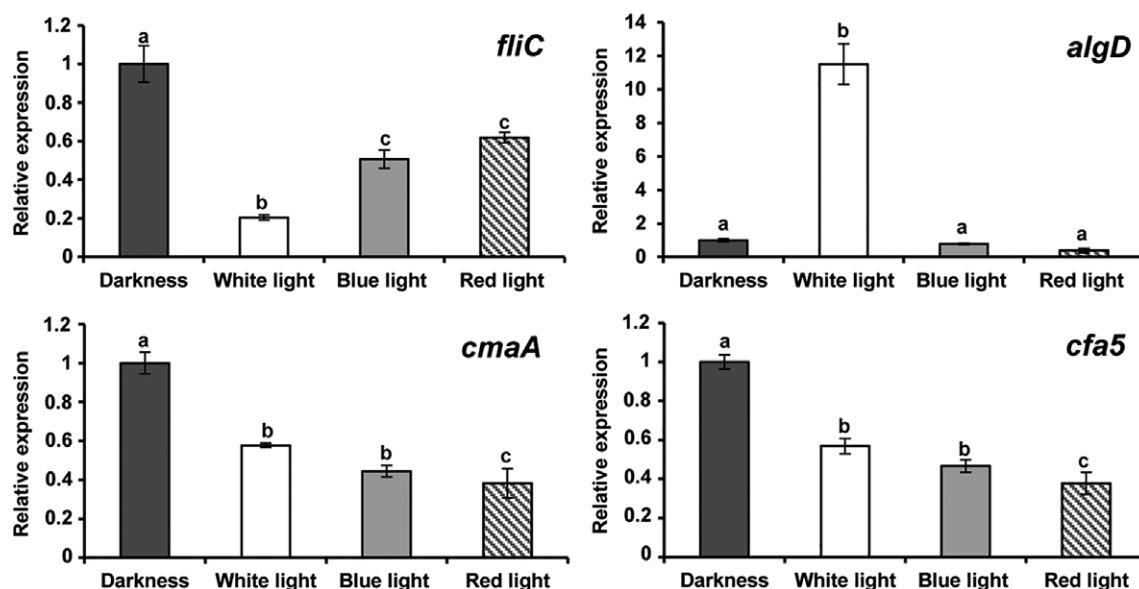


Fig. 4. Motility and coronatine biosynthesis genes, but not an alginate biosynthesis gene, were expressed more in the dark than the light. Differential gene expression was evaluated by qRT-PCR after cells were maintained in the dark (dark grey bars) or were subjected to a 10 min treatment with white light (white bars), blue light (light grey bars), or red light (striped bars). Bars represent the relative expression changes of the target genes in the light-treated cells with respect to the darkness-treated cells. Data represent the means and standard errors of three replicates, with different letters indicating significant differences among treatments ($p < 0.05$, Fisher's LSD test).

the photoreceptors were required for nocturnal priming. Although photoreceptors can help cells sense light, they can also help sense the absence of light (Yu *et al.*, 2016). This concept was advanced in studies with the phytochrome FphA of *Aspergillus nidulans*, where the kinase activity of FphA that initiates signal transduction was shown to increase under dark conditions (Yu *et al.*, 2016). These data suggest a similar phenomenon in the case of the *PsPto* photoreceptors, and are consistent with the photoreceptors affecting traits in the dark that subsequently benefit virulence. Actually the expression level of *fliC* (although is not the only determinant for motility) and *cmaA* is down regulated in the mutant strains after the darkness treatment (Supporting Information Fig. S4). These results thus collectively provide evidence for a role for the *PsPto* photoreceptors in dark-priming of virulence, and highlight the importance of photoreceptor-mediated dark-priming during the initial stages of infection.

When *PsPto* cells were exposed to white or blue light and then inoculated at subjective dawn, the photoreceptor mutants were less virulent than the wild-type strain (Fig. 5B, C). This difference supports a role for the photoreceptors in sensing light as well as sensing the absence of light, and likely involves light-mediated modulation of gene expression based on our microarray analyses. The photoreceptors mutants were similar in virulence to the wild-type when pre-exposed to white or blue light and inoculated at subjective dusk (Fig. 5B, C), which is a time when the stomata are generally closed. This similarity

may be due, at least in part, to the pre-treatment with light reducing wild-type coronatine biosynthesis gene expression (Fig. 4) which could lead to a coronatine level insufficient to re-open stomata on leaves in the dark (Panchal *et al.*, 2016).

The distinct photoreceptor mutants were qualitatively similar to one another in behavior following the dark, white-light and blue-light treatments, possibly due to crosstalk among the photoreceptors during light perception (Wu *et al.*, 2013; Djouani-Tahri, 2014). However, exposure of the *PsPto* strains to red light prior to inoculation at subjective dusk showed that mutants lacking *bphP1* or *bphP2* were more virulent than the wild-type and *lov* mutant (Fig. 5D). Based on this finding and the negative effect of red light on coronatine production genes (Fig. 2), we propose that *PsPto* bacteriophytochromes could mediate the repression of coronatine production under red light. This would reduce the ability of wild-type *PsPto* cells to re-open the stomata that close following subjective dusk, thus reducing entry into the leaves; moreover, the lack of *bphP1* or *bphP2* would prevent this repression, thus resulting in greater coronatine production, greater entry and larger populations (Fig. 5D). This prediction is supported by the significantly greater expression of the coronatine biosynthesis genes *cmaA* and *cfa5* in the *bphP* mutants than in the wild-type after red light treatment both in KB media and in hrp inducing medium (Fig. 6A, Supporting Information Fig. S3). Furthermore, when we analysed the average stomatal aperture on tomato leaves that had been

