


Light prevents pathogen-induced aqueous microenvironments via potentiation of salicylic acid signaling

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Gaële Lajeunesse^{1,2}, Charles Roussin-Léveillé^{1,2}  , Sophie Boutin¹,
Élodie Fortin¹, Isabelle Laforest-Lapointe¹  & Peter Moffett¹  

Many plant pathogens induce water-soaked lesions in infected tissues. In the case of *Pseudomonas syringae* (*Pst*), water-soaking effectors stimulate abscisic acid (ABA) production and signaling, resulting in stomatal closure. This reduces transpiration, increases water accumulation, and induces an apoplastic microenvironment favorable for bacterial growth. Stomata are sensitive to environmental conditions, including light. Here, we show that a period of darkness is required for water-soaking, and that a constant light regime abrogates stomatal closure by *Pst*. We find that constant light induces resistance to *Pst*, and that this effect requires salicylic acid (SA). Constant light did not alter effector-induced accumulation of ABA, but induced greater SA production, promoting stomatal opening despite the presence of ABA. Furthermore, application of a SA analog was sufficient to prevent pathogen-induced stomatal closure and water-soaking. Our results suggest potential approaches for interfering with a common virulence strategy, as well as providing a physiological mechanism by which SA functions in defense against pathogens.

The outcome of a plant-pathogen interaction is determined by multiple factors. These include environmental conditions, as well as plant defense mechanisms, and the ability of pathogens to avoid or overcome the latter^{1,2}. Plants encode a large variety of pattern recognition receptors that can detect microbe-associated molecular patterns and induce pattern-triggered immunity (PTI)^{2–4}. Early PTI responses include acidification of the apoplast, production of reactive oxygen species (ROS) and transcriptional reprogramming^{2–4}. Later responses include biosynthesis of defense-related phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene^{2,4}. In turn, a key virulence mechanism used by pathogens is the translocation of effector proteins into host plant cells, many of which have been shown to inhibit PTI⁵. Plant pathogenic bacteria possess a needle-like structure, commonly referred to as the type-3 secretion system (T3SS), that delivers effector proteins which promote disease progression in their hosts^{6,7}. However, inhibition of defense responses is not sufficient for optimal pathogen proliferation and additional effector activities are

required for the establishment of favorable microenvironments in the apoplast^{8–12}.

Plant stomata are often targeted by pathogenic effector proteins or toxins as these structures represent natural breaches for pathogen entry^{12,13}. Indeed, an important aspect of early response to pathogens involves stomatal immunity, wherein PTI responses result in stomatal closure^{13,14}. To counteract stomatal immunity, *Pseudomonas syringae* (*Pst*) produces a JA-mimicking phytotoxin called coronatine (COR). COR has been reported to induce stomatal reopening to allow the pathogen to gain access to the apoplastic space^{13,15,16}. COR appears to contribute more significantly to *Pst* virulence at night in *Arabidopsis thaliana* (hereafter, *Arabidopsis*)¹⁵, suggesting either a time of day and/or an effect of light on bacterial interaction with stomata.

In contrast to early events in infection that promote stomatal opening, pathogens appear to induce stomatal closure in later stages^{9,10}. A frequent feature of pathogen infection in plants is the creation of an aqueous environment in the plant apoplast, visually

¹Centre SÈVE, Département de Biologie, Université de Sherbrooke, Sherbrooke, QC, Canada. ²These authors contributed equally: Gaële Lajeunesse, Charles Roussin-Léveillé.  e-mail: charles.roussin-leveillee@usherbrooke.ca; peter.moffett@usherbrooke.ca

noticeable as water-soaked lesions, which are crucial for virulence¹¹. *Pst* encodes two highly conserved effector proteins, HopMI and AvrEI, that induce water-soaking lesions^{9–11}. Notably, these water-soaking effectors stimulate host abscisic acid (ABA) biosynthesis and signaling pathways to induce stomatal closure, resulting in a loss of transpiration and accumulation of water in the apoplast^{9,10}.

Stomatal status is strongly influenced by diurnal light cycles, and light also affects plant responses to pathogens^{17,18}. Moreover, SA regulates phytochromes (PHY) proteins, which act as photoreceptors¹⁸, and PHYA and PHYB have been reported to be required for the induction of SA-responsive genes^{19,20}. PHYTOCHROME-INTERACTING FACTORS (PIFs) are rapidly degraded during red-light perception by PHYs and are also involved in light-dependant modulation of immune signaling²¹. Furthermore, functional circadian cycles are required to respond to many abiotic and biotic stresses²², including responses to infection²³. Indeed, plant susceptibility to pathogens varies, depending on the time of day of infection^{24,25}. It has been reported that plants are less susceptible in the subjective morning because defense responses are regulated by the circadian clock²⁶. Accumulation of SA has been reported to be circadian-gated, with levels increasing throughout the day and decreasing throughout the night²⁷. Moreover, the circadian regulators CCA1 and LHY play key roles in defense against *Pst*, as they promote stomatal closure in early defense responses in a SA-independent manner²⁵. However, as circadian cycles are tightly linked to light perception, it is important to determine if a given phenomenon is related to the presence of light or to circadian mechanisms per se.

In this study, we describe the involvement of light in modulating plant defense responses. Constant light treatment prevents bacterial infection by abrogating the ability of *Pst* to induce stomatal closure, thereby inhibiting the accumulation of water in the apoplast. This effect is due to a potentiation of SA signaling, which induces stomatal opening despite an increase in ABA biosynthesis and signaling that would normally induce stomatal closure. In addition to highlighting the interplay between abiotic and biotic stress, our results shed light on a physiological mechanism by which SA contributes to plant defense against pathogens.

Results

Constant light confers protection against *Pst* DC3000

To study the influence of light on the outcome of a bacterial infection in plants, we evaluated the virulence of *Pst* on *Arabidopsis* Col-0 wild-type (WT) plants under different light regimes. Plants were grown under 12 h light/dark cycles before being challenged with *Pst* and placed under either constant light (LL), 12 h light/dark (LD) or constant dark (DD) regimes for 3 days. We found that *Pst*-infected *Arabidopsis* plants kept in LL showed a drastic reduction in disease symptoms compared to those kept in LD or DD over the course of the infection (Fig. 1a). This observation was reflected in bacterial titers, as plants kept under LL accumulated 10-fold less *Pst* compared to other conditions (Fig. 1b). Interestingly, constant light had no additional protective effect against *Pst* DC3000 *hrcC*, which lacks a T3SS, suggesting that this regime affects effector protein actions (Fig. 1b, c).

Circadian rhythms and light regulation have been shown to impact bacterial virulence^{24,25,28}. Therefore, we evaluated whether plants subjected to different light regimes for a 24-h period displayed irregular modulation of immunity following treatment with the immunogenic peptide flg22. We first explored the production of apoplastic reactive oxygen species (aROS), a commonly used early marker of immune responses. We found that plants subjected to LL for 24 h prior to treatment with flg22 produced significantly more aROS than plants kept under LD or DD regimes (Fig. 1d, e). Notably, under LL, *Arabidopsis* plants displayed a moderate, but persistent second wave of aROS, as opposed to other light conditions (Fig. 1d). In contrast, plants that were subjected to DD treatment for a single day produced much less aROS in response to flg22, compared to plants kept under

LD conditions (Fig. 1e). Hence, light appears to prime *Arabidopsis* plants to produce enhanced and more sustained aROS after immune elicitation, potentially contributing to disease resistance.

We evaluated the expression of two early PTI-responsive genes, *FRKI* and *WRKY29*, to assess early PTI responsiveness. Plants were subjected to the three different light regimes for 24 h before being challenged with flg22 for 4 h. Although not significantly altered, these genes were less expressed in DD compared to LD or LL (Fig. 1f, g). Interestingly, constant light by itself resulted in increases in both *FLS2* and *RBOHD* expression levels in the absence of flg22, although only the latter was statistically significant (Supplementary Fig. 1a, b). Contrary to this observation, *FLS2* and *RBOHD* expression levels were not upregulated upon flg22 treatment under constant dark (Supplementary Fig. 1a, b). These results suggest that light perception and/or signaling is critical for the establishment of a proper early PTI response.

