

LOV-domain photoreceptor, encoded in a genomic island, attenuates the virulence of *Pseudomonas syringae* in light-exposed *Arabidopsis* leaves

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SUMMARY

In *Arabidopsis thaliana*, light signals modulate the defences against bacteria. Here we show that light perceived by the LOV domain-regulated two-component system (Pst–Lov) of *Pseudomonas syringae* pv. tomato DC3000 (Pst DC3000) modulates virulence against *A. thaliana*. Bioinformatic analysis and the existence of an episomal circular intermediate indicate that the locus encoding Pst–Lov is present in an active genomic island acquired by horizontal transfer. Strains mutated at Pst–Lov showed enhanced growth on minimal medium and in leaves of *A. thaliana* exposed to light, but not in leaves incubated in darkness or buried in the soil. Pst–Lov repressed the expression of principal and alternative sigma factor genes and their downstream targets linked to bacterial growth, virulence and quorum sensing, in a strictly light-dependent manner. We propose that the function of Pst–Lov is to distinguish between soil (dark) and leaf (light) environments, attenuating the damage caused to host tissues while releasing growth out of the host. Therefore, in addition to its direct actions via photosynthesis and plant sensory receptors, light may affect plants indirectly via the sensory receptors of bacterial pathogens.

Keywords: light, LOV domain, two-component system, plant-pathogen interaction, *Pseudomonas syringae* pv. tomato DC3000, *Arabidopsis thaliana*.

INTRODUCTION

In addition to its action through photosynthesis, light perceived by photo-sensory receptors has profound effects on plant growth and development (Kami *et al.*, 2010), including the regulation of plant defences against bacterial attack (Genoud *et al.*, 2002; Faigón-Soverna *et al.*, 2006; Wu and Yang, 2010). Light signals perceived by non-photosynthetic bacteria may alter their interactions with hosts, including plants. For instance, light-activated bacterial two-component systems enhance infection of macrophages by *Brucella abortus* (Swartz *et al.*, 2007), the ability of the opportunistic pathogen *Acinetobacter baumannii* to kill cells of the fungus *Candida albicans* (Mussi *et al.*, 2010), the invasiveness of *Listeria monocytogenes* for Caco-2 enterocytes (Ondrusch and

Kreft, 2011), the nodulation of *Rhizobium leguminosarum* in plant roots (Bonomi *et al.*, 2012), and the adhesion of *Xanthomonas axonopodis* pv. citri strains to the leaves of orange (*Citrus sinensis*) trees (Kraiselburd *et al.*, 2012). The effects of light on *A. baumannii* are mediated by a blue light-sensing-using flavin (BLUF) domain protein (Mussi *et al.*, 2010); those on *L. monocytogenes* are mediated by a LOV (light, oxygen, voltage) domain linked to a C-terminal domain typical of sulfate transporters and anti-sigma factor antagonists (Ondrusch and Kreft, 2011); those on *B. abortus*, *R. leguminosarum* and *X. axonopodis* are mediated by sensor histidine kinases bearing a LOV domain (Swartz *et al.*, 2007; Bonomi *et al.*, 2012; Kraiselburd *et al.*, 2012). A locus

encoding a LOV sensor histidine kinase and a single-domain response regulator is a negative regulator of the general stress pathway in *Caulobacter crescentus*, but attempts to modulate transcription of the general stress pathway of *C. crescentus* using various light stimuli under standard growth conditions have been unsuccessful (Foreman *et al.*, 2012).

The benefits of enhancing virulence (Swartz *et al.*, 2007; Mussi *et al.*, 2010; Ondrusch and Kreft, 2011), nodulation (Bonomi *et al.*, 2012) or adhesion (Kraiselburd *et al.*, 2012) in the light but not in darkness are not clear (McBride, 2010; Gomelsky and Hoff, 2011). For instance, light may prepare the *B. abortus* present in the aborted placenta for infection of the next host, and light reduction inside the host may make the pathogen less aggressive, favouring long-term survival (Swartz *et al.*, 2007; Gomelsky and Hoff, 2011). Alternatively, bacterial pathogens may be more virulent at night, or there may eventually be enough light inside the body of the animal host to promote virulence. Testing these ideas requires comparison of wild-type and mutant bacterial strains in the actual tissues in which they proliferate inside and outside the host body, which is experimentally challenging in many systems. Given the available knowledge concerning light as a source of information for plants, and the routine techniques developed for plant light treatments, the plant–bacteria system appears an ideal model to investigate the functional significance of bacterial light responses in general.

Pseudomonas syringae pv. tomato is the causative agent of bacterial speck disease, which severely reduces the yield and marketability of tomato crops (Pedley and Martin, 2003). The open reading frame (ORF) *PSPTO2896* from *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) encodes a LOV-domain protein of 534 amino acids (Pst–Lov), corresponding to a molecular mass of 58.9 kDa (Cao *et al.*, 2008). The full-length Pst–Lov protein contains an N-terminal LOV domain that binds a flavin chromophore, a histidine kinase motif and the receiver domain (not the output domain) of a response regulator, which form a blue light-inducible two-component signal transduction system (Cao *et al.*, 2008). *Arabidopsis thaliana* accession Columbia is susceptible to *Pst* DC3000, and this plant–pathogen system has become a model for molecular and genetic studies (Whalen *et al.*, 1991; Preston, 2000; Katagiria *et al.*, 2002). Here we provide insight into the biological function of Pst–Lov by addressing its evolutionary origin, and its impact on transcript patterns and on plant–pathogen interactions in this model system.

