# Light Regulation and Daytime Dependency of Inducible Plant Defenses in Arabidopsis: Phytochrome Signaling Controls Systemic Acquired Resistance Rather Than Local Defense[1]

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We have examined molecular and physiological principles underlying the light dependency of defense activation in Arabidopsis (*Arabidopsis thaliana*) plants challenged with the bacterial pathogen *Pseudomonas syringae*. Within a fixed light/dark cycle, plant defense responses and disease resistance significantly depend on the time of day when pathogen contact takes place. Morning and midday inoculations result in higher salicylic acid accumulation, faster expression of pathogenesis-related genes, and a more pronounced hypersensitive response than inoculations in the evening or at night. Rather than to the plants' circadian rhythm, this increased plant defense capability upon day inoculations is attributable to the availability of a prolonged light period during the early plant-pathogen interaction. Moreover, pathogen responses of Arabidopsis double mutants affected in light perception, i.e. *cryptochrome1cryptochrome2* (*cry1cry2*), *phototropin1phototropin2* (*phot1phot2*), and *phytochromeA-phytochromeB* (*phyAphyB*) were assessed. Induction of defense responses by either avirulent or virulent *P. syringae* at inoculation sites is relatively robust in leaves of photoreceptor mutants, indicating little cross talk between local defense and light signaling. In addition, the blue-light receptor mutants *cry1cry2* and *phot1phot2* are both capable of establishing a full systemic acquired resistance (SAR) response. Induction of SAR and salicylic-acid-dependent systemic defense reactions, however, are compromised in *phyAphyB* mutants. Phytochrome regulation of SAR involves the essential SAR component FLAVIN-DEPENDENT MONOOXYGENASE1. Our findings highlight the importance of phytochrome photoperception during systemic rather than local resistance induction. The phytochrome system seems to accommodate the supply of light energy to the energetically costly increase in whole plant resistance.

To successfully adapt to a changing environment, plants must simultaneously perceive and appropriately respond to a variety of different biotic and abiotic stimuli. Upon attempted infection by microbial pathogens, plants induce a multitude of defense responses to combat the attacking intruders (Dangl and Jones, 2001). At infection sites, these responses often include rapid production of reactive oxygen species (ROS), biosynthesis of low-molecular-weight defense signals such as salicylic acid (SA) and jasmonic acid (JA), accumulation of phytoalexins, increased expression of pathogenesis-related (PR) proteins, and hypersensitive cell death (hypersensitive response [HR]). A localized contact of leaf tissue with pathogenic or nonpathogenic microbes can further lead to systemic acquired resistance (SAR), a state of enhanced, broad-spectrum resistance at the whole plant level that protects against subsequent pathogen attack (Durrant and Dong, 2004; Mishina and Zeier, 2007). Plant SA levels rise systemically during SAR, and this increase is required for induced expression of SA-dependent *PR* genes and systemic enhancement of disease resistance (Ryals et al., 1996; Métraux, 2002).

Inducible plant defenses and resistance against pathogens can be affected by changing environmental conditions (Colhoun, 1973). Light is the major external factor influencing plant growth and development, and an appropriate light environment is also required for the establishment of a complete set of resistance responses in several plant-pathogen interactions (Roberts and Paul, 2006). In tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), and Arabidopsis (*Arabidopsis thaliana*), HR-associated programmed cell death triggered by bacterial and viral pathogens is light dependent (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Chandra-Shekara et al., 2006). Similarly, the constitutive cell death phenotype of Arabidopsis *acd11* and *lsd1* mutants is only evident when light of a certain quantity or duration is present (Brodersen et al., 2002; Mateo et al., 2004). Pathogen-induced activation of phenylpropanoid biosynthesis is another major defense pathway controlled by light. Deposition of lignin-like polymers in *Xanthomonas*

[1] This work was supported by the Deutsche Forschungsgemeinschaft (project no. B2, Graduiertenkolleg 1342).