Pst DC3000 requires darkness to induce water-soaking lesions

The virulence of *Pst* is greatly enhanced by its ability to induce water-soaking lesions by closing stomata under high humidity conditions^{9,10}. As light modulates stomatal aperture²⁹, we questioned whether a pathogen's ability to induce stomatal closure could be affected by diurnal light oscillations. We observed that, under LL conditions, *Pst* could no longer induce water-soaking lesions in *Arabidopsis* leaves, in contrast to LD or DD (Fig. 2a). The latter was not due to differences in levels of bacteria, as inoculations were carried with a high concentration of bacteria (1×10^8 CFU/ml) (Fig. 2b). Consistent with the lack of water soaking, we found that stomata of inoculated *Arabidopsis* plants kept under LL conditions were still fully open 24 h post inoculation (Fig. 2c). In contrast, stomata of inoculated plants kept under LD conditions were closed, as expected, as were those of plants kept under DD conditions (Fig. 2c).

To consolidate our findings, we designed an experiment where *Arabidopsis* plants were infected with *Pst* and either kept under LL or LD conditions for the first 16 h of the infection. At this timepoint, LD, but not LL treated plants underwent water soaking (Fig. 2d). Plants were then placed in darkness to determine if water soaking could be reappearing. Indeed, we found that the water-soaking phenotype could be fully restored after keeping LL treated plants in darkness for 6 h (Fig. 2d). Stomatal behavior analysis further supported the observation that constant light prevents *Pst* from inducing stomatal closure, but that this phenomenon is swiftly reversed once under dark conditions (Fig. 2e).

Pst manipulates its host ABA machinery via secretion of its conserved effectors HopMI and AvrEI to induce stomatal closure leading to water-soaking^{9,10}. We designed experiments to test whether a deficiency in apoplast hydration was the main reason behind reduced *Pst* aggressivity under LL. Since the *Arabidopsis aba2-1* mutant was shown to prevent *Pst*-induced water-soaked lesions^{9,10}, we compared the bacterial levels of these plants infected under LL and LD conditions. We found that, while bacterial population levels differed between LL and LD conditions in WT plants, they were unaltered in *aba2-1* plants (Supplementary Fig. 2). Moreover, under LL, *Pst* grew to levels similar to *Pst hopMI/avrEI* (*h/a*), which cannot induce water-soaking (Supplementary Fig. 3). Furthermore, the pathogen's ability to manipulate ABA appeared unaffected as transcript levels of the ABA biosynthesis and signaling marker genes, *NCED3* and *RD29A*, respectively, in WT infected plants were similar under all light conditions (Supplementary Fig. 4). These results suggest that the lack of apoplastic fluid could at least partly explain the growth deficiency of *Pst* under LL. Thus, it appears that pathogens might benefit from natural light cycles to establish a favorable microenvironment inside host leaves.

SA is required to prevent water-soaking under continuous light

SA is a key immune signaling molecule and has been reported to antagonize multiple actions driven by ABA signaling^{30–32}. However,

how SA and ABA signaling interact with each other with respect to stomatal control during late phases of pathogen infection is unknown. We investigated whether plants subjected to a constant light cycle for 24 h would be altered in SA signaling or accumulation. First, we analyzed expression levels of SA responsive-genes, *ICS1* (*ISOCHORISMATE SYNTHASE 1*) and *PR1* (*PATHOGENESIS-RELATED PROTEIN 1*), which are often used to assess SA biosynthesis and responsiveness, respectively. We observed that the expression levels of these two genes were increased under LL conditions relative to LD or DD regimes, although the increase of *ICS1* relative expression was not significant (Fig. 3a, b). Consistent with this, we observed, using ultra performance liquid chromatography-mass spectrometry (UPLC-MS), that upon treatment with flg22, plants kept under LL conditions showed an increase in SA levels compared to LD and DD

treated plants (Fig. 3c). Likewise, upon infiltration of plants with *Pst*, plants grown in LL conditions showed a greater induction of *ICS1* and accumulation of SA than plants grown in LD or DD (Supplementary Fig. 5). As such, constant light appears not only to modulate early PTI responses, but also SA-related responses.

In agreement with our findings, a recent report revealed that SA-related responses are primed by photoperiodic stress³³. Considering that SA can antagonize ABA responses, we investigated whether SA is necessary for light-mediated prevention of water-soaked lesions. We inoculated *Arabidopsis* mutants, *npr1-1* and *sid2-2*, which are compromised in SA signaling and biosynthesis, respectively, with *Pst* under different light conditions. Interestingly, we observed water-soaking in the inoculated leaves of *npr1-1* and *sid2-2* mutant plants at 24 h post-infection (hpi) under both LD and LL conditions, whereas

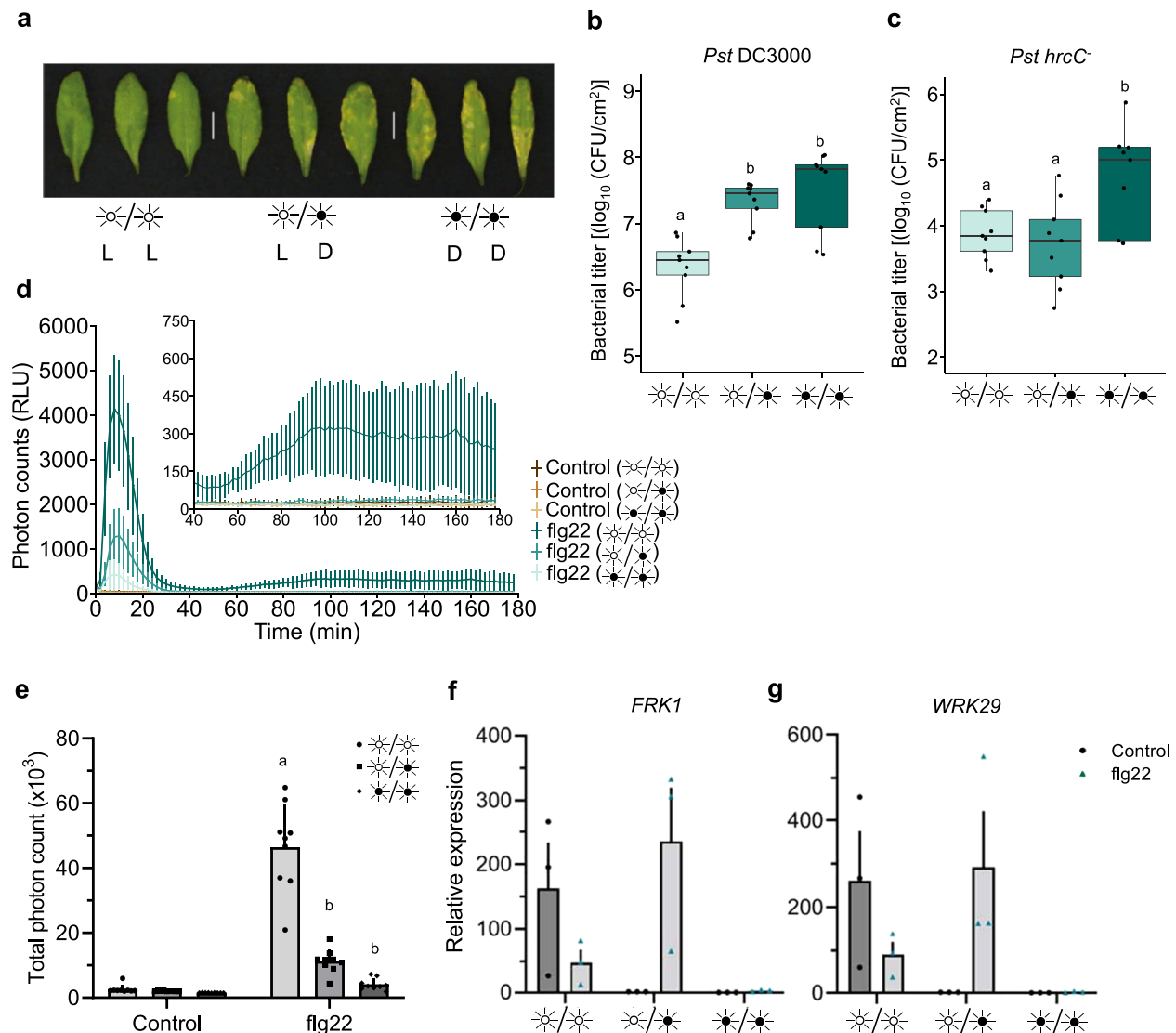


Fig. 1 | Constant light confers protection against *Pst*. **a** Disease phenotypes in *Arabidopsis* leaves syringe-infiltrated with *Pst* DC3000 (1×10^5 CFU/ml). WT Plants were either kept under constant light (LL), 12 h light/dark (LD), or constant dark (DD) light cycles for 3 days after inoculation. Bacterial titers from WT leaves syringe-infiltrated with *Pst* DC3000 (**b**) or *Pst hrcC*⁻ (**c**) at 3 days post-infection (dpi) under light regimes described in **a** ($n = 3$ biologically independent replicates). **d** Apoplastic oxidative burst triggered by 1 μ M flg22 in WT *Arabidopsis* leaf punches. Leaf punches were kept in water under LL, LD or DD for 24 h prior to stimulation with flg22. Values are averages \pm se ($n = 27$). **e** Total photon counts measured over 180 min from the experiments in **d**. Expression levels of the early PTI

marker genes *FRK1* (**f**) and *WRKY29* (**g**) treated with 1 μ M flg22 in plants that were previously placed in the indicated light settings and measured by qRT-PCR. Samples were kept under these light settings for 6 h after control or flg22 treatment after which they were harvested ($n = 3$ biologically independent replicates). Different letters indicate statistically significant differences, $p < 0.05$, ANOVA (**b**, **c**) or $p < 0.0001$, ANOVA followed by Tukey's range test (**e**). All statistical tests are two-sided. In box plots, box edges delineate lower and upper quartiles, the center line represents the median, and whiskers extend to 1.5 times the interquartile range (IQR). In non-box-plot graphs, boxes and error bars represent mean \pm S.E.M.