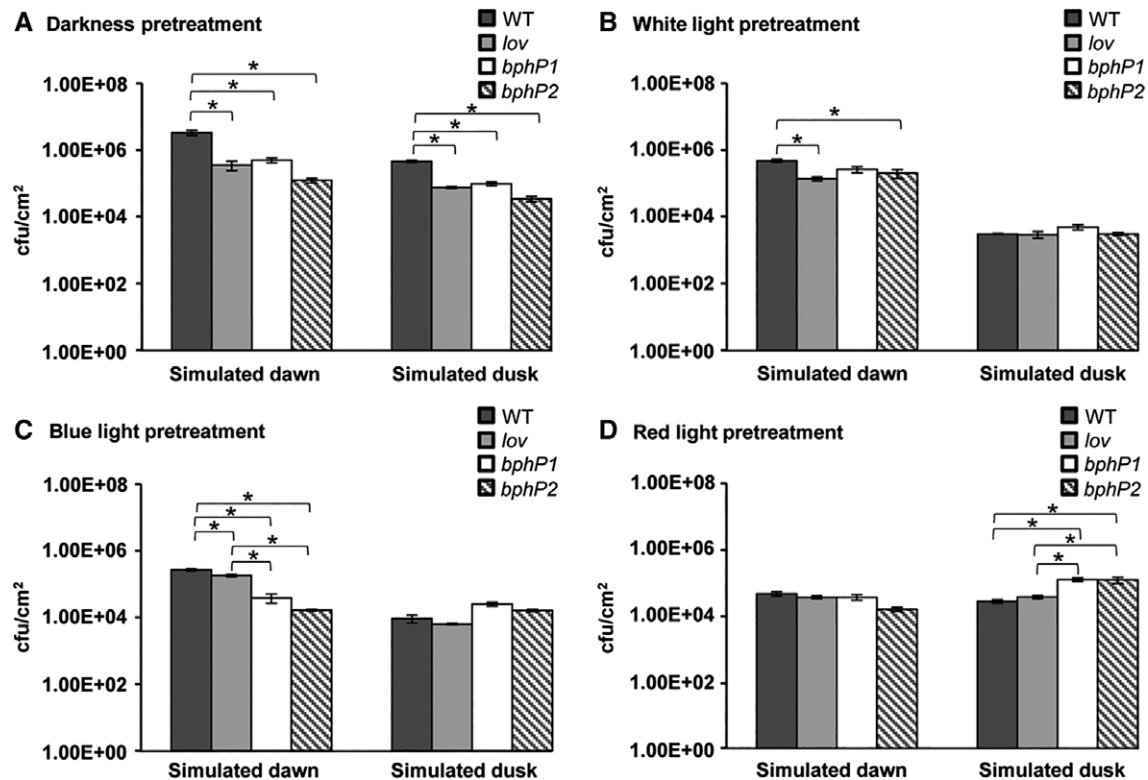


Fig. 5. *PsPto* photoreceptors contribute to both dark- and light-mediated effects on *PsPto* virulence and these effects depend on the infection time in the diurnal cycle. The virulence of wild-type *PsPto* (WT) and *PsPto* mutants insertionally inactivated in the genes for the photoreceptor proteins LOV, BphP1 and BphP2 were evaluated using cells that were (A) maintained in the dark prior to inoculation, or were exposed to (B) white light, (C) blue light, or (D) red light for 10 min prior to inoculation. Plants were infected as described in Fig. 3. The population sizes at 6 dpi are shown for the wild-type (dark grey bars) and the *lov* (light grey bars), *bphP1* (white bars) and *bphP2* (striped bars). Data represent the means and standard errors of three replicates, with different letters indicating significant differences among treatments (* $p < 0.05$, ** $p < 0.01$, Fisher's LSD test). The results are representative of three independent experiments.

inoculated with the red light-treated *bphP* mutants, we observed a significantly enhanced ability of the mutants to reopen the stomata at night (Fig. 6B). These results are consistent with the ability of *PsPto* to reopen stomata at night (Panchal *et al.*, 2016), and with the involvement of a *Xanthomonas campestris* pv. *campestris* phytochrome in the down-regulation of virulence factors including xanthan exopolysaccharide production and biofilm formation (Bonomi *et al.*, 2016). To highlight the relevance of the red light control over the traits involved in the entry to the apoplast we performed an inoculation assay by syringe infiltration. This assay bypasses the need of both motility and stomatal aperture to entry into the apoplast. Data showed that the difference in bacterial population of bacteria treated with red light respect to bacteria under darkness disappears (Fig. 6C).

In order to corroborate the involvement of photoreceptors in the control of the expression of motility and coronatine biosynthetic genes we performed q-RT-PCR assays. Results showed that expression of *flhC* and *cmaA* is under the control of light perception through the photoreceptor functions, as differences in the expression level

respect to the wild-type can be observed in the mutant strains after different light treatments (Supporting Information Fig. S4).

Overall, these results illustrate a potent influence of the diurnal cycle on both plant and pathogen physiology and a consequent impact on virulence outcomes. *PsPto* may be especially cued into light signals, as it is a poorer epiphyte than other *P. syringae* pathogens, such as strain B728a, and therefore likely depends more on entry into the apoplast for effective leaf colonization (Freeman *et al.*, 2013).