RESULTS AND DISCUSSION

Incongruence between LOV-domain ORF-based trees and 16S rRNA-based trees in Proteobacteria

Determination of the phylogenetic relationship among well-characterized species belonging to the phylum Proteobacteria based on the LOV-domain ORFs revealed incon-

gruence with 16S rRNA-based trees (Yarza *et al.*, 2010). The γ -proteobacteria *Pst* DC3000 and *Xanthomonas axonopodis* pv. citri str. 306 clustered within α -proteobacteria, and the α -proteobacterium *Rhizobium leguminosarum* biovar viciae strain 3841 clustered within β -proteobacteria (Figure 1a). The LOV domains of all *Pseudomonas syringae* and *Xanthomonas* spp. strains tested here clustered together (>90% amino acidic identity, Figure S1). The incongruent pattern is not due to sampling error, as indicated by the general congruence with the 16S rRNA-based trees (except for the indicated incongruence), which would be unlikely by chance ($P < 3 \times 10^{-77}$) (Li, 1997), and the high bootstrap values (mostly >80). The incongruence was also observed in phylogenetic trees constructed using either the neighbour-joining method (Figure 1a) or maximum-parsimony method (Figure S2). These observations suggest that ancestral strains of the species *Pseudomonas syringae* and the genus *Xanthomonas* may have obtained the same LOV-domain gene by horizontal transfer.

The *PSPTO2896* gene of *Pst* DC3000 is within a genomic island

The *PSPTO2896* gene was found within a large mobile genetic element (57 586 bp) that is different from other genomic islands described previously for *Pst* DC3000 (Alfano *et al.*, 2000; Pitman *et al.*, 2005), and which we named LOV–GI (LOV-domain gene genomic island). We were unable to identify a mobile genetic element containing the LOV-domain locus in *Xanthomonas* or in *P. syringae* other than *Pst* DC3000, which is not surprising given the intrinsic instability of genomic islands (Van Der Meer and Sentchilo, 2003). The LOV–GI is located in the chromosome region 3 212 683–3 270 268 (NC_004578.1), and contains 46 putative ORFs. LOV–GI is flanked by a 14 bp (5'-CCA-GAACGCCACGC-3') direct repeat, which is part of its site-specific recombination site (DR in Figure 1b, and Table S1). Several LOV–GI regions have G + C contents that are significantly higher (up to 65%) or lower (up to 42%) than the mean for the *Pst* DC3000 chromosome (58.4%) (Buell *et al.*, 2003) (Figure 1b and Table S1), and this pattern is consistent with horizontal transfer. LOV–GI encodes proteins typical of mobile elements such as an integrase homologous to XerC, which is involved in the excision and integration of genomic islands (Toussaint *et al.*, 2003; Ayub *et al.*, 2007), a DNA helicase and two transposases (Toussaint *et al.*, 2003) (Figure 1b and Table S1). In addition to Pst–Lov, LOV–GI encodes other genes involved in circadian timing (LabA-like protein; Taniguchi *et al.*, 2007) or light responses (TspO protein; Yeliseev and Kaplan, 1995).

LOV–GI contains putative virulence factors

LOV–GI contains several putative virulence factors such as tellurite and vancomycin resistance proteins (Lopez *et al.*, 2011; Ponnusamy *et al.*, 2011), proteins with similarity to