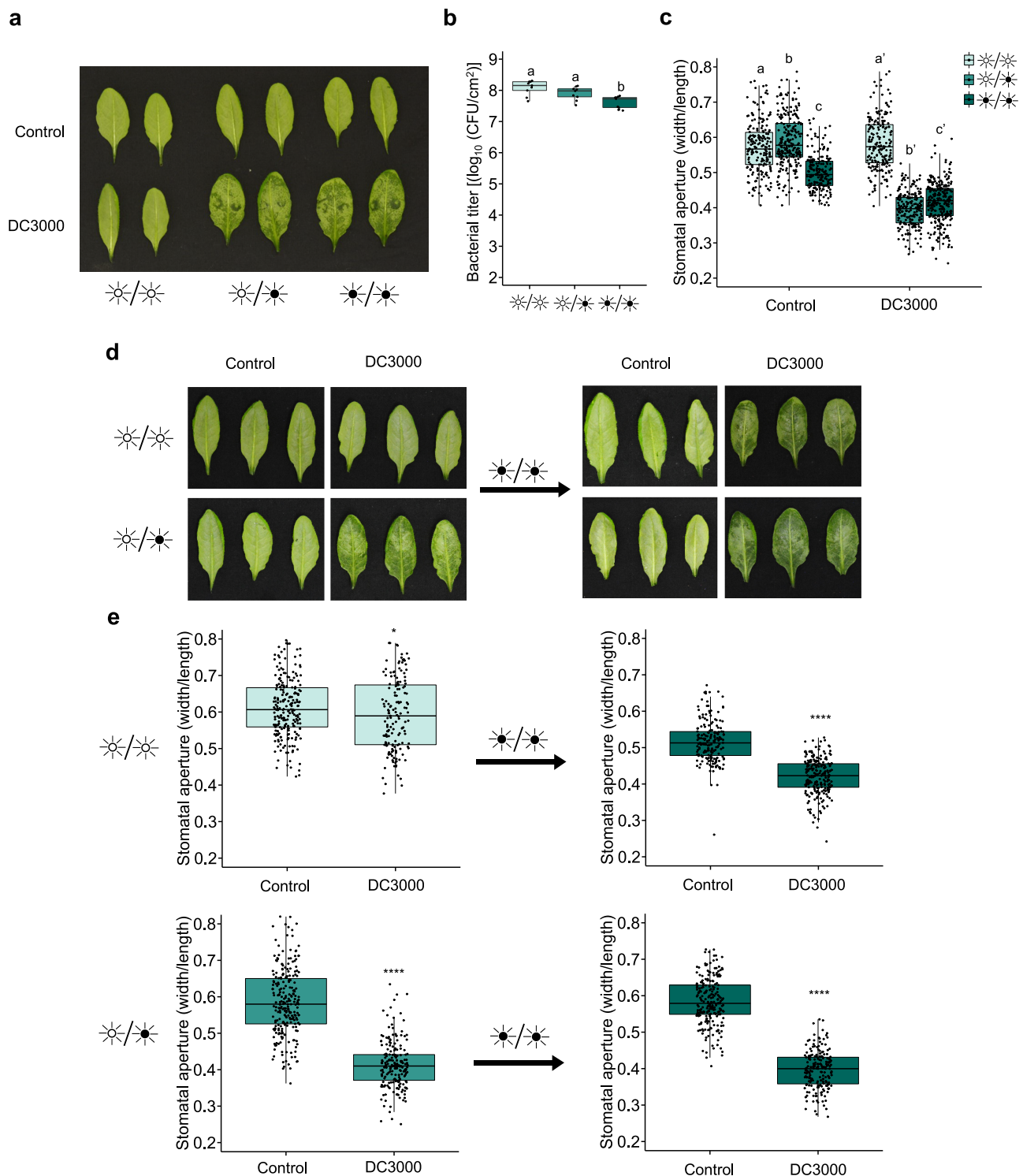


Fig. 2 | *Pst* requires darkness to induce stomatal closure and water-soaking.

a Water-soaking phenotypes in *Arabidopsis* leaves syringe-infiltrated with *Pst* DC3000 (1×10^8 CFU/ml) at 24 hours post-infection (hpi) under LL, LD or DD conditions. **b** Bacterial titers from plants described in **a** ($n = 3$ biologically independent replicates). **c** Stomatal aperture measurements in plants described in **a** by epifluorescence microscopy ($n = 194$ stomata for LL Control, $n = 190$ stomata for LL DC3000, $n = 229$ stomata for LD Control, $n = 196$ stomata for LD DC3000, $n = 185$ stomata for DD Control, $n = 262$ stomata for DD DC3000). **d** Water-soaking phenotypes in *Arabidopsis* leaves that were syringe-infiltrated with *Pst* DC3000 (1×10^8 CFU/ml) at 24 hpi under LL or LD conditions (left panel) and in plants treated in the same way followed by transferred to DD for 6 h (right panel). **e** Stomatal aperture measurements from leaves treated as in **d** ($n = 220$ stomata for

Mock and $n = 156$ stomata for DC3000 [upper left panel], $n = 182$ stomata for Mock and $n = 227$ stomata for DC3000 [upper right panel], $n = 252$ stomata for Mock and $n = 195$ stomata for DC3000 [bottom left panel], $n = 229$ stomata for Mock and $n = 195$ stomata for DC3000 [bottom right panel]). Different letters indicate statistically significant differences, $p < 0.05$, ANOVA (**b**) or Kruskal–Wallis test (**c**). Asterisks indicate statistically significant differences compared to control, $*p < 0.05$, $****p < 2.2 \times 10^{-16}$, Student's *t* test (**e**, upper right panel) or Wilcoxon–Mann–Whitney test (**e**, upper left, lower right and lower left panels). All statistical tests are two-sided. In box plots, box edges delineate lower and upper quartiles, the center line represents the median, and whiskers extend to 1.5 times the interquartile range (IQR).