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.119503

*oryza*-treated rice leaves decrease when light is absent during the first hours after inoculation (Guo et al., 1993). Moreover, Arabidopsis plants inoculated in darkness with an avirulent strain of *Pseudomonas syringae* are not able to substantially accumulate the phenolic metabolite SA and fail to induce expression of the key phenylpropanoid pathway enzyme Phe ammonia lyase (PAL; Zeier et al., 2004). Light is not only required for SA biosynthesis, but also controls SA perception, because treatment of Arabidopsis leaves with exogenous SA in dim light or in the dark results in strongly reduced expression of the SA-induced defense gene *PR-1* (Genoud et al., 2002). Both impaired production and perception of SA therefore account for the observation that *PR-1* expression in *P. syringae*-treated Arabidopsis leaves is completely suppressed in dark-situated plants (Zeier et al., 2004).

The HR- and SA-associated defenses are effective means to restrict invasion of biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). Thus, light-controlled activation of these responses can explain why resistance of plants to many bacterial and viral pathogens is attenuated in the dark (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Chandra-Shekara et al., 2006). It is noteworthy, however, that several inducible plant defenses occurring at sites of pathogen inoculation do not require the presence of light. In Arabidopsis, these responses include biosynthesis of the phytoalexin camalexin, accumulation of the oxylipin-derived signal JA, and expression of the ROS-associated glutathione-*S*-transferase *GST1* (Zeier et al., 2004). Similarly, in tomato (*Solanum lycopersicum*), activation of lipoxygenase and lipid peroxidation are not light dependent (Peever and Higgins, 1989). Induction of resistance at the whole plant level during SAR and associated systemic elevation of SA levels and *PR-1* gene expression in Arabidopsis, by contrast, strictly depend on the presence of a light period during the first 2 d after pathogen contact (Zeier et al., 2004).

The molecular mechanisms by which responses to light and biotic stress interact are only poorly understood (Roberts and Paul, 2006). Through photosynthesis, light can directly provide energy, reduction equivalents, and metabolic precursors for the production of defense metabolites. Light also acts as a signal to regulate many aspects of plant growth, development, and physiology. Regulatory light signals are perceived and transduced into cellular responses by different photoreceptor families: the cryptochromes and phototropins, which both absorb UV-A and blue light, the phytochromes, which sense red/far-red light, and as yet unidentified UV-B receptors (Gyula et al., 2003). Whether and how specific light-induced signaling pathways interact with defense pathways has only scarcely been investigated. Genoud et al. (2002) have demonstrated cross talk between phytochrome signaling and both SA perception and HR development in Arabidopsis upon inoculation with avirulent *P. syringae*. The light-dependent HR triggered by *Turnip crinkle virus* and resistance to viral infection, on the other hand, proved to be phytochrome independent (Chandra-Shekara et al., 2006).

In this work, we study the principles underlying light dependency of inducible plant defenses in the Arabidopsis-*P. syringae* model interaction at the molecular level. Our data indicate that light regulation of defense responses manifests itself not only during artificial dark treatments but is also relevant within naturally occurring light/dark cycles. Furthermore, employing Arabidopsis photoreceptor double mutants, we show that inducible defense responses at inoculation sites are not or only moderately altered when cryptochrome, phototropin, or phytochrome photoperception is impaired. SAR, by contrast, strongly depends on phytochrome photoperception and can be established without functional cryptochrome or phototropin signaling pathways.

## RESULTS

### Plant Defenses and Resistance Depend on the Daytime of Inoculation

To study the influence of light on inducible plant defenses and disease resistance, we previously compared resistance responses of ecotype Columbia of Arabidopsis (Col-0) plants situated in conventional 9-h light/15-h dark photoperiodic conditions with those of plants transferred to continuous darkness before pathogen inoculation. The HR-inducing bacterial strain *Pseudomonas syringae* pv. *maculicola* ES4326 carrying the avirulence gene *avrRpm1* (*Psm avrRpm1*) was used in these experiments. In summary, we observed that induction of a specific subset of plant defense responses, which includes SA-associated responses and the HR, depends on the presence of light after pathogen inoculation (Zeier et al., 2004). To examine whether light regulation of defense reactions is relevant not only during artificial darkening experiments but also within a light/dark cycle that naturally occurs during the course of a day, we inoculated Col-0 plants at defined daytimes with *Psm avrRpm1*, i.e. in the morning (9 AM), at midday (1 PM), in the evening (7 PM), and in the night (1 AM), and scored resistance responses at constant times after each treatment. As in previous experiments (Zeier et al., 2004), the applied day/night cycle in the growth chamber consisted of a 9-h light period (photon flux density = 70 $\mu$mol $m^{-2}$ $s^{-1}$) starting from 9 AM until 6 PM, and a dark period during the remaining daytime (Fig. 1A).