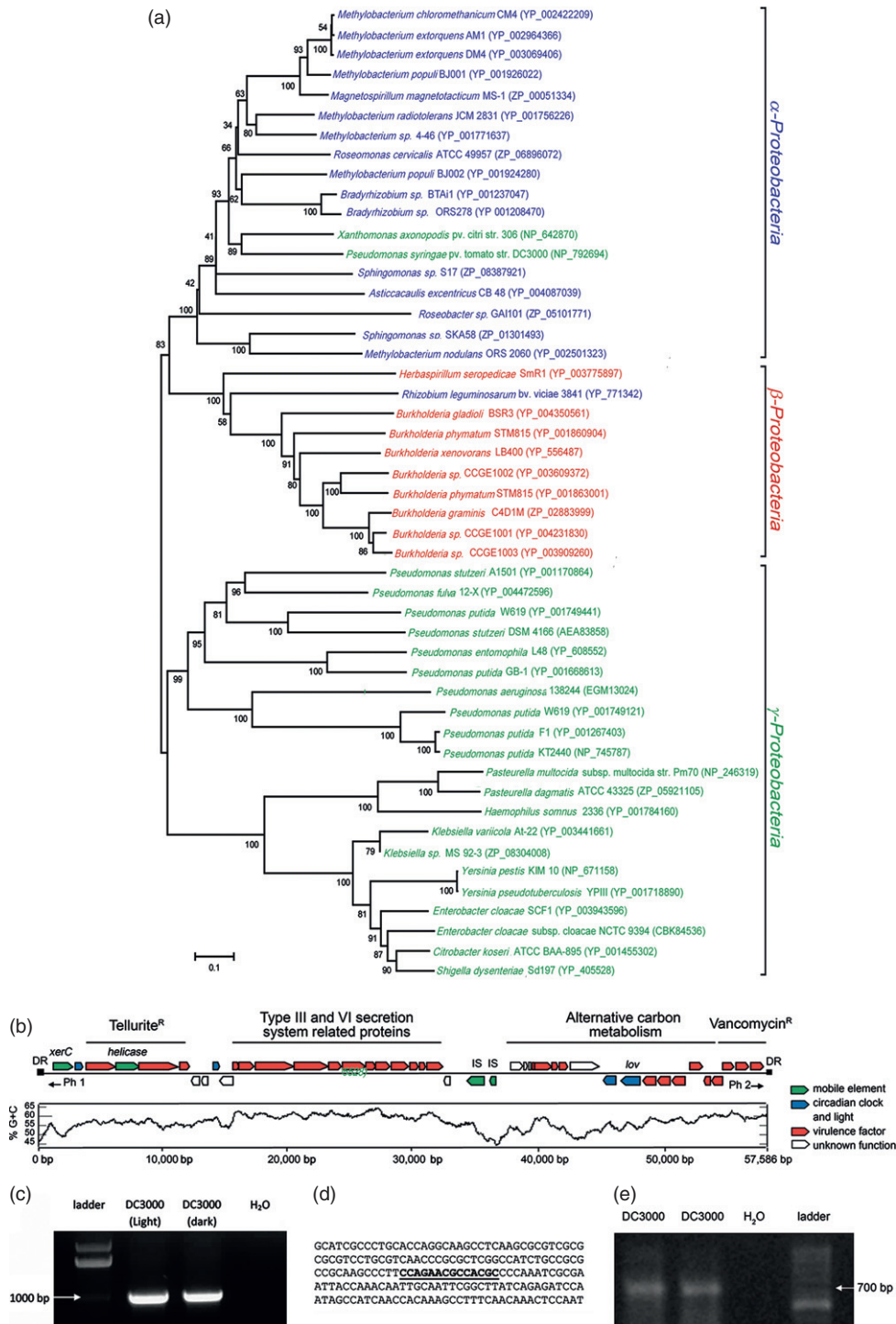


Figure 1. *Pseudomonas syringae* acquired the LOV-domain gene by horizontal transfer of a virulence island. (a) Incongruence between LOV-domain ORF-based trees and 16S rRNA-based trees in Proteobacteria. Phylogenetic relationships between representative taxa of the phylum Proteobacteria based on neighbour-joining analysis of the LOV-domain ORFs. The α , β and γ sub-classes of Proteobacteria based on the 16S analysis (Yarza *et al.*, 2010) are shown in blue, red and green, respectively. Bootstrap percentages are indicated at the branch points. (b) Genetic organization of LOV-GI. Boxes indicate the position of direct repeats (DR). The percentage G + C content was calculated from a 1000 bp window moved along the sequence by 10 bp steps. Gene *PSPTO 2895* is labeled as *lov*. (c) Detection of a circular episome in *Pst* DC3000 by PCR. (d) Sequence of the PCR product showing the region containing the 14 bp overlap defining the DR site where circularization occurred (bold and underlined). (e) *PSPTO2895* and *PSPTO2896* belong to the same operon. The two left lanes show PCR products obtained using primers specific for each locus (independent biological samples) and the third lane corresponds to the mix without DNA.

type III and VI secretion system proteins (Schmidt and Hensel, 2004; Filloux *et al.*, 2008; Munkvold *et al.*, 2009), antioxidant proteins, and proteins associated with alternative carbon metabolism (Van Der Meer and Sentchilo, 2003) (Figure 1b and Table S1). Therefore, LOV-GI shares the common features of pathogenicity islands (Schmidt and Hensel, 2004). In the phylogenetic analysis, Pst-Lov of *Pseudomonas syringae* grouped closer to plant-associated strains of the α -proteobacteria genera *Methylobacterium* and *Bradyrhizobium* than to non-pathogenic species of *Pseudomonas*. The presence of *PSPTO2896* within a pathogenicity island is consistent with the idea that *Pseudomonas syringae* acquired Pst-Lov by horizontal transfer, probably simultaneously with the ability to become a plant pathogen.

LOV-GI is functional

Genomic islands are often integrated and excised via an episomal circular intermediate (Pitman *et al.*, 2005). The existence of circularized forms of the excised DNA was examined using primers ph1 and ph2 (Figure 1b) directed outwards from the internal boundaries of LOV-GI. We obtained a PCR product of 1000 bp from light- and dark-grown cultures of *Pst* DC3000 (Figure 1c), and DNA sequencing confirmed the presence of a circular molecule (Figure 1d). This indicates that LOV-GI is a functional genomic island, a feature that is important for the evolution of bacterial virulence in plants (Pitman *et al.*, 2005).

The Pst-Lov-permease operon

PSPTO2896 encodes Pst-Lov, a hybrid protein bearing a LOV domain, a histidine kinase domain and the receiver domain of a response regulator but not the output domain (Cao *et al.*, 2008). The adjacent *PSPTO2895* locus encodes a putative permease of the major facilitator superfamily (<http://img.jgi.doe.gov>). There are examples in which permeases become activated by the receiver domain of response regulators that lack an output domain (Maeda *et al.*, 2006) and may control virulence gene expression (Hung and Miller, 2009). To investigate whether *PSPTO2895* and *PSPTO2896* belong to the same operon, we used primers directed to sequences specific for each locus. Using cDNA, we obtained an RT-PCR product of the expected size (Figure 1e), which confirms that both loci are part of the same operon. As a control, a similar analysis involving primers for *PSPTO2896* and *PSPTO2897* failed to give a product. Pst-Lov may operate via the adjacent locus encoding a putative permease, but testing this hypothesis will require specific experiments.

Light-specific Pst-Lov-mediated repression of bacterial growth in minimal L medium

To investigate the function of Pst-Lov, we used two independent mutants of *PSPTO2896* (Δ *pst-lovA* and Δ *pst-lovB*), and the Δ *pst-lovB* mutant complemented with the

wild-type *PSPTO2896* gene under the control of its own promoter. All strains showed a similar growth pattern in aerobic cultures in minimal L medium at 28°C in darkness (Figure 2a). However, under white light, the wild-type and the complemented strain showed a longer initial lag than the Δ *pst-lovA* and Δ *pst-lovB* mutant strains (Figure 2a). When exposed to continuous blue light, Pst-Lov reduced the growth of *Pst* DC3000 at high irradiances but not at the lowest irradiance (Figure 2b). Therefore, Pst-Lov inhibits the growth of *Pst* DC3000 in a strictly light-dependent manner. The mutant strains retained part of the growth inhibition caused by light, and this may reflect the action of other photoreceptors such as phytochrome (Shah *et al.*, 2013) or harmful effects of light (Beattie and Lindow, 1995).