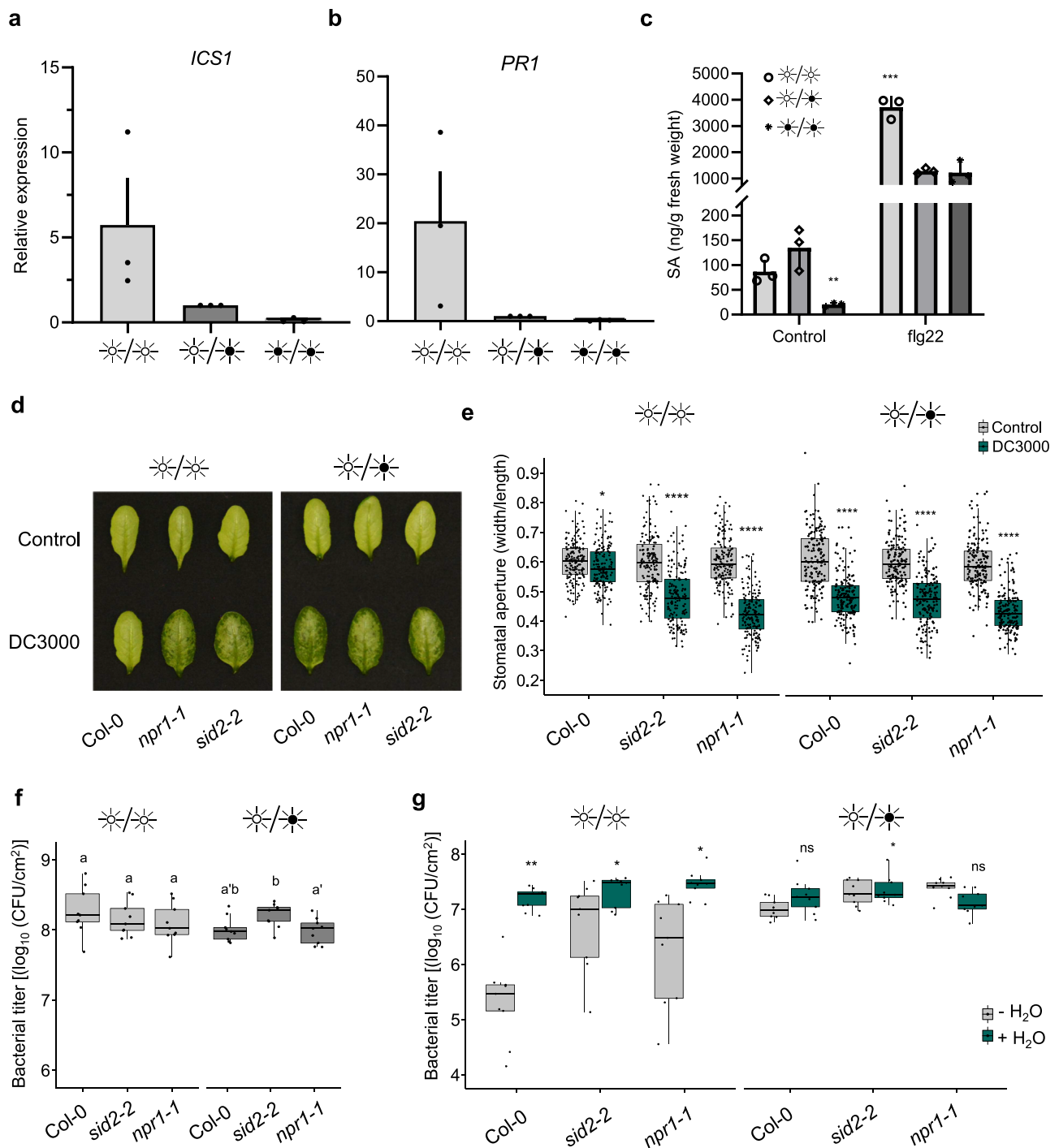


Fig. 3 | Salicylic acid is required for preventing stomatal closure and disease progression under constant light. **a, b** Expression levels, measured by qRT-PCR, of SA biosynthesis (*ICS1*) and signaling (*PRI*) marker genes in WT *Arabidopsis* plants treated with 1 μ M flg22 after having previously been placed in the identified light settings. Samples were harvested at 24 hpi ($n = 3$ biologically independent replicates). **c** Quantification of SA in WT *Arabidopsis* plants mock inoculated (control) or inoculated with 1 μ M flg22 under the same experimental settings as in **a** measured by UPLC-MS at 24 hpi ($n = 3$ biologically independent replicates). **d** Water-soaking phenotypes in *Arabidopsis* WT (Col-0), *sid2* and *npr1* mutant plants syringe-infiltrated with *Pst* DC3000 (1×10^8 CFU/ml) at 24 hpi under LL, LD or DD. **e** Stomatal aperture measurements from infected leaves displayed in **d** ($n = 159$ stomata for all conditions). **f** Bacterial titers from *Arabidopsis* WT (Col-0), *sid2* and *npr1* mutant plants infected with *Pst* DC3000 (1×10^8 CFU/ml) at 24

hpi ($n = 3$ biologically independent replicates). **g** Bacterial titer of *Arabidopsis* WT (Col-0), *sid2* and *npr1* mutant plants infected with *Pst* DC3000 (1×10^8 CFU/ml) in which infiltrated leaves were allowed to return to a pre-infiltrated state ($-H_2O$) or in plants that were immediately domed following infiltration ($+H_2O$). Bacterial titers were measured at 3 dpi ($n = 3$ biologically independent samples). Asterisks indicate statistically significant differences compared to control, ns non-significant, $*p < 0.05$, $**p < 0.001$, $****p < 2.2 \times 10^{-16}$, ANOVA followed by Tukey's range test compared to LD (**c**), Student's *t* test (**e, f**) or Wilcoxon–Mann–Whitney test (**e, g**). All statistical tests are two-sided. In box plots, box edges delineate lower and upper quartiles, the center line represents the median, and whiskers extend to 1.5 times the interquartile range (IQR). In non-box-plot graphs, boxes and error bars represent mean \pm S.E.M.

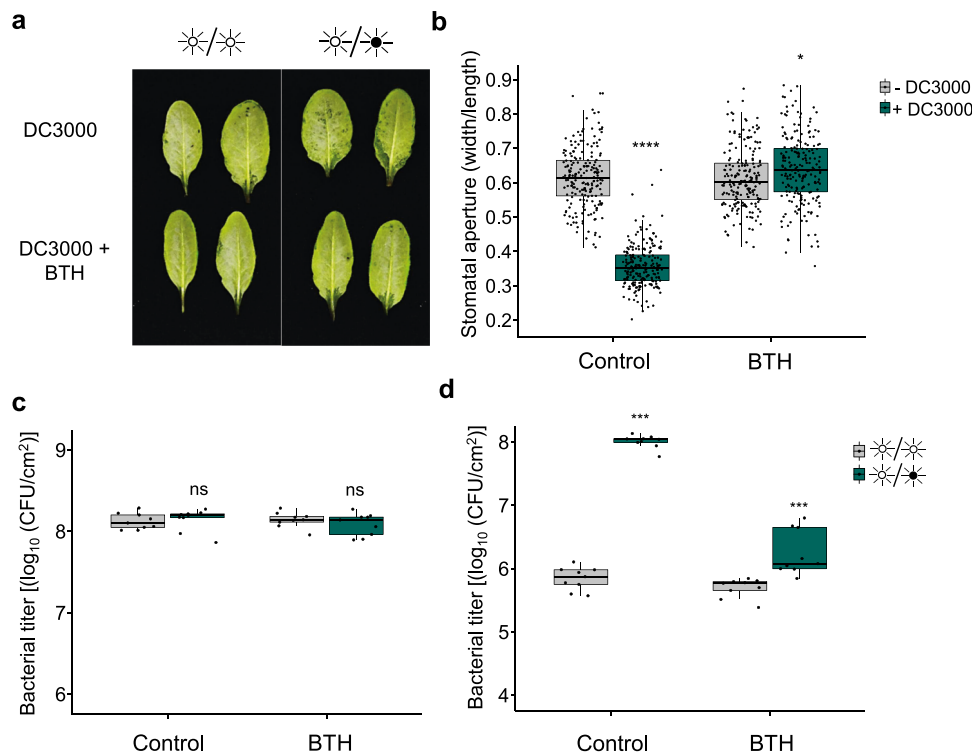


Fig. 4 | BTH provides protection against bacterial induction of water-soaking. **a** Water-soaking phenotypes in WT *Arabidopsis* infiltrated with DMSO (0.1%) or BTH (50 μ M) prior to syringe-infiltration with *Pst* DC3000 (1×10^8 CFU/ml) 24 h later. Photos were taken 24 h post-infection. **b** Stomatal aperture in WT *Arabidopsis* treated as in **a** and in which MgCl₂ (10 mM; -DC3000) or *Pst* (1×10^8 CFU/ml; +DC3000) was syringe-infiltrated. Plants were left under light/dark conditions and stomatal apertures assessed at 24 h post infiltration (hpi) ($n = 202$ stomata for all conditions). **c** Bacterial titers from plants treated in **a** measured 24 hpi ($n = 3$ biologically independent replicates). **d** Bacterial titers from *Arabidopsis* leaves that were infiltrated with DMSO (0.1%) or BTH (50 μ M) 24 h prior to being challenged

with *Pst* DC3000 (1×10^8 CFU/ml). Plants were placed under the indicated light conditions after infection. Bacterial titers were assessed at 3 days post-infection and plants were kept under the indicated light conditions throughout the infection process ($n = 3$ biologically independent replicates). Asterisks indicate statistically significant differences compared to control, ns non-significant, * $p = 0.001$, *** $p < 0.0001$, **** $p < 2.2 \times 10^{-16}$, Student's *t* test (**b** for Control, **c**) or Wilcoxon–Mann–Whitney test (**b** for BTH, **d**). All statistical tests are two-sided. In box plots, box edges delineate lower and upper quartiles, the center line represents the median, and whiskers extend to 1.5 times the interquartile range (IQR).

wild-type plants showed water-soaking only under LD conditions (Fig. 3d). Consistent with these results, we observed that *Pst* inoculation induced stomatal closure at 24 hpi in all genotypes under LD conditions (Fig. 3e). Furthermore, apoplastic hydration was elevated in LL in SA-related mutants, but not in Col-0 under LD (Supplementary Fig. 6). However, constant light did not hamper the ability of the pathogen to induce stomatal closure in the *npr1-1* mutant, or (to a lesser degree) in the *sid2-2* mutant, as it does in WT plants (Fig. 3e). These altered responses were not due to differences in bacterial densities, as bacterial titers did not differ in mutant versus wild-type plants at this time point (Fig. 3f).