In Col-0 leaves, biosynthesis of SA is induced during the first 4 to 8 h after pressure infiltration of *Psm avrRpm1* suspensions (Mishina et al., 2008). When applying bacteria at different daytimes, we found that the amount of total (sum of free and glucosidic) SA produced within the first 10 h postinoculation (hpi) strongly depends on the inoculation daytime, with SA accumulating to 8.0 $\mu$g $g^{-1}$ fresh weight (FW), 4.5 $\mu$g

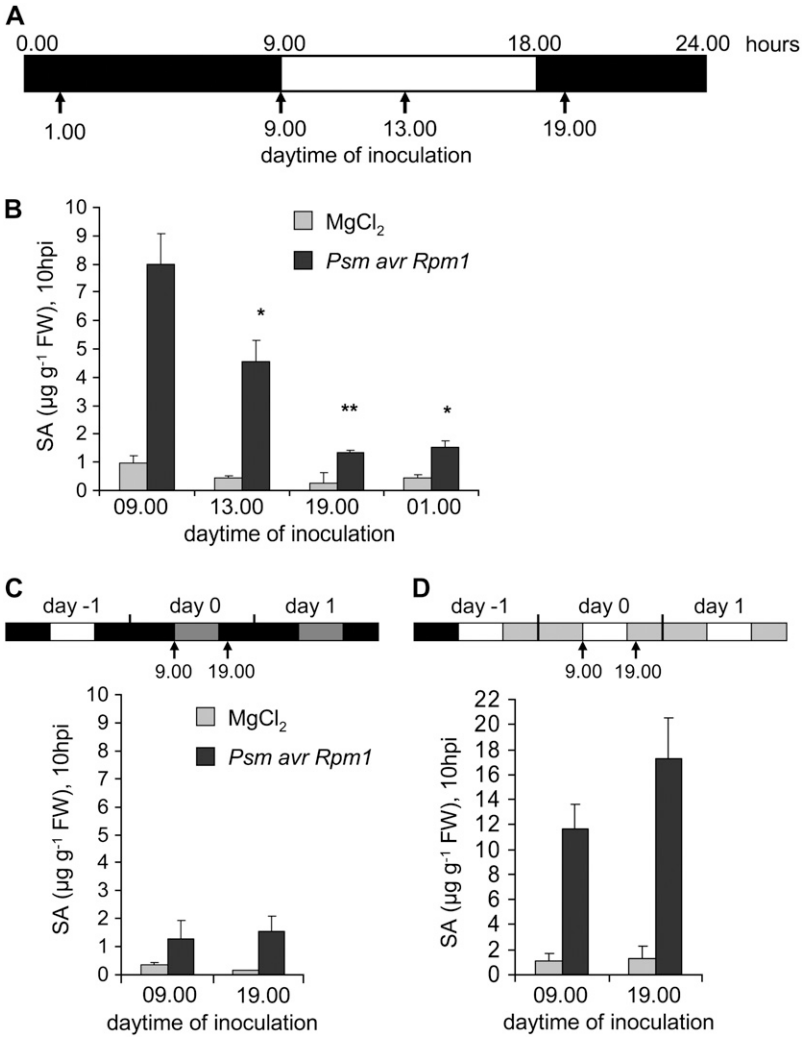


**Figure 1.** SA accumulation in Arabidopsis depends on the daytime of pathogen inoculation. A, Daytimes of *Psm avrRpm1* inoculation and light/dark regime in the plant growth chamber. Black and white boxes correspond to dark and light periods, respectively, during a normal growth chamber day. Arrows and bottom numbers indicate the four different inoculation times. B, SA accumulation in Arabidopsis Col-0 leaves at 10 hpi with *Psm avrRpm1* (OD = 0.005) following the experimental setup described in A. Control samples were infiltrated with 10 mM $MgCl_2$. Values of free and glycosidic SA were added to yield total SA levels. Bars represent mean values (± SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisks denote values with statistically significant differences to the 9 AM value (*, $P < 0.05$, **, $P < 0.001$; Student's *t* test). Light bars, $MgCl_2$-treatment; dark bars, *Psm avrRpm1* inoculation. C, Accumulation of total SA in continuous darkness after inoculation at 9 AM and at 7 PM. The top illustration indicates light regime and inoculation times during three consecutive days around the beginning of the experiment (day 0). Until day −2, normal light/dark cycles (depicted in A) were applied. Dark gray boxes correspond to dark phases with subjective day character. D, Accumulation of total SA in continuous light after inoculation at 9 AM and at 7 PM. The top illustration is according to C except that light gray boxes indicate light periods with subjective night character.

$g^{-1}$ FW, 1.3 $\mu g\ g^{-1}$ FW, or 1.5 $\mu g\ g^{-1}$ FW after morning, midday, evening, and night inoculations, respectively (Fig. 1B). The differences in leaf SA accumulation between morning, midday, and evening/night inoculations were statistically significant ($P < 0.02$), and the trend for total SA depicted in Figure 1B was similarly observed for the levels of both free and glucosidic SA (data not shown). The amounts of SA produced during the first 10 hpi thus correlated with the number of light hours (9 h for morning, 5 h for midday, 0 h for evening, and 2 h for night inoculations, respectively) during this early infection period.