Swarming motility in PsPto is regulated by light quality through the photosensory proteins

Active motility is required for *PsPto* to enter the apoplast (Río-Álvarez *et al.*, 2014) and is therefore important to establishing infections. Swarming motility is a form of surface motility that requires flagella and surfactant production (Berti *et al.*, 2007; Burch *et al.*, 2010; Xu *et al.*, 2012). It has been previously demonstrated that

swarming motility in *PsPto* (Río-Álvarez *et al.*, 2014) and *Pss* B728a (Wu *et al.*, 2013) depends on light quality and that flagellar gene expression is down-regulated under white and red light (Fig. 2). Measuring swarming motility can be challenging due to its extreme sensitivity to relative humidity and temperature (Kearns, 2010; Hockett *et al.*, 2013). Since our previous report (Río-Álvarez *et al.*, 2014), we have optimized our assay for measuring *PsPto* swarming motility for more robust reproducibility. When this assay was used to evaluate the impact of distinct light wavelengths on swarming motility, we observed that *PsPto* exhibited greater swarming in the dark than under any of the wavelengths tested (Fig. 7). We similarly observed white- and blue-light inhibition of *PsPto* swarming motility in an earlier study (Río-Álvarez *et al.*, 2014), although *PsPto* showed greater swarming under the conditions tested here. However, we previously observed red-light enhancement (Río-Álvarez *et al.*, 2014) rather than red-light inhibition of *PsPto* wild-type swarming motility (Fig. 7). Collectively, these results indicate that all of the wavelengths tested reduced swarming motility.

The colony surface area of motility displayed by photoreceptor mutants under darkness conditions is not significantly different from that of the wild-type, however a distinctive morphology on swarming motility can be observed. At it has been previously stated, swarming motility requires active flagella and biosurfactant production. When analysing the expression of *flhC* in the photoreceptor mutants under darkness condition (Supporting Information Fig. S4) we observed a down-regulation of *flhC* in all of the mutant backgrounds with respect to the wild-type which could explain the observed different morphology of swarming motility and support the function of these photoreceptors on sensing the absence of light.

Loss of the LOV photoreceptor reduced *PsPto* swarming motility under blue and red light, indicating that this photoreceptor positively regulates motility and is involved in the response to both blue and red wavelengths. The findings with blue light are consistent with previous results with *Pss* B728a, which provided evidence that LOV positively regulates swarming motility in blue light by attenuating bacteriophytochrome-mediated repression of swarming motility (Wu *et al.*, 2013). Unlike B728a, however, *PsPto* also showed a role for LOV in the presence of red light. Although LOV is widely known for sensing blue light (Losi *et al.*, 2015), studies with a LOV-histidine kinase in the picoalga *Ostreococcus tauri* (Djouani-Tahri, 2014) provided a precedent for the involvement of a LOV photoreceptor in regulation under red light.

Whereas red light inhibits swarming motility of the wild-type, potentially due to down-regulation of flagellar synthesis by red light (Figs. 2 and 4), the *bphP1* mutant exhibited increased motility under blue, red and white