The impact of SA on plant responses to infection may be due to multiple effects. Given the effect of SA on water-soaking and stomatal control (Fig. 3d, e), we tested if the effect of SA might be related to the establishment of an aqueous environment, which is beneficial to bacteria. To do so, we employed an inoculation method wherein plants are immediately placed in a >95% humidity environment after inoculation without allowing liquid from the inoculum to evaporate. Under these conditions, leaves remain saturated with water, mimicking at least in part the phenomenon of water-soaking. Artificial induction of water soaking had no effect on bacterial growth of either wild-type or mutant plants under LD conditions (Fig. 3g). Keeping plants under LL conditions resulted in a dramatic decrease in bacterial growth in WT plants, while this growth defect was partially rescued in *sid2-2* and *npr1-1* mutants (Fig. 3g). Importantly, artificial water-soaking resulted in a dramatic increase of bacterial growth under LL conditions, with both WT and mutant plants showing bacterial levels similar to LD grown

plants (Fig. 3g). These results indicate that the induction of resistance induced by constant light requires SA signaling and that the effects of SA are, at least in part, related to its effects on inhibiting stomatal closure. This, in turn, inhibits the pathogen's ability to create an ideal aqueous environment in the apoplast.

BTH suppresses *Pst*-induced water-soaking lesions

Benzothiadiazole (BTH) is an analog of SA and, like SA, triggers systemic acquired resistance in plants and confers protection against *Pst*^{34,35}. Since SA biosynthesis and signaling are essential for preventing water-soaking under LL (Fig. 3d), we explored the potential of using BTH to prevent this phenomenon. Plants that were pre-treated with BTH 24 h prior to a *Pst* challenge did not display water-soaking lesions, in contrast to control-treated plants (Fig. 4a). Most importantly, BTH prevented *Pst*-induced water-soaking lesions under a LD regime, in which water-soaking lesions are normally visible. Consistent with this result, *Pst* did not induce stomatal closure in LD-grown plants pre-treated with BTH (Fig. 4b). These effects were independent of the number of bacteria, as bacterial titers in leaves did not differ between treatments at the time of stomatal evaluation (Fig. 4c).

We next tested the ability of BTH to mitigate a *Pst* infection under either LL or LD in locally pre-treated leaves. Interestingly, levels of bacteria in control or BTH pre-treated leaves were similar under constant light, suggesting that BTH does not induce more resistance the effect of a 24-h period of light alone (Fig. 4d). However, a clear difference was observed between control and BTH pre-treated plants under LD conditions (~100-fold less bacteria). At the same time, LD-

grown plants pre-treated with BTH showed a protection similar to albeit statistically significantly different from that accorded by growing under LL (5.69 ± 0.15 CFU/cm² (LL); 6.25 ± 0.36 CFU/cm² (LD)) (Fig. 4d). These results suggest that BTH provides protection similar to that provided by a constant light treatment, and further indicates that the effects of constant light in this context are due to the actions of SA.

Constant light allows plants to recover from bacterial infection

Given the role of light in preventing disease symptoms and pathogen growth, we evaluated whether constant light could mitigate the progression of the pathogen in plants that were already infected. To test this, plants were inoculated and left under a LD cycle for a day to allow the pathogen to establish an infection. Plants were then transferred to LL conditions for 2 days, then again to a LD cycle for two additional days. Control plants were similarly inoculated but kept under a LD cycle for the duration of the experiment (Fig. 5a). No disease symptoms were observed on plant leaves that had been subjected to the 2-day LL treatment (Fig. 5b). Bacterial titer assays were also performed and a decrease in bacterial load was observed when plants were kept under LL conditions compared to plants kept under LD conditions for the entire experiment (Fig. 5c). These results suggest that constant light treatment allows plants to recover from a *Pst* infection.

We also assessed stomatal aperture over the course of a 5-day infection period in the same experiment. As expected, infiltration with *Pst* induced stomatal closure in LD conditions within 24 h compared to control plants (Fig. 5d). At 3 dpi, plants kept under LD conditions maintained this phenotype to an even greater degree, presumably due to increased bacterial numbers (Fig. 5c, d). In contrast, moving plants to LL conditions resulted in stomatal opening similar to uninoculated plants (Fig. 5d). Consistent with a report showing that phytochemicals induce reopening of stomata at late stages of infection³⁶, we observed that previously closed stomata reopened at 5 dpi even when plants were returned to a LD regime (Fig. 5d). This is consistent with observations that water-soaking lesions are present in the early phases of *Pst* infection, and disappear in later phases¹¹. Nonetheless, our results show that it is possible to revert pathogen-induced stomatal closure to prevent disease progression by placing infected plants under a constant light regime for 2 days.

PIFs may modulate disease resistance through SA signaling

We investigated the implication of light photoreception and signaling in susceptibility to bacterial infection under LL or LD regimes. For this, we used the *Arabidopsis* blue-light insensitive (*blus1*) mutant as well as a quadruple mutant for phytochrome-interacting factors (*pifq*), which is responsible for integrating red and far/red light signaling. The *Arabidopsis blus1* mutant behaved similarly to wild-type plants in terms of water-soaking under LD and LL (Fig. 6a). However, *pifq* mutants displayed a reestablishment of water-soaked lesions 24 h after inoculation with *Pst* (Fig. 6a). These observations were consistent with measurements of stomatal aperture, wherein only *pifq* mutants infected with *Pst* had closed stomata under constant light (Fig. 6b). Interestingly, all three genotypes showed similar increases of induction of expression of *ICS1* upon infection with *Pst*, with somewhat higher levels in LL versus LD (Fig. 6c). However, upon inoculation with *Pst*, WT and *blus1* plants showed much higher levels of induction of *PRI* expression in LL versus LD conditions, whereas *pifq* plants did not (Fig. 6d). At the same time, upon inoculation with *Pst*, *pifq* mutant plants kept under LL conditions showed no statistically significant difference (Wilcoxon Rank Sum test) in *PRI* induction to WT plants grown in a LD regime (Fig. 6d). Thus, the levels of *PRI* induction and stomatal closure in LL-grown *pifq* plants resemble those of LD-grown WT plants, further underlining the link between SA signaling, stomatal closure, and water-soaking. Light photoreception mutants displayed no significant differences in susceptibility toward a *Pst* infection compared to WT plants under different light regimes (Supplementary Fig. 7). This can be explained by the fact

that water-soaking symptom appearance in *pifq* mutant requires a high load of bacteria upon infiltration, while a low load is used for bacterial growth assays. These results thus suggest that PIFs might not be absolutely required for, but may be involved in, reinforcing the SA module during constant light after immune elicitation.

Discussion

Induction of a water-soaked apoplast is crucial for disease development in plants^{11,37–39}. At the same time, it is known that this can be affected by light. For example, in an early study of fire blight, it was observed that “as the sun came out, many of these areas (water-soaked lesions) cleared up completely”⁴⁰. In this study, we provide evidence that diurnal light cycles are intimately linked with the ability of a bacterial pathogen to establish an aqueous apoplast. We show that a period of darkness is required to induce water-soaking and that constant light prevent induction of stomatal closure mediated by water-soaking effectors. Light appears to counteract the stomatal-closing effects of ABA through potentiation of SA responses. Furthermore, BTH, a SA analog, was sufficient to prevent bacterial induction of water-soaking lesions. Whether SA signaling directly inhibits some aspect of ABA action in this case remains to be determined. Considering how common the induction of water-soaked lesions is as an initial step in microbial pathogenesis³⁷, finding non-invasive and easily applicable solutions to prevent them is of great practical interest.