Light-specific repression of expression of sigma factor genes and their downstream targets by Pst-Lov

To analyse the molecular phenotype, we investigated the expression of selected genes in wild-type, the Δ *pst-lovA* mutant, the Δ *pst-lovB* mutant and the complemented strain in minimal L medium at 28°C under white light by real-time RT-PCR. To standardize expression levels, we used *gap1* (Schechter *et al.*, 2006; Vencato *et al.*, 2006; Swingle *et al.*, 2008; Filiatrault *et al.*, 2010) and *gyrB* (Ortiz-Martin *et al.*, 2010; Schreiber and Desveaux, 2011) as control genes, as their expression levels were unaffected by light or by the mutation (Figure S3). The standardized expression levels were similar for both control genes (Figure 1 and Figure S4). Taking into account the fact that the *PSPTO2896* gene is situated within LOV-GI with features of a pathogenicity island (Figure 1) and inhibits growth in minimal L medium (Figure 2a), the list of genes included *rpoD*, which is the principal sigma factor gene and is highly expressed during exponential growth, the alternative sigma factor genes *rpoS*, *rpoN* and *hrpL*, which are involved in the general stress response and secondary metabolite production (*rpoS*), the nutrient-scavenging stress response and expression of *hrpL* (*rpoN*) and production of the type III secretion system and the effectors secreted by this pathway (*hrpL*) (Fujita *et al.*, 1994; Chatterjee *et al.*, 2003). In darkness, these four sigma factor genes showed high expression that was unaffected by the presence or absence of Pst-Lov (Figure 2c). However, Pst-Lov significantly reduced expression of these genes under white light as indicated by comparison of the wild-type and the complemented strain against the Δ *pst-lovA* and Δ *pst-lovB* mutants (Figure 2c). These effects on expression of the principal and alternative sigma factors suggest that Pst-Lov is a master negative regulator of gene expression in *Pst* DC3000. In fact, the *GacS/A* regulon genes that operate upstream of *rpoN* and *rpoS* (Chatterjee *et al.*, 2003) also showed reduced expression in the presence of Pst-Lov under white light (Figure 2c). We also analysed the expression of the type III secretion component *hrpE* and

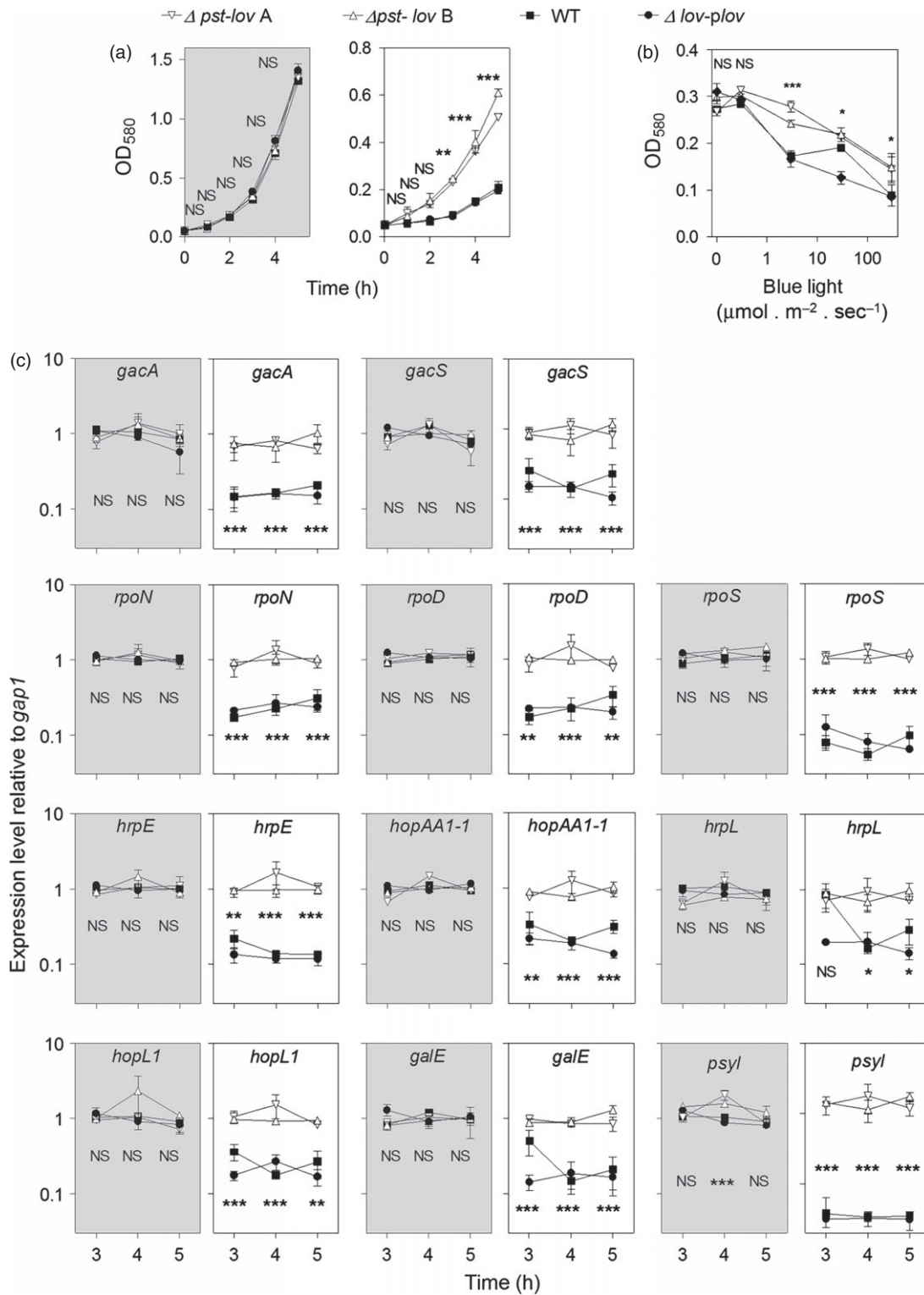


Figure 2. Pst–Lov inhibits growth and the expression of sigma factor and downstream genes in *Pst* DC3000 in the light. (a) Growth at 28°C in L medium under white light. Time = 0 indicates the start of the culture and the start of irradiation for the light-treated samples. (b) Growth at 28°C in L medium under blue light plotted against irradiance. (c) Gene expression at 28°C in L medium under white light normalized to *gap1* expression. In (a) and (c), boxes with grey or white backgrounds correspond to samples grown in full darkness or under white light, respectively. Values are means \pm SE of three (a, c) or two (b) biological replicates. The significance of the difference between lines with or without Pst–Lov is indicated (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant; ANOVA).