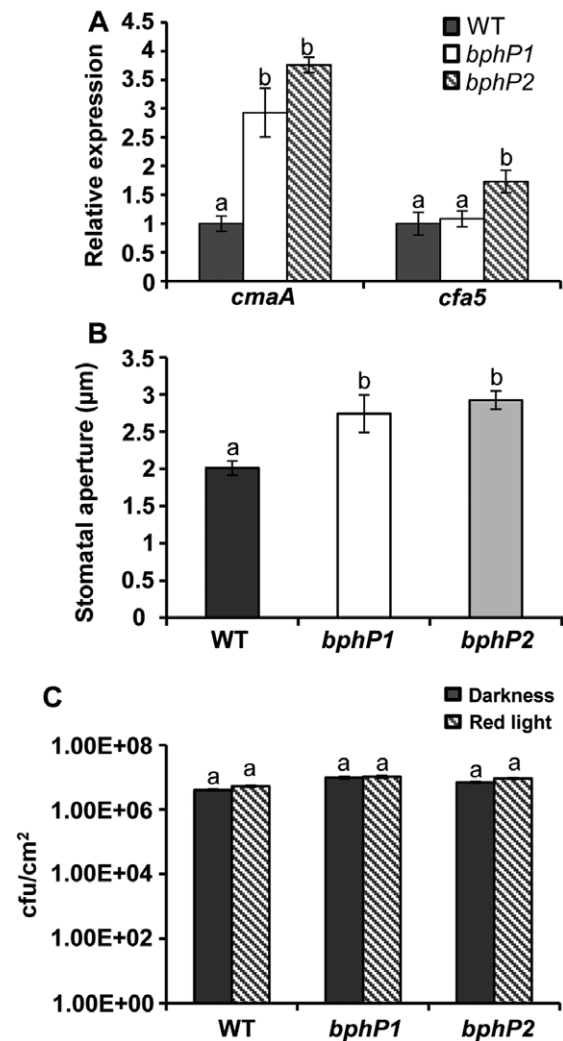


Fig. 6. *PsPto* phytochromes are involved in the red light-dependent regulation of coronatine gene expression that leads to an increased stomatal opening. **A.** Expression of coronatine biosynthetic genes in the wild-type and *bphP* mutants as evaluated by qRT-PCR after a 10 min treatment with red light. Bars represent the relative expression changes of *cmaA* and *cfa5* genes in the *bphP* mutants with respect to the wild-type strain. Data represent the means and standard errors of three replicates, with different letters indicating significant differences among treatments ($p < 0.05$, Fisher's LSD test). **B.** Stomatal aperture on tomato leaves infected at dusk with the wild-type or the phytochrome mutants previously treated with red light for 10 min, and then incubated for 3 h in the dark. The aperture width of 30 randomly-selected stomata on a leaf was measured, and three independent leaves were examined for each strain. Significant differences among the wild-type (dark grey bar), *bphP1* mutant (white bar) and *bphP2* mutant (light grey bar) were determined based on a Fisher's LSD test (p -value < 0.05). **C.** Bacterial population was observed on tomato leaves at 3 dpi after syringe infiltration with *PsPto* wild-type or the *bphP* mutants, treated with red light (striped bar) or maintained in the dark (dark grey bar) for 10 min prior to inoculation. Plants were infiltrated at the subjective dusk with immediate transfer to the dark. The means and standard errors of three replicates are shown. Significant differences between treatments were determined according to Student's t -test ($p < 0.05$).

light conditions but not in the dark. These results illustrate that this photoreceptor functions as a repressor of motility under light conditions, as was previously reported in *Pss*

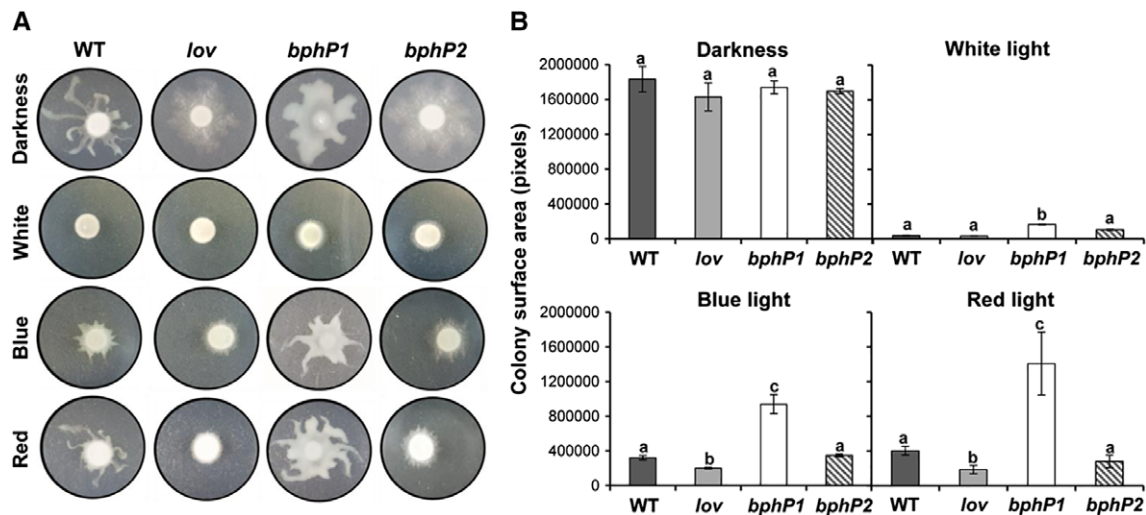


Fig. 7. *PsPto* swarming motility is regulated by changes in light quality detected through photoreceptors. The wild-type *PsPto* and three photoreceptor mutants were grown on soft KB agar under dark conditions or white, blue, or red light. Five replicates of each strain were placed on a single plate and were examined after 16 h. A. Photographs of representative swarm colonies. B. Quantification of lateral colony surface area in digital images of the colonies. Data represent the means and standard errors of the five replicates, with different letters indicating significant differences among the strains ($p < 0.05$, Fisher's LSD test). The results are representative of three independent experiments.

B728a (Wu *et al.*, 2013). Motility assays with the *bphP2* mutant showed no difference in swarming motility from the wild-type (Fig. 7). Studies with *P. syringae* B728a similarly found that loss of the bacteriophytochrome BphP2 did not detectably alter swarming motility (Wu *et al.*, 2013). These studies on the impacts of light on *PsPto* gene expression, virulence and swarming motility together support a model in which *PsPto* responds to diurnal changes in light to optimize invasion of leaves (Fig. 8). In particular, *PsPto* motility genes are maximally expressed at night, which could promote an active colonization of the leaf surface when the stomata are closed. Nocturnal priming of virulence could optimize the infection process at dawn. Nocturnal priming involves the expression of selected virulence-related traits in the absence of light. These traits include the expression of coronatine biosynthetic genes, which could support a successful infection at dawn when stomatal opening is tempered by PAMP-induced stomatal closure (Melotto *et al.*, 2006; Panchal *et al.*, 2016) and the apoplast humidity is high. Motility, which is necessary to enter the plant apoplast, is also primed by the absence of light. Blue, white and red light all reduce the expression of coronatine biosynthesis genes, but red light has the strongest repressive effect.

Although the biological significance of this sensitivity to red light is not clear, red light dominates the light spectrum at dawn and thus may ensure a down-regulation of the expression of coronatine biosynthetic genes, which could lead to a reduction in coronatine production. Coronatine induces ROS in a light-dependent manner and coronatine-induced ROS are necessary for necrotic cell

death in bacterial speck disease (Ishiga *et al.*, 2009). Thus, the prevalence of red light during the first stages of infection at dawn may provide a signal to minimize early coronatine-mediated ROS generation; alternatively, the greater penetrating effect of red/far-red light over blue light into the leaf tissue may reduce coronatine production within the apoplast.