In studies of the circadian clock machinery plants are often placed in constant light to study oscillations in gene regulation and other phenomena⁴¹. As such, much of our understanding of circadian-gated regulation of phytohormone networks rely heavily on data obtained under constant light regimes^{27,42,43}. However, light can affect molecular processes independently of circadian mechanisms. Indeed, *Arabidopsis* plants with mutations in key circadian cycle-regulating genes are protected against infection when grown under LL conditions compare to LD. Importantly, the degree of protection is similar to that seen with WT plants (Supplementary Fig. 8), suggesting that the effects of constant light are not dependant on the circadian cycle.

Our data suggest that, under constant light regimes, plant immune responses are augmented or primed. Indeed, apoplastic ROS bursts following *flg22* perception are more intense under LL conditions and less intense under DD (Fig. 1d). These results are consistent with the elevated levels of expression of *FLS2*, *RBOHD*, *ICS1* and *PRI* under LL conditions prior to *flg22* treatment (Supplementary Figs. 1 and 3a, b). Furthermore, the expression levels of early PTI-responsive genes, *FRK1* and *WRKY29*, were significantly increased under constant light conditions prior to, and after, treatment with *flg22* (Fig. 1f, g). Nonetheless, consistent with increased aROS production, plants grown under LL conditions appear to respond with greater intensity to *flg22* in terms of SA accumulation (Fig. 3c). How light affects the responsiveness to immune elicitors remains to be investigated. However, our results showing that it affects aROS production and SA-related responses, but not early PTI responses, suggest that constant light affects specific modules in plant immunity. At the same time, our results are consistent with a recent study showing that, under photo-periodic stress, the *Arabidopsis* transcriptional signature resembles that of a response to pathogens³³. Notably, SA signatures increased in plants kept under constant light and were reduced in dark-grown plants.

It has been reported that pathogen aggressiveness is increased in infections occurring in the shade⁴⁴. However, whether this involves host stomatal responses and is related to pathogen-induced water-soaking lesions has not been investigated. Diurnal light changes affect stomatal aperture, where stomata are open during the day and partially close during the night⁴⁵. As such, a synergistic interaction may occur in which the mechanism(s) behind dark-induced stomatal closure, while not well defined, may act as facilitators for effector-mediated induction of stomatal closure. It is important to note that, in

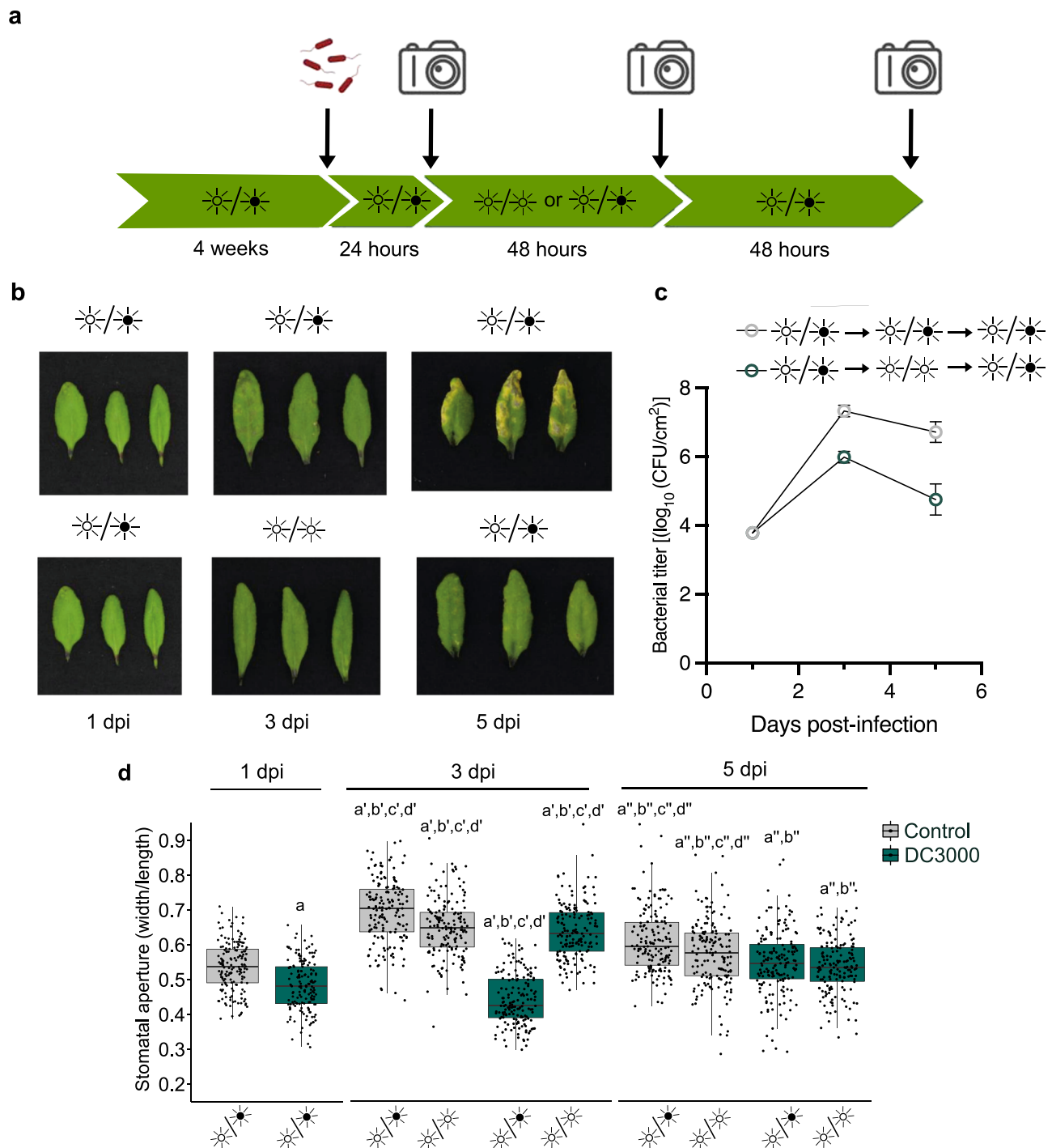


Fig. 5 | Constant light can prevent disease development. **a** Schematic diagram representing treatments and phenotype assessment of light-mediated disease prevention. This figure was created with BioRender.com. **b** Disease symptoms in *Arabidopsis* plants infected with 1×10^5 CFU/ml under different light settings, as indicated. **c** Bacterial titers from plants shown in **b** and harvested at the indicated timepoints ($n = 3$ biologically independent replicates). Bacterial titers were significantly different between plants subjected to a LL treatment versus control (Student's *t* test, $p = 0.0024$) **d** Stomatal aperture measurements of leaves evaluated as in

c ($n = 160$ stomata for 1 dpi LD control and DC3000, 3 dpi LL control and LD DC3000; $n = 161$ stomata for 3 dpi LL DC3000 and LD control; $n = 163$ stomata for all data at 5 dpi). Letters indicate statistically significant differences compared to control with Student's *t* test (1 dpi) or Wilcoxon–Mann–Whitney test (3 and 5 dpi). Data points and error bars represent mean \pm S.E.M. In box plots, box edges delineate lower and upper quartiles, the center line represents the median, and whiskers extend to 1.5 times the interquartile range (IQR).

infected plants, light-induced inhibition of stomatal closure and water-soaking can be rapidly reversed. That is, non-water-soaked infected plants with LL and SA-dependent open stomata undergo water-soaking after a 4-h dark treatment (Fig. 2d, e). This suggests that dark conditions may be a prerequisite for the induction of water soaking. Current understanding of the mechanism behind darkness-induced stomatal

closure suggest that ABA signaling and metabolism are important for the response speed, but not essential for closure, suggesting ABA-dependent and independent mechanisms⁴⁶. Thus, we speculate that the balance between the differential effects of ABA and SA on stomatal aperture may be affected by additional light-dependent mechanisms (Fig. 7). Whether this is related to previously described antagonistic