the type III effector *hopAA1-1*, which are virulence genes and downstream targets of RpoN/HrpL (Chatterjee *et al.*, 2003; Munkvold *et al.*, 2009). As expected, based on their control by RpoN/HrpL, expression of *hrpE* and *hopAA1-1* was repressed by Pst-Lov in a strictly light-dependent manner (Figure 2c). A similar pattern was observed for *psyl*, the N-acyl homoserine lactone synthase-encoding gene that is involved in generation of a quorum sensing signal and controlled by GacA and RpoS (Chatterjee *et al.*, 2007) (Figure 2c).

As expected from the higher expression of *gacA* and *rpoS* (Chatterjee *et al.*, 2003) in the light, the $\Delta pst-lovA$ and $\Delta pst-lovB$ mutants showed enhanced resistance to oxidative stress in the light [inhibition zone (mm). darkness: WT = 24 ± 1 ; $\Delta pst-lovA$ = 25 ± 1 ; $\Delta pst-lovB$ = 25 ± 0 ; light: WT = 35 ± 1 ; $\Delta pst-lovA$ = 25 ± 0 ; $\Delta pst-lovB$ = 24 ± 1 ; means \pm SD, $n = 3$].

Pst-Lov also repressed the expression of *hopL1*, a putative type III effector gene without an Hrp box (Schechter *et al.*, 2006), and *galE*, a UDP-galactose 4'-epimerase gene involved in biofilm formation (Niou *et al.*, 2009), in light-exposed cultures (Figure 2c). Thus, *PSPTO2896* acts as a negative regulator *in trans* (*hrpE* and *hopAA1-1*) as well as *in cis* (*hopL* and *galE*), probably due to its effects on global regulators.

The expression of the genes reported here showed no significant responses to light in the $\Delta pst-lovA$ and $\Delta pst-lovB$ mutants, indicating that other photoreceptors or harmful effects of light did not obviously contribute to their patterns (Figure 2c).

Light-specific Pst-Lov-mediated repression of bacterial proliferation *in planta*

To investigate the kinetics of the bacterial population in the complex leaf environment (Beattie and Lindow,

1999), *A. thaliana* plants were inoculated by dipping their shoots in a suspension containing the wild-type, the $\Delta pst-lovA$ mutant or the $\Delta pst-lovB$ mutant strain of *Pst* DC3000, and transferred to either continuous darkness or continuous white light. Light is known to penetrate the leaves (Vogelmann *et al.*, 1996). The blue light (400–500 nm) irradiance reaching the upper face of the leaves was $31.2 \pm 1.6 \mu\text{mol m}^{-2} \text{sec}^{-1}$, and that transmitted through the leaf and emerging at its lower face was $1.7 \pm 0.1 \mu\text{mol m}^{-2} \text{sec}^{-1}$. This indicates that the internal leaf tissues and the bacteria they contain received blue light within these upper and lower limits. This range is largely within the range of irradiances that affect bacterial growth in minimal L medium (Figure 2b). The shape and magnitude of the bacterial growth curves were similar to those in previous reports (Rojo *et al.*, 2004). In darkness, the number of colony-forming units was unaffected by the mutation (Figure 3a). Under white light, Pst-Lov reduced proliferation as indicated by the higher number of colony-forming units in the $\Delta pst-lovA$ and $\Delta pst-lovB$ mutants 2 or 3 days after inoculation (Figure 3a).

To focus on the ability to grow within the plant and to exclude potential effects of Pst-Lov on the ability to enter host tissue (Beattie and Lindow, 1999), we injected the bacterial suspension through the abaxial stomatal pores by using a syringe without a needle. The leaves were either left attached to the plant or detached and buried in wet sterilized soil, i.e., two ecological conditions that differ with respect to the light environment. No detectable light reached buried leaves (Tester and Morris, 1987). Pst-Lov reduced bacterial proliferation in attached, light-exposed leaves, but had no effect in leaves buried in the soil (Figure 3b).

Figure 3. Pst-Lov inhibits growth of *Pst* DC3000 in light-exposed *A. thaliana* leaves.

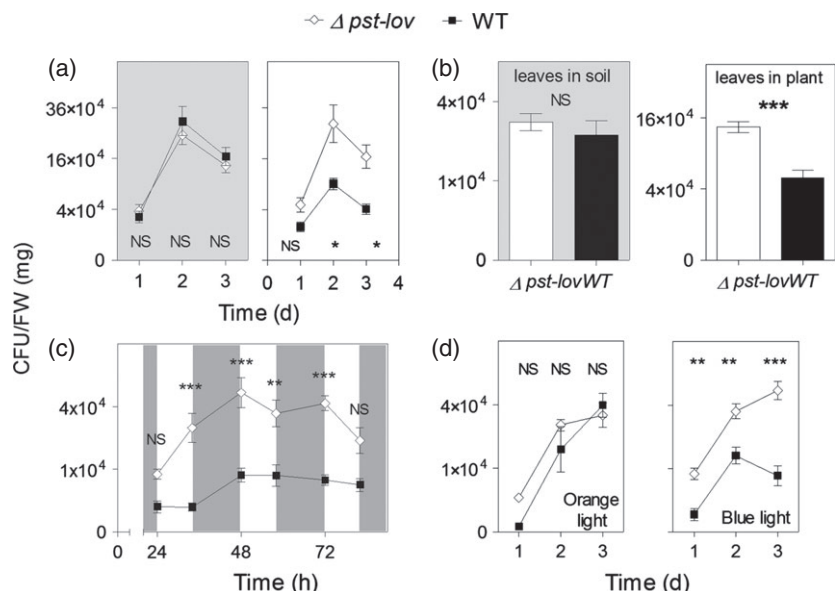
(a) Growth of wild-type, $\Delta pst-lovA$ or $\Delta pst-lovB$ strains of *Pst* DC3000 in *A. thaliana* leaves after dip inoculation at time = 0. Plants were exposed either to continuous white light or continuous darkness. Data from both mutants were pooled to increase accuracy.