In our model, the increased quantity of light of all wavelengths by mid-day would ensure the up-regulation of genes associated with environmental stress tolerance, including genes for the production of alginate, a polysaccharide that provides hydration and promotes adhesion to leaf surfaces, anti-oxidative enzymes such as catalase, and compatible solutes for tolerating water limitation. Our results are consistent with *PsPto* prioritizing the need to cope with light-associated stressful conditions over active stomatal entry during the day, despite that stomata are open. Interestingly, the expression of the *PsPto* T3SS was strongest in the presence of blue light. The amount of blue light that reaches the earth's surface increases at mid-day, and although the intensity of blue light reaching the apoplast would be attenuated via absorption by chlorophyll, the intensity of blue light would be greatest at mid-day even in the apoplast. Bacterial expression of the T3SS at mid-day would be commensurate with *PsPto*-plant interactions that suppress basal defences and support *PsPto* multiplication in the apoplast. By dusk when the stomata are mostly closed, the long period of light-mediated repression of coronatine synthesis and motility would have greatly lowered the probability of stomatal entry, with the red light-enrichment

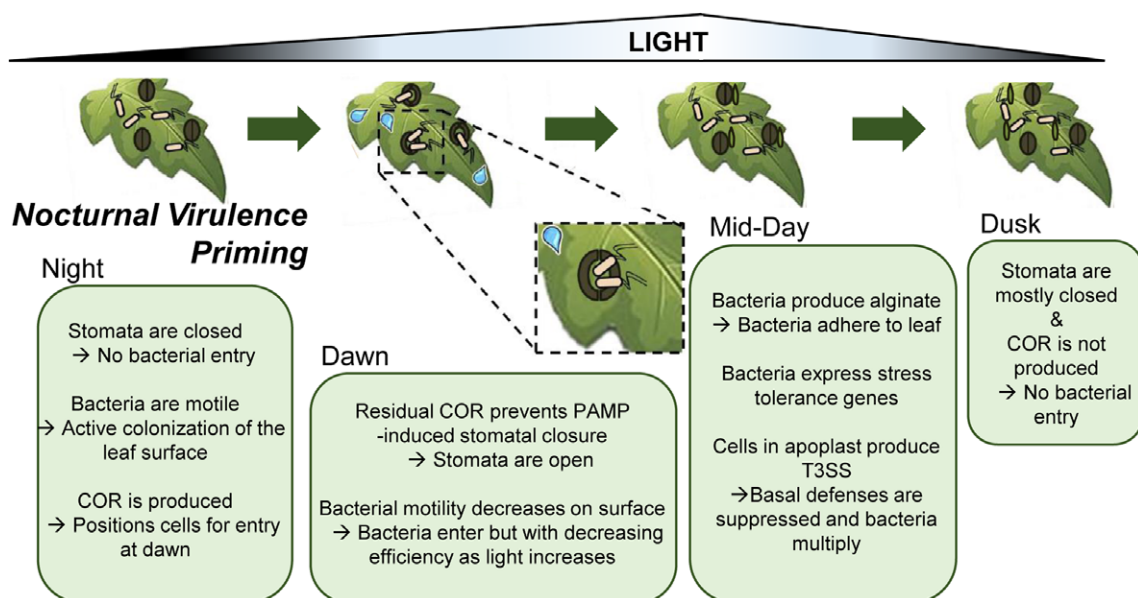


Fig. 8. Model illustrating the impact of *PsPto* light-sensing at various times through the diurnal light cycle on virulence and colonization of tomato leaves. See Conclusion section for a detailed description of the model.

associated with dusk contributing to repression of coronatine synthesis.

Our results with the photoreceptor mutants are consistent with this model. They illustrate an active role for the photoreceptors in nocturnal virulence priming and provide evidence for dark-mediated photoreceptor activities. They also illustrate a role for white and blue light-mediated photoreceptor activities in promoting virulence following inoculation at subjective dawn, and for red light-mediated photoreceptor activities in reducing virulence following inoculation at subjective dusk. Our experiments demonstrated that the latter was due, at least in part, to the key role of a bacteriophytochrome in repressing coronatine biosynthetic gene expression in response to red light.

Altogether these results illustrate that *PsPto* has the ability to integrate distinct monochromatic light signals over the course of a day, and by regulating the expression of large numbers of genes, *PsPto* exploits diurnal changes in light to maximize its virulence. Thus, just as plants exploit light cues to maximize their resistance to pathogens (Roden and Ingle, 2009; Kangasjärvi *et al.*, 2012), bacteria can exploit light cues to maximize their virulence and survival on plants.

Experimental procedures

Microbiological methods

Bacterial strains and plasmids used in this study are listed in Supporting Information Table S1. *PsPto* derivatives were grown at 28 °C in King's B (KB) medium (King *et al.*, 1954). *Escherichia coli* derivatives were grown at

37 °C in Luria-Bertani (LB) medium (Bertani, 1951). When appropriate, antibiotics were added to the medium at the following concentrations (μg/ml): rifampicin, 25; streptomycin, 20; kanamycin, 50; nitrofurantoin, 5; ampicillin, 100; chloramphenicol, 10.