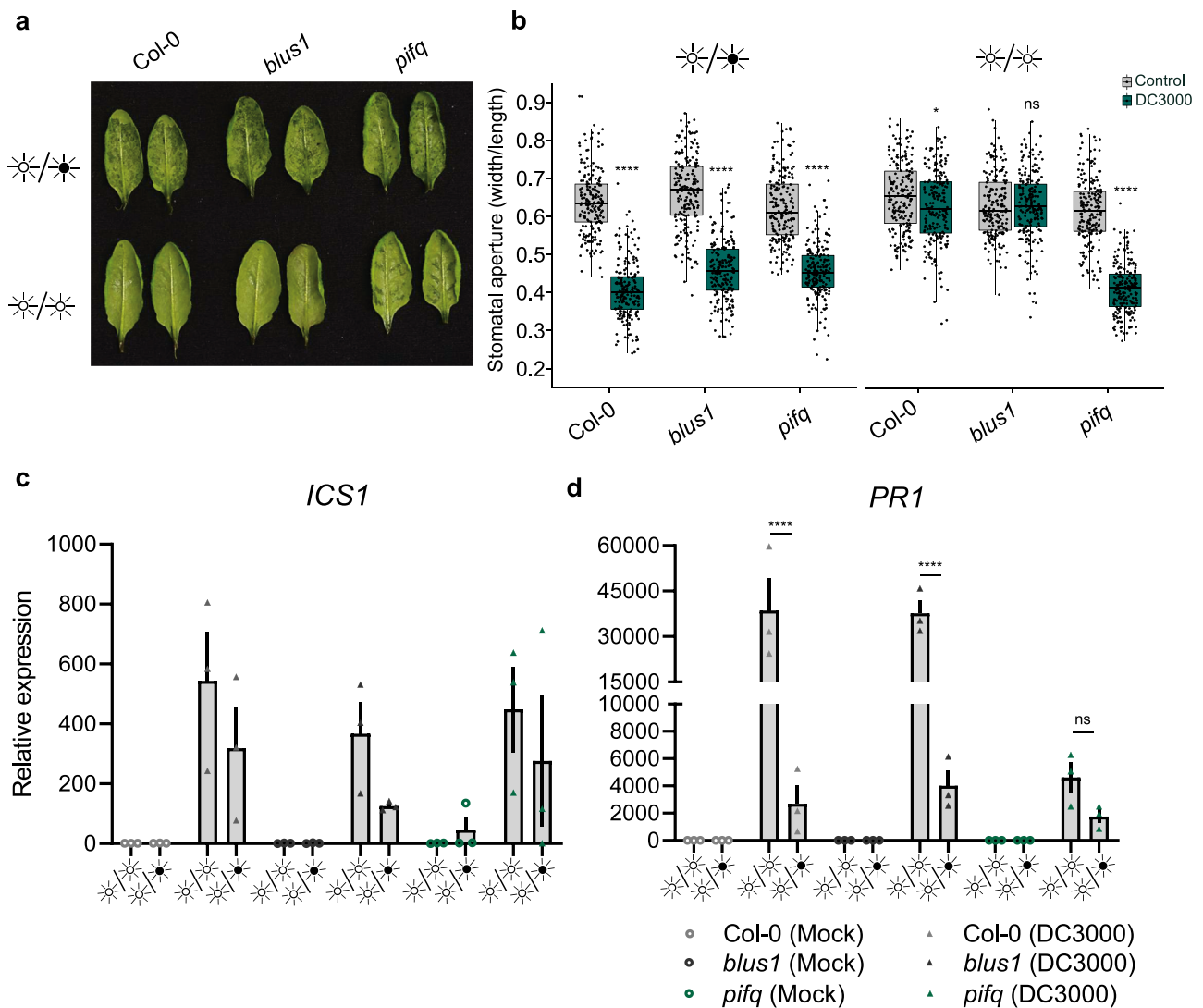


Fig. 6 | Potential implication of PIFs in preventing bacterial induction of water-soaked lesions under light. **a** Water-soaking phenotypes of *Arabidopsis* WT (Col-0), *blus1* and *pifq* mutant plants syringe-infiltrated with *Pst* 3000 (1×10^8 CFU/ml) under the indicated light settings. Photos were taken at 24 h post infiltration (hpi). **b** Stomatal aperture measurements from infected leaves displayed in **a** ($n = 192$ stomata for all LD DC3000 data, $n = 196$ stomata for all control data, $n = 199$ stomata for all LL DC3000 data). Expression levels, assessed by qRT-PCR, of *ICS1* (**c**) and *PR1* (**d**) marker genes in *Arabidopsis* WT (Col-0), *blus1* and *pifq* mutant plants infected with *Pst* DC3000 (1×10^8 CFU/ml) or with control solution (MgCl_2

10 mM) in plants that were placed in the identified light settings. Samples were harvested at 24 hpi ($n = 3$ biologically independent replicates). Asterisks indicate statistically significant differences compared to control, ns non-significant, $*p < 0.05$, $****p < 2.2 \times 10^{-16}$, Wilcoxon–Mann–Whitney test (**b**) or $****p < 0.0001$, Student's *t* test (**d**). All statistical tests are two-sided. In box plots, box edges delineate lower and upper quartiles, the center line represents the median, and whiskers extend to 1.5 times the interquartile range (IQR). In non-box-plot graphs, boxes and error bars represent mean \pm S.E.M.

effects of SA on ABA signaling^{30–32} remains to be determined. Likewise, this could include regulation of ABA levels, which increase substantially within a 4-h period of darkness⁴⁷.

Water-soaking effectors of *Pst* modulate ABA-related responses^{9,10}. The induction of expression of ABA biosynthesis and signaling pathways by *Pst* was unaltered between all light regimes tested (Supplementary Fig. 4). Indeed, SA and ABA mutants do not show large differences in the accumulation of ABA and SA, respectively upon bacterial infection, suggesting that the reported SA antagonism of ABA-related responses may be downstream of ABA-responsive targets^{30–32}. Our observations are reminiscent of a previous report showing that SA accumulation antagonizes ABA responses, including stomatal closure, even if ABA over accumulates and certain ABA-responsive genes are activated at the transcriptional level⁵². Whether SA-mediated antagonism of ABA responses is solely responsible for the pathogen's inability to induce an aqueous apoplast remains to be investigated.

ABA is a major susceptibility factor to most known ABA producing or ABA inducing plant pathogens⁴⁸. In addition to *Pst*, exposure to photoperiodic stress leads to enhanced disease resistance to the hemibiotrophic fungus *M. oryzae* and to the necrotrophic fungus *B. cinerea*^{49,50}. Interestingly, these two fungal pathogens produce ABA and induce water-soaking lesions early on during the infection process. Whether water-soaking contributes to fungal pathogenicity is not clear, but ABA was found to be a key virulence component for *M. oryzae* and *B. cinerea*^{51,52}. It is thus interesting to speculate that the reduced virulence of these fungal pathogens in plants exposed to photoperiodic stress is caused by their inability to create an aqueous apoplast as well. Therefore, exposing plants to constant light for a short amount of time could be a very impactful strategy to reduce infections from pathogens from different kingdoms of life.

We are still in the dark about the specific wavelengths of light that affect the defense responses described herein. However, it has been

reported that red light treatment improves resistance against *Pst* and that this treatment increased SA signaling as well as transcription of genes involved in redox homeostasis, such as *RBOH* and *GSTs*⁵³. Our results provides further evidence for the role of red light in mediating resistance⁵⁴, as a quadruple mutant of PIF genes, which are involved in red light signaling, displays water-soaking in constant light, whereas this is not observed in the *Arabidopsis* blue light signaling mutant *blus1* (Fig. 6a). *Arabidopsis pifq* mutant displayed less SA potentiation upon-pathogen inoculation, which could be the reason behind the induction of water-soaking in this mutant. Further research will be required to dissect how red-light circuits are integrated into immune responses.

In summary, our study better defines a physiological mechanism for disease resistance and adds to the growing trend demonstrating that plant defenses involve starving pathogens for water and nutrients^{11,55}. We provide a proof of concept showing that altering light conditions for relatively short periods of time can limit pathogen damage (Fig. 5) and suggest that this could have broad applications. While controlling photoperiods to prevent disease outbreaks is not as applicable for field production, this simple method could be used to controlled environment food production, which is growing quickly and is linked to urban food sovereignty. At the same time, our current and previous work^{9,10,39} indicate that any treatment that leads to increased stomatal opening and transpiration leads to decreases in pathogen virulence. As such, strategies to prevent pathogen-induction of water-soaking could lead to broad-spectrum disease resistance.

Methods

Plant material

Arabidopsis plants were grown in Promix™ soil (PremierTech) in growth chambers either with 12 h light/dark, 24 h light or 24 h dark photoperiod, with relative humidity of ~60% at 21 °C. Light intensity was measured at 180 $\mu\text{mol}/\text{m}^2/\text{s}$ in all growth chambers when lights were on. Four- to 5-week-old *Arabidopsis* plants were used for all experiments described.