(b) Growth in syringe-inoculated *A. thaliana* leaves either left attached to the plant or detached and buried in sterilized soil. Samples were collected 2 days after inoculation.

(c) Growth in syringe-inoculated leaves of *A. thaliana* plants exposed to day/night cycles.

(d) Growth in syringe-inoculated leaves of *A. thaliana* plants exposed to orange or blue light.

CFU, colony-forming units. 1 cm² is approximately 12 mg fresh weight (FW). Values are means \pm SE of at least 15 (a, c) or eight (b, d) biological replicates. The significance of the difference between lines with or without Pst-Lov is indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant; ANOVA).



Pst-Lov also reduced bacterial proliferation in leaves exposed to 10 h white light/14 h darkness cycles (Figure 3c), which are more representative of natural conditions than continuous light. The dark hours of the night were not enough to promote bacterial growth compared to daytime light conditions (Figure 3c and Figure S5).

The above results demonstrate that the $\Delta pst-lovA$ and $\Delta pst-lovB$ mutant phenotypes require light. However, as Pst-Lov is predicted to be a specific blue light photoreceptor, we compared the effectiveness of blue and orange light at equal irradiance *in planta*. The $\Delta pst-lovA$ and $\Delta pst-lovB$ mutations enhanced the number of colony-forming units under continuous blue light but not under continuous orange light (Figure 3d).

Pst-Lov attenuates the virulence of *Pst* DC3000 against *A. thaliana*

To compare the aggressiveness of wild-type and $\Delta pst-lov$ mutant strains of *Pst* DC3000, we performed infectivity titration experiments, in which leaves of *A. thaliana* were infiltrated with a series of doses of pathogen and the bacterial cell counts and the levels of chlorophyll were recorded 2 days later. The leaf symptoms were more intense in the $\Delta pst-lov$ mutant strains than in wild-type *Pst* DC3000 (Figure S6). The invasiveness (defined as the capacity of a pathogen to grow and spread within the host; Cabrefiga and Montesinos, 2005) was reduced by Pst-Lov (Figure 4a). The infectiveness (defined as the efficiency of the pathogen inoculum in terms of development of infections, which is inversely proportional to the number of pathogen cells required to cause an infection or to kill a susceptible host; Cabrefiga and Montesinos, 2005) was largely unaffected, as the decay of chlorophyll approximately followed the increase in bacterial cell numbers (Figure 4b).

In contrast to previous observations in *B. abortus* (Swartz *et al.*, 2007), *A. baumannii* (Mussi *et al.*, 2010) and *L. monocytogenes* (Ondrusch and Kreft, 2011), in which photoreceptors enhance virulence, Pst-Lov attenuates the virulence of *Pst* DC3000 by reducing invasiveness in leaf tissues (Figure 4). The LOV-domain receptor of *X. axonopodis* pv. citri enhances adhesion to the leaves of orange trees, but reduces their subsequent damage by unknown

mechanisms (Kraiselburd *et al.*, 2012). Attenuation of *Pst* DC3000 virulence by Pst-Lov may be the result of low expression of all genes of the *gacS-gacA-rpoN-hrpL* pathway (with *rpoN-hrpL* encoding alternative sigma factors), and its downstream virulence factor genes *hopAA1-1* and *hrpE* (Chatterjee *et al.*, 2003) (Figure 2c). The reduced expression of *hopL1*, a putative type III effector gene without an Hrp box (Schechter *et al.*, 2006), *galE*, a UDP-galactose 4'-epimerase gene related to biofilm formation (Niou *et al.*, 2009), *psyl*, an -acyl homoserine lactone synthase-encoding gene involved in generation of a quorum sensing signal (Chatterjee *et al.*, 2007) and *rpoD*, the principal sigma factor-encoding gene linked to exponential growth (Fujita *et al.*, 1994) (Figure 2c) may also contribute to lower virulence in the light.

Functional significance of Pst-Lov

It is noteworthy that virulence genes and the gene encoding Pst-Lov, which attenuates virulence, are both present in LOV-GI. *P. syringae* pv. tomato survives as a saprophyte in plant debris, soil and on leaf surfaces (Preston, 2000), but its population may decline rapidly in both natural soil and buried host debris, particularly at high temperatures (McCarter *et al.*, 1983). *Pst* DC3000 may infect roots through the soil, but the formation of biofilms and the secretion of surfactin by other micro-organisms impose a barrier to root colonization (Bais *et al.*, 2004). Light penetrates only the upper few millimetres of the soil (Tester and Morris, 1987). Whilst Pst-Lov reduced *Pst* DC3000 proliferation in light-exposed leaves, it had no effects on soil-buried leaves (Figure 3). We speculate that, on the one hand, the lack of light/Pst-Lov-imposed growth restriction may keep the number of cells as high as possible given the availability of resources, thus reducing the chances of stochastic extinction of the soil population. On the other hand, the attenuated proliferation caused by blue light perception by Pst-Lov (Figure 3) reduces *Pst* DC3000 damage to the light-exposed host leaf tissue (Figure 4), increasing the time available for dispersal to new hosts (Beattie and Lindow, 1999; Alizon *et al.*, 2009; Jackson *et al.*, 2011).