Illumination conditions

White light was provided using fluorescent tubes (Osram, Munich, Germany) at an intensity of 70 μE/m²s. Red and blue lights were provided using GreenPower LED HF red (660 nm) and GreenPower LED HF blue (458 nm) lights (Philips, Amsterdam, The Netherlands), respectively, each at an intensity of 20 μE/m²s. The LED spectra are similar to gaussians centered on the nominal wavelength and with a full width at half maximum of FWHM = 20 nm. LED lighting was chosen because this lighting does not produce heat in the form of infrared radiation. To maintain dark conditions, plates and flasks were covered with two layers of aluminium foil.

For the determination of white light spectrum, a portable fibre-fed spectrometer (JAZ EL200-XR1 Extended-Range UV-VIS Spectrometer, Ocean Optics, Dunedin, Florida) was used. The intensities of 10 exposures were averaged to avoid possible blinking of the lamps. Light intensity was determined using two photometers, a Sekonic C-700-U SpectroMaster Colour Meter (Sekonic Corporation, Japan) and a LI-COR LI-250A Light Meter (LI-COR Biosciences, Lincoln, Nebraska). The photometers were placed side-by-side with the optic fibre to verify appropriate calibration. The portable spectrograph was

calibrated in flux using a standard tungsten lamp as reference. The wavelength measurements were calibrated based on the spectra of thorium-neon and thorium-argon lamps using standard procedures.

Molecular biology techniques

Bacterial growth was monitored based on optical density measured at 600 nm (OD_{600}) using a Jenway 6300 spectrophotometer. Plasmid DNA was isolated with the NucleoSpin Plasmid Kit (Macherey-Nagel, Düren, Germany). DNA restriction, dephosphorylation, ligation and electrophoresis were performed using standard protocols (Sambrook *et al.*, 1989) and manufacturer instructions. DNA fragments were recovered from agarose gels using the GenJet Gel Extraction Kit (Thermo Scientific, Waltham, Massachusetts). Competent cells were prepared and transformations were carried out using standard procedures (Sambrook *et al.*, 1989). KAPA HiFi DNA polymerase (Kapa Biosystems, Wilmington, Massachusetts) was used for the amplification of DNA fragments.

Construction of *PsPto* *bphP1* and *bphP2* mutants

To generate the *bphP1* mutant, an internal 442-bp fragment of PSPTO_1902 (corresponding to nucleotides 44–485 of the coding sequence) was amplified by PCR from the *PsPto* genome using primers 1902XbaF (5'-ATTGTCTAGA TGAGCCCATTCAG-3') and 1902EcoRIR (5'-CTCTG AATCCACGGACACTG-3') and cloned into pCHESI- Ω Km (Llamas *et al.*, 2003). For the construction of the *bphP2* mutant, an internal 618-bp sequence of PSPTO_2652 (corresponding to nucleotides 35–652 of the coding sequence) was amplified by PCR from the *PsPto* genome using primers 2652EcoRIF (5'-GAATCGAATTCAGGCGATACA-3') and 2652XbaR (5'-CAGCTCTAGAAGCAGCGCTTG-3') and cloned into pCHESI- Ω Km. The resulting plasmids were introduced into *PsPto* by triparental mating (De Lorenzo and Timmis, 1994) using *E. coli* CC118 λ pir (for *bphP1*) or *E. coli* DH5 α (for *bphP2*) as donors and *E. coli* HB101 (pRK600) as a helper strain. As pCHESI- Ω Km cannot replicate in *PsPto*, single crossover cointegrates were selected based on resistance to kanamycin. Nitrofurantoin was added to the selection plates to counter-select the donor strain. Plasmid integration was confirmed using PCR and a Southern Blot.

RNA extraction

Bacterial cells grown overnight in the dark at 28 °C on KB agar were resuspended in 40 ml of liquid KB medium and adjusted to an OD_{600} of 0.05. These bacterial suspensions were incubated in the dark at 28 °C with shaking at 150 r.p.m to an OD_{600} of 0.5. Cells placed in flasks

were then treated for 10 min under white light (70 μ E/m²s), red light (20 μ E/m²s), blue light (20 μ E/m²s) or maintained in the darkness. These treatments were replicated four times. Total RNA was extracted separately from each biological replicate from each treatment using the TRI Reagent (Ambion) as recommended by the manufacturer. The RNA samples were pre-treated with RNase-free DNase I (Roche, Basel, Switzerland) and RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen Corp., Carlsbad, California), followed by purification with RNeasy columns (Qiagen, Hilden, Germany). The RNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA), their integrity was assessed by agarose gel electrophoresis, and their quality was evaluated using an Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, California).

Microarray hybridization experiments

For each treatment, the four biological replicates were independently hybridized onto custom-designed microarrays containing the complete *PsPto* genome (Agilent *PsPto* Oligo Microarrays 8x15K; #042459, Agilent Technologies). Fluorescently-labelled cDNA for microarray hybridization was obtained using the superscript Indirect cDNA labeling system (Invitrogen Corp.). In brief, 15 μ g of total RNA were converted into cDNA using superscript III reverse transcriptase and random hexamers as primers, and including aminoallyl-modified nucleotides in the reaction mixture. After cDNA purification, the Cy3 fluorescent dyes (Amersham Biosciences Corp, Piscataway, New Jersey) were coupled to the amino-modified first-strand cDNA, and the labelling efficiencies were assessed using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). Hybridization of these probes to the microarray was performed as described (One-Colour Microarray Based Gene Expression Analysis Manual Ver. 6.5, Agilent Technologies). Briefly, for each hybridization, 600 ng of the Cy3 probes were combined with 5 μ l of 10X blocking agent and nuclease-free water in a 25 μ l reaction, which was then combined with 25 μ l of 2X GExHybridization buffer and placed on ice, and then loaded onto a microarray and allowed to hybridize at 65 °C for 17 h. The microarrays were then washed in GE wash buffer 1 at room temperature for 1 min and in GE wash buffer 2 at 37 °C for 1 min.