Bacterial disease assays

Pseudomonas syringae pv. *tomato* DC3000 WT and mutant strains were cultured overnight at 28 °C in Luria-Bertani (LB) media containing 50 mg/l of rifampicin. On the day of the infection, fresh LB media was inoculated with 0.5 μl of the overnight culture and bacteria were collected when OD_{600} reached between 0.8–1. Bacteria were centrifuged for 10 min at 4000 $\times g$ and the pellet resuspended in MgCl_2 10 mM. Bacterial density was adjusted to 0.2 (1×10^8 CFU/ml) prior to further dilutions. Bacterial infections were carried between 14:00–15:00, or a zeitgeber time of 6:00–7:00.

All inoculations of *Arabidopsis* leaves were performed by syringe-infiltration. Infiltrated plants were all kept under ambient humidity levels for 1–2 h to allow water to evaporate, then domed with a plastic unit to maintain high humidity (>95% RH), unless stated otherwise.

Bacterial growth in planta was monitored by harvesting infected *Arabidopsis* leaves, surface sterilizing in 80% ethanol and rinsing in sterile water twice. Leaf disks were taken from three leaves from the same plant (one per leaf; total of three leaf disks) using a cork borer (0.6 mm in diameter) and ground in sterile 10 mM MgCl_2 . Three biological replicates were performed for each experiment. Colony-forming units (CFU) were determined by making serial dilutions (10^0 – 10^{-6}) and plating on LB plates containing 50 mg/l of rifampicin. Each dilution was plated in three technical replicates. Experiments were repeated at least three times and values from replicates are tabulated in the Source Data File.

ROS quantification

Leaf disks from 4-week-old *Arabidopsis* plants were collected using a 4 mm diameter biopsy punch and placed into white 96-well plates

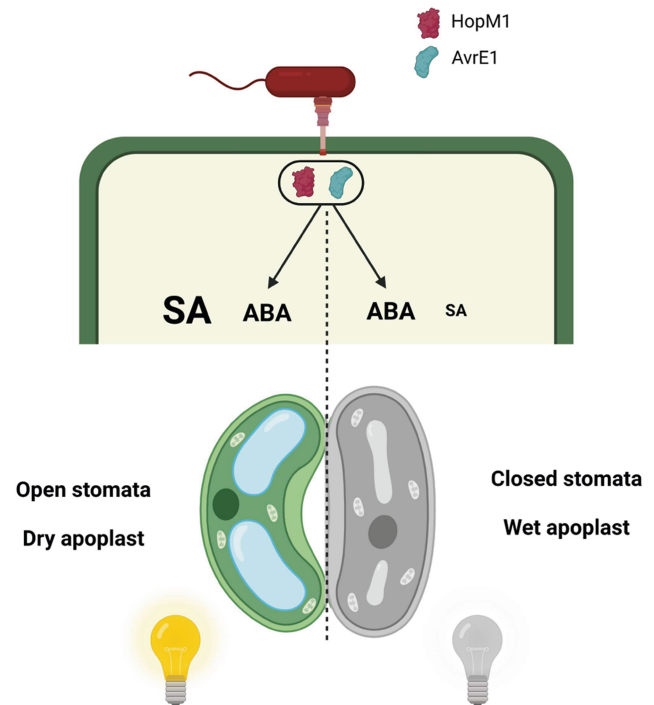


Fig. 7 | Model for light-induced disease resistance in plants. In continuous light (left), HopM1 and AvrE1-mediated stimulation of ABA biosynthesis and signaling occurs. However, elevated SA accumulation in continuous light conditions ensures that stomata remain open despite the pro-closure influence of ABA, thus preventing water-soaking. When plants experience a period of darkness (right), the pro-closure influence of ABA predominates, possibly due to reduced SA accumulation, leading to water-soaking. This figure was created with BioRender.com.

(Corning) containing 100 μl of distilled water for 16 h (overnight). Prior to ROS quantification, the water was removed and replaced with ROS assay solution (100 μM Luminol [Millipore-Sigma], 20 $\mu\text{g}/\text{ml}$ horseradish peroxidase [Millipore-Sigma] with or without immune elicitors). Light emission was measured using a TECAN Spark® plate reader. Experiments were repeated three times and values from replicates are tabulated in the Source Data File.

Stomatal aperture assays

In LL and LD experiments, plants were sampled during the subjective day (i.e., light conditions). In DD experiments, plants were sampled from dark conditions. Leaves were cut at the base of the petiole and immediately immersed in the stomatal fixation solution (formaldehyde 4% and rhodamine 6 G 0.5 μM) for 1 min to stop stomatal movement. A quarter of each leaf was cut with a razor blade and stomata were observed by epifluorescence microscopy. Stomatal apertures were measured using OMERO software and the degree of the stomatal opening was measured as a ratio of stomatal width to length. Between 150 to 250 stomata were measured for each data point. Data points represented in stomatal aperture measurement figures originated from at least three leaves from three different plants. Data collection and analysis was performed by using double-blinded standards to avoid bias. Values from replicates are tabulated in the Source Data File.

Chemical treatments

To assess plant immune responses, *Arabidopsis* leaves were treated with flg22 (1 μM ; BioBasic) by syringe-infiltration. For BTH protection assay, *Arabidopsis* leaves were syringe-infiltrated with BTH (50 μM ; Millipore-Sigma).

RNA extraction and real-time quantitative PCR

RNA was extracted from frozen and ground leaf tissue using QIAzol (QIAGEN) reagents, followed by on-column DNase treatment (QIAGEN), according to the manufacturer's protocol. RNA purity was assessed with a spectrophotometer and quality by gel electrophoresis. cDNA was generated by using MMuLV-RT (Service de purification des protéines—Université de Sherbrooke).

Quantitative real-time PCR was performed with a Bio-Rad CFX96 machine. Each reaction contained 1X SYBR mix (Service de purification des protéines—Université de Sherbrooke), specific primers and a 1:20 dilution of 500 ng of cDNA stock. Amplification cycle protocols were as follows: 2 min at 95 °C; 40 cycles of 6 s at 95 °C and 30 s at 60 °C. Melting curves were verified at the end of 40 cycles for confirmation of primer specificities. All reactions were repeated in three technical and biological replicates. Average C_q values were normalized by $\Delta\Delta C_T$ formula against *ACTIN2* expression. Oligos used in this study can be found in Supplementary Table 1. Experiments were repeated at least three times and values from replicates are tabulated in the Source Data File.

Apoplast extraction

Three fully expanded leaves per plant from three plants were excised and apoplast extracted as previously described⁵⁶, with some modifications. Briefly, the initial weight of freshly excised leaves was measured before being infiltrated with distilled water and weighed again once leaves were fully saturated with water. Leaves were centrifuged at 4000 rcf in 2 ml microcentrifugation tubes containing glass beads at the bottom to prevent tissue collapse during the centrifugation. Leaves were weighed post centrifugation and apoplast hydration determined as previously described⁵⁶. Experiments were repeated at least three times and values from replicates are tabulated in the Source Data File.

Salicylic acid extraction and quantification

Fully expanded 4-week-old *Arabidopsis* leaves were harvested and weighed for fresh weight calculation and immediately flash-freeze in liquid nitrogen. Tissues were ground with a plastic pestle and phytohormones were extracted overnight using 0.5–1 ml of ice-cold extraction buffer (methanol: water [80:20 v/v], 0.1% formic acid, 0.1 g/l butylated hydroxytoluene and 100 nM ABA-d₆ as an internal standard). Extracts were filtered using centrifugal filter units.

Filtered extracts were quantified using an Acquity Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA) as described previously¹⁰. SA was quantified based on a standard curve to calculate sample concentration (nM), which was converted to ng using the molecular weight of each specific compound and the extraction volume used. All data was normalized to initial fresh weight in grams. Experiments were repeated three times and values from replicates are tabulated in the Source Data File.

Statistical analysis

Statistical analyses were performed in GraphPad Prism 8.0 for all RT-qPCR experiments, photon counts, and SA quantification. For all other experiments (bacterial counts and stomatal assays), the bioinformatic software R was used. *p* values greater than 0.05 were considered non-significant. Sample sizes, statistical tests used, and *p* values are stated in figure legends. All statistical test assumptions such as normality and homoskedasticity were tested. When not respected, non-parametric equivalent tests were performed. In multiple comparison tests, Bonferroni corrections were used. Data points represented in stomatal aperture measurement figures originated from at least three leaves from three different plants.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source data are provided with this paper.

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Author contributions

C.R.-L. and P.M. conceived the study. G.L. and C.R.-L. performed all experiments, data analysis and the creation of figures. S.B. and E.F. contributed to some experiments and data collection. I.L.-L. contributed to the statistical analysis. G.L. and C.R.-L. wrote the manuscript with support from P.M. and I.L.-L. All authors revised and agreed with the content written in this manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Charles Roussin-Léveillé or Peter Moffett.

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