Light is important for plants as a source of energy and as a source of information perceived by sensory receptors (Kami

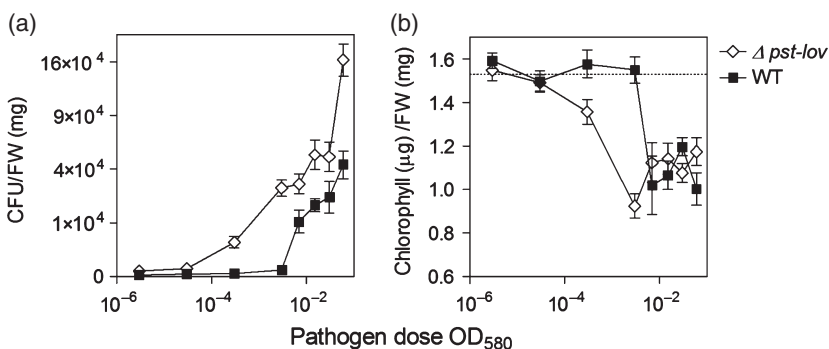


Figure 4. Pst-Lov attenuates virulence of *Pst* DC3000 against *A. thaliana*.

Leaf population of *Pst* DC3000 (a) and leaf chlorophyll levels (b) in infectivity titration experiments with wild-type, $\Delta pst-lovA$ or $\Delta pst-lovB$ strains. Data from both mutants were pooled to increase accuracy. CFU, colony-forming units. In (b), the dotted line indicates chlorophyll levels in control, non-inoculated plants. Values are means \pm SE of at least eight plant replicates.

et al., 2010). Here we show that the light environment also affects plants indirectly, via perception of these signals by their bacterial pathogens (e.g. chlorophyll levels in the plant depend on Pst-Lov activity in bacteria, Figure 4 and Figure S6).

EXPERIMENTAL PROCEDURES

Bioinformatics

Sequence searches were performed using BLASTP tools (<http://www.ncbi.nlm.nih.gov/blast>). The G + C content was calculated for a 1000 bp window moved along the sequence in 10 bp steps using EMBOS FREAK (<http://emboss.bioinformatics.nl/cgi-bin/emboss/freak>). Phylogenetic analysis was performed using MEGA version 5.0 (Tamura *et al.*, 2011). Protein sequences were aligned using the CLUSTALW program (settings for pairwise alignment: gap opening penalty = 10, gap extension penalty = 0.1; settings for multiple alignment: gap opening penalty = 10, gap extension penalty = 0.2; other settings: protein weight matrix set at 'gonnet', residue-specific penalties and hydrophobic penalties set 'on', gap separation distance = 4, end gap separation and use negative matrix set 'off', delay divergent cut-off 30%, keep predefined gaps set 'no'). Phylogenetic trees were constructed using the neighbour-joining and maximum-parsimony methods. In the neighbour-joining analysis, genetic distances were computed using a pairwise deletion Poisson model. The maximum-parsimony analysis was performed using close neighbor interchange (CNI) (level = 1) with initial tree obtained by random addition (ten repetitions), with a phylogeny test involving 500 bootstrap replicates and root on midpoint.

Analysis of the episomal circular intermediate and Pst-Lov-permease operon from LOV-GI

In order to examine the presence of an episomal circular intermediate from LOV-GI, exponentially growing cells ($OD_{580\text{ nm}} = 0.5$) were harvested by centrifugation (16 000 g, 22°C). Then genomic DNA was prepared using a Wizard genomic DNA purification kit (A1120, Promega, www.biodynamics.com.ar) and used for PCR amplification (Table S2). This fragment was sequenced. To analyse the Pst-Lov-permease operon, we used cDNA and RT-PCR with primers for *PSPTO2895*, *PSPTO2896* and *PSPTO2897* (Table S2).

Wild-type, knock-out mutant and complemented bacterial strains

The wild-type *Pst* DC3000 was kindly provided by Robin Buell (Institute for Genomic Research, Rockville, MD, USA). The gene encoding full-length Pst-LOV (*PSPTO2896*) was amplified from genomic DNA by PCR and cloned into the pJET1.2 cloning vector (Fermentas, www.biodynamics.com.ar). The amplified region contained a BstZ17I restriction site, which was used to insert a kanamycin resistance cassette (isolated from pBSL15, www.atcc.org, by *Sma*I digestion) either in the reading direction of the polymerase (Δ *pst-lovA*) or in the opposite orientation (Δ *pst-lovB*). A linear, double-stranded DNA fragment was generated by *Pst*I restriction digestion, and used for electroporation of *Pst* DC3000 cells. This method produced a much higher yield of transformants than the normally used homologous recombination technique [the mean efficiency (percentage of selected colonies of transformed cells yielding a positive, gene-disruption signal) was >30%]. Δ *pst-lovA* and Δ *pst-lovB* knockout mutant strains were obtained and confirmed by genomic DNA sequencing. RT-PCR

analysis using primers amplifying either the whole gene or a region of 459 bp encompassing part of the LOV domain yielded the expected product for *Pst* DC3000 and no product in the mutants, thus confirming inactivation of the gene (Shah, 2011).

To complement the Δ *pst-lovB* mutant, a 2 kb fragment containing the *PSPTO2896* locus with intergenic regions was amplified by PCR (Table S2). This fragment was cloned into pCR2.1-TOPO (Invitrogen, www.invitrogen.com.ar) and sequenced. The 2 kb fragment was then digested with *Xba*I and *Sac*I, and cloned into the bacterial broad-host-range vector pBBR1MCS-3 (ncbi accession number U25059). This plasmid was introduced by transformation into competent cells of the Δ *pst-lovB* mutant (Ayub *et al.*, 2009). Transformants were selected by plating on LB agar containing 5 μ g ml⁻¹ tetracycline.