Images from the Cy3 channel were equilibrated and captured with a High-Resolution Microarray Scanner (Agilent Technologies) and the fluorescence in each spot was quantified using Feature Extraction Software (Agilent Genomics, Santa Clara, California, USA). Background correction and normalization of expression data were performed using LIMMA (Smyth and Speed, 2003; Smyth,

2004). LIMMA is part of Bioconductor, an R language project (Ihaka and Gentleman, 1996). For local background correction and normalization, the methods “normexp” and loess in LIMMA were used, respectively (Smyth, 2004). To have similar distribution across arrays and to achieve consistency among the arrays, the log-ratio values were scaled using the median-absolute-value as a scale estimator (Smyth and Speed, 2003). Differentially-expressed genes were determined using methods for linear models. Each probe was tested for changes in expression by using an empirical Bayes moderated *t*-statistic (Smyth, 2004). To control the false discovery rate (FDR), *p*-values were corrected using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). The expected FDR was controlled to be less than 1%; thus, genes were declared to be differentially expressed if they exhibited a fold-change ≥ 2 and an FDR ≤ 0.01 . Hybridizations and statistical analyses were performed by the Genomics Facility at Centro Nacional de Biotecnología. Hierarchical clustering was performed using the average linkage method and Pearson's correlation coefficient as a measure of similarity in the Multiexperiment Viewer (MeV) v.s. 4.3 software (www.mev.tn4.org), thus grouping the samples based on the degree of similarity in gene expression.

Assignment and analysis of gene representation in functional categories

The *PsPto* genes were assigned to 69 functional categories (Supporting Information Table S4), following the procedure described by Yu *et al.* (2013). Each gene was assigned to a single functional category or to no category if it could be placed into multiple categories or its function was unknown. For each functional category, we computed the number of differentially-expressed and non-differentially-expressed genes in that category, as well as the number of differentially-expressed and non-differentially-expressed genes not in that category. Genes that were enriched in a given functional category were identified by performing a hypergeometric test (*p*-value ≤ 0.05) using the R function ‘phyper’. The whole analysis was performed using R version 3.3.1 (R Core Team, 2014; <http://www.R-project.org/>) with *ad hoc* scripts (available upon request).

Quantitative real time Polymerase Chain Reaction

Total RNA isolated for the microarray analyses described previously, was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). Primers were designed to amplify fragments of approximately 100 bp for all genes of interest (Supporting Information Table S5),

including *rpoD*, which was used as an internal control for normalization. This gene has been described as an invariant housekeeping gene for *P. syringae* (Sawada *et al.*, 1999). Real-time PCR amplifications were carried out on a ABI PRISM 7300 RT PCR System (Applied Biosystems) using a SYBR Green PCR Master Mix (Applied Biosystems). Thermal cycling conditions were as follows: one cycle at 95 °C for 10 min; 50 cycles at 95 °C for 15 s and 65 °C for 1 min; and a final cycle at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. The relative gene expression ratio was calculated using the comparative critical threshold ($\Delta\Delta C_t$) method (Pfaffl, 2001; Rotenberg *et al.*, 2006).

Tomato virulence assays

PsPto strains were grown at 28 °C for 24 h on KB agar under dark conditions. Cells were resuspended in 10 mM MgCl₂ and diluted to 10⁸ cfu/ml with all activities performed under dark conditions (less than 0.05 $\mu\text{E}/\text{m}^2\text{s}$). Prior to inoculation, the bacterial suspensions placed in flasks were either subjected to a light treatment (white, red or blue light) for 10 min or maintained in the darkness. Silwet L-77 was added to the bacterial suspensions at a final concentration of 0.02% (vol/vol) immediately before inoculation.

Three-week-old tomato plants (*Solanum lycopersicum* cv Moneymaker) were collected either at the end of a 12 h night cycle or at the end of a 12 h day cycle; inoculations of these plants corresponded to inoculation at the subjective dawn or subjective dusk, respectively. Plants were inoculated by immersing all of the leaves in a bacterial suspension (10⁸ cfu/ml) for 30 s. Plants were incubated in a growth chamber at 25 °C and 60% RH with a daily light period (100 $\mu\text{E}/\text{m}^2\text{s}$) of 12 h. Six days after inoculation, the leaf symptoms were recorded and bacterial populations from three plants were enumerated by sampling five 1-cm leaf disks per plant. The infected leaf disks were washed twice with 10 mM MgCl₂ before homogenized to eliminate the bacteria from the leaf surface. Plant material was homogenized in 10 mM MgCl₂, and drop plated on KB agar supplemented with the appropriate antibiotics. The average number of bacteria per cm² isolated from five infected tomato leaves were determined based on log-transformed data, and the treatments were compared using generalized linear models (GzLM), when variances were different, or one way analysis of variance (ANOVA) when variances were equal; followed by Fisher's least-significant-difference (LSD) *post hoc* test for multiple comparisons, performed using the statistical software package SPSS 22.0 (SPSS, Chicago, Illinois).

For the infiltration assays, leaves were infiltrated with a suspension containing 3×10^4 (cfu/ml), and three leaves were mock-infiltrated with 10 mM MgCl₂. The bacterial

populations were enumerated at 3 dpi following the same protocol described above for the dipping assay.

Stomatal assays

Tomato plants were collected at the subjective dusk. Individual leaves were detached and placed abaxial side down on a 300- μ L drop of bacterial suspension (10^8 cfu/ml) on a microscope slide; this was to ensure bacterial contact. The leaves were cut to ensure that they remained flat on the slide. The leaves in contact with the bacteria were then incubated in the dark in a growth chamber for 3 h. We measured stomatal aperture as described previously (Melotto *et al.*, 2006; Chitrakar and Melotto, 2010; Panchal *et al.*, 2016), except that leaves were directly analysed without propidium iodide staining. In particular, we imaged the abaxial surface of the leaves using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Stomatal apertures were measured using the open-source software ImageJ, specifically the Fiji distribution (Schindelin *et al.*, 2012). For each treatment, we imaged three independent leaves and measured 30 stomata on each leaf. Samples were kept under the described treatments exactly the same time considering also the time spent during the microscopy analysis. Significant differences were determined based on a Fisher's LSD test (p -value < 0.05), performed using the statistical software package SPSS 22.0.

Swarming motility assays

PsPto strains were grown at 28 °C for 24 h on KB agar under dark conditions. Cells were resuspended in KB medium to an OD₆₀₀ of 1. Five microliters of the bacterial suspension were spotted onto soft KB agar (0.5% agar, wt/vol). Plates were incubated for 16 h at 28 °C and 80% RH under white light (70 μ E/m²s), red light (20 μ E/m²s), blue light (20 μ E/m²s) or dark conditions. Three replicate plates were prepared for each treatment, and three replicate experiments were performed. Swarm colonies were photographed, the surface area of each colony was quantified using the area selection tool of Adobe Photoshop software with readings in pixels, and treatment comparisons were made using Fisher's LSD test (p -value < 0.05).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Spectrum of the fluorescent lamp (white light). The spectrum is dominated by mercury lines. The resulting light from the fluorescent is white with a correlated colour temperature CCT = 2700 K. Blue and red lines representing the wavelength of the blue (458 nm) and red (660 nm) LED lights used in this study are also included.

Fig. S2. Luciferase activity of *PsPto* derivative harbouring the *luxCDABE* operon. Bioluminescence was determined on *PsPto* cultures that had been pretreated for 10 min either with white light, blue light or red light and compared to the darkness-treated cells. Luciferase activity is given in relative light units (RLU) per OD_{600nm}. Data represent the means and standard errors of three replicates ($p < 0.05$, Student's *t*-test). Bacterial cells grown overnight in the dark at 28 °C on KB agar were resuspended in liquid KB medium and adjusted to an OD₆₀₀ of 0.05. These bacterial suspensions were incubated in the dark at 28 °C with shaking at 150 r.p.m to an OD₆₀₀ of 0.5. Cells placed in flasks were then treated for 10 min under white light (70 µE/m²s), red light (20 µE/m²s), blue light (20 µE/m²s) or maintained in the darkness. For quantification of bioluminescence, cells were diluted to an OD₆₀₀ of 0.05 and the luciferase activity was recorded immediately thereafter in a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific). Bioluminescence was also determined 30 min after the light treatments (AT) and 1 h AT.

Fig. S3. Expression of *PsPto* genes in hrp-inducing medium after light treatments. Bacterial suspensions were incubated in the dark at 24 °C in hrp-inducing medium (Huynh et al.,) with shaking at 150 r.p.m to an OD₆₀₀ of 0.5. Cells placed in flasks were then treated for 10 min under blue light (20 µE/m²s), red light (20 µE/m²s) or maintained in the darkness. (A) Differential expression of *hrpR* and *avrPto* genes was evaluated by qRT-PCR after a 10 min treatment with blue light or maintained in the darkness. Bars represent the relative expression changes in the blue-treated wild-type strain with respect to the wild-type strain maintained in the

darkness. (B) Differential expression of *cmaA* gene was evaluated by qRT-PCR after a 10 min treatment with red light in the *bphP1* mutant with respect to the wild-type strain. The means and standard errors of three replicates are shown. Data represent the means and standard errors of three replicates, with different letters indicating significant differences among treatments ($p < 0.05$, Fisher's LSD test).

Fig. S4. Expression of *fliC* and *cmaA* is regulated by light perception through the photoreceptor functions. Differential expression of *fliC* gene (A) and *cmaA* gene (B) was evaluated by qRT-PCR after a 10 min treatment either with white light, blue light, red light or maintained in the darkness in the *lov* (light grey bars), *bphP1* (white bars) and *bphP2* (striped bars) mutants with respect to the wild-type strain (dark grey bars). Bars represent the relative expression changes in the

mutants with respect to the wild-type strain. The means and standard errors of three replicates are shown. Data represent the means and standard errors of three replicates, with different letters indicating significant differences among treatments ($p < 0.05$, Fisher's LSD test).

Table S1. Bacteria and plasmids used.

Table S2. Functional categories overrepresented after the white, blue or red light treatments.

Table S3. Differentially expressed genes in the overrepresented functional categories after the white, blue or red light treatments.

Table S4. *PsPto* genes that were assigned to each functional category.

Table S5. qRT-PCR primers used in this study.