Pst DC3000 growth in L medium

Cultures were performed in 125 ml Erlenmeyer flasks containing 25 ml L medium, and incubated at 28°C with shaking (250 rpm). Preparatory overnight cultures grown at 28°C were used to inoculate the medium to obtain an initial optical density ($OD_{580\text{ nm}}$) of 0.05. Growth was monitored by measuring the $OD_{580\text{ nm}}$ for 6 h. L medium comprises 7.5 mM KH₂PO₄, 17.22 mM K₂HPO₄, 3.42 mM NaCl, 7.57 mM (NH₄)₂SO₄, 2 mM MgSO₄·7H₂O, 3.7 mM FeCl₃·6H₂O, 0.1 mM CuCl₂·2H₂O, 0.1 mM ZnSO₄·7H₂O, 0.73 mM MnCl₂·H₂O, 1 mM CaCl₂·2H₂O, 0.21 mM NaMoO₄, 3.4 mM citric acid, 28 mM glucose and 100 mg l⁻¹ yeast extract, pH 7.

Gene expression

Total RNA was extracted using TRIzol reagent (Life Technologies, www.invitrogen.com.ar), 2 μ g were resuspended in milli q water, treated with RQ1 RNase-free DNase (Promega), and used in combination with random hexamers and AMV retrotranscriptase (Promega) to synthesize cDNA. The cDNAs were amplified using FastStart Universal SYBR Green Master Mix (Roche, www.siste-masanaliticos.com.ar) on a 7500 real-time PCR system cyclor (Applied Biosystems, www.invitrogen.com.ar). Gene expression was normalized to that of *gap1* (Filiatrault *et al.*, Schechter *et al.*, 2006; Swingle *et al.*, 2008; Vencato *et al.*, 2006) and *gyrB* (Ortiz-Martin *et al.*, 2010; Schreiber and Desveaux, 2011). The oligonucleotide primers were designed using Beacon Designer version 5.01 (<http://www.premierbiosoft.com>), and the annealing temperature for all primer pairs was 60°C (Table S2).

Plant material

Single plants of *Arabidopsis thaliana* accession Columbia were grown in pots (110 cm³) containing perlite (Perlome), peat moss (Finca Don Calvino) and vermiculite (Intersum) (2:2:1), and watered twice a week using a solution containing 1 g l⁻¹ Hakaphos R (Compos) (all from Agroquímica Larrocca, <http://www.agroquimicalarocca.com.ar>). The photoperiod comprised 8 h white light (70 μ mol m⁻² sec⁻¹) provided by fluorescent tubes (Osram Universal, www.osram.com). The temperature was 22–25°C.

Pst DC3000 growth in leaves

Arabidopsis thaliana plants were infected by dipping the shoot into a 10 mM MgCl₂ solution containing 0.2% Silwet-20 and a bacterial suspension ($OD_{600\text{ nm}} = 0.3$, approximately 1.5×10^8 colony-forming units ml⁻¹) for 1 min (Tornero and Dangel, 2001). Alternatively, leaves were infiltrated through their abaxial stomata with a bacterial suspension ($OD_{600\text{ nm}} = 0.03$, approximately 1.5×10^7 colony-forming units ml⁻¹) by using a syringe without a needle (Griebel and Zeier, 2008).

In some experiments, infiltrated leaves were buried 2 cm beneath the surface in sterilized soil moistened with distilled water and contained in plastic pots. Either entire dip-infected leaves or two 6.3 mm diameter disks per infiltrated leaf were harvested at the indicated times, weighed and incubated for 60 min in 1 ml of 10 mM MgCl₂. Appropriate dilutions were plated on King's B medium (King *et al.* 1954). The number of colonies was counted after incubating the plates at 28°C for 48 h. Data were square root-transformed for statistical analysis. To assess the damage caused by bacterial attack, in some experiments, chlorophyll levels were measured with a portable chlorophyll meter (SPAD-502, <http://www.konicaminolta.com>) calibrated against *N,N*-dimethylformamide extracts (Moran, 1982).

Light treatments

White, blue or orange light (100 μmol m⁻² sec⁻¹, unless indicated otherwise) were provided by fluorescent tubes (Osram Universal), low-pressure sodium lamps (Philips SOX, www.philips.com) or blue light-emitting diodes (<http://www.cavadevices.com>), respectively. The irradiance provided by the various light treatments was measured using an Li-188B sensor (Li-Cor, <http://www.licor.com>). The sensor was buried to the same depth that leaves were buried in experiments with soil. To measure the irradiance reaching the upper face of the leaf and that transmitted through the leaf in the range of blue light (400–500 nm) for plants grown under white light, we used an Ocean Optics USB4000 UV-Vis spectrometer (<http://www.oceanoptics.com>) configured with a DET4-200-850 detector and QP600-2-SR optical fibre.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Incongruence between LOV-domain ORF-based trees and 16S rRNA-based trees in *Pseudomonas syringae* and *Xanthomonas spp.* strains.

Figure S2. Incongruence between LOV-domain ORF-based trees and 16S rRNA-based trees in Proteobacteria based on maximum-parsimony analysis.

Figure S3. Internal controls used for quantitative PCR are unaffected by light/darkness or genotype.

Figure S4. Pst-Lov inhibits the expression of sigma factor and downstream genes in *Pst* DC3000 in the light.

Figure S5. The dark hours of the night are not enough to promote growth of *Pst* DC3000.

Figure S6. Symptoms in leaves infected with wild-type or Δ *pst-lov* mutant *Pst* DC3000.

Table S1. Localization and annotation of ORFs of LOV-GI from *Pst* DC3000.

Table S2. Primers used in this work.

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