Because pathogen defense has previously been linked with the circadian rhythm (Sauerbrunn and Schlaich, 2004), we examined a possible contribution of the circadian clock to the observed daytime effect on SA accumulation. Conventionally grown plants were therefore placed into continuous darkness from dusk of day −1 (the day before the pathogen experiment was started), and leaves were inoculated with *Psm avrRpm1* the following day (day 0) at 9 AM or at 7 PM (Fig. 1C). In both cases, lower SA levels comparable with those accumulating in leaves of evening inoculated plants experiencing the normal light/dark cycle (Fig. 1B) were detected at 10 hpi, suggesting that the contribution of the circadian rhythm to the daytime effect is negligible, and indicating that the differences in SA accumulation observed during the daytime experiment (Fig. 1B) essentially resulted from distinctive lengths of the light period during the early plant-pathogen interaction. Conversely, we also placed plants into continuous light from dawn of day −1 onwards, treated leaves with *Psm avrRpm1* at 9 AM or at 7 PM of day 0, and scored SA accumulation at 10 hpi (Fig. 1D). High SA levels (11.7 $\mu g\ g^{-1}$ FW), which exceeded the 9 AM value (8.0 $\mu g\ g^{-1}$ FW) from the normal daytime experiment (Fig. 1B), accumulated after the 9 AM inoculation at continuous light. Although circadian clock regulation of SA production would imply a lower SA value for the 7 PM inoculation under continuous light, we detected an even higher mean value of 17.2 $\mu g\ g^{-1}$ FW than for the 9 AM treatment. This again emphasizes that the circadian clock does not regulate pathogen-induced SA production.

Although differences between both daytimes under continuous light were statistically not different ($P = 0.07$), the tendencies observed in Figure 1, B and D, might suggest that the duration of the light period just before bacterial inoculation has an influence on the amount of accumulating SA.

We next assessed whether expression of the SA-inducible defense gene *PR-1* and HR cell death, two responses that had previously been shown to be light regulated (Genoud et al., 2002; Zeier et al., 2004), would also depend on inoculation daytime. Whereas a morning or midday treatment of Col-0 leaves with *Psm avrRpm1* induced a distinct *PR-1* expression already at 10 h after pathogen contact, evening or night inoculation did not result in induction of the defense gene at 10 hpi (Fig. 2). Thus, like SA accumulation, early expression of *PR-1* depends on the presence of a light period immediately after pathogen inoculation. Later, at 24 hpi, *PR-1* was strongly expressed under each of the experimental conditions. The hypersensitive cell death response induced by *Psm avrRpm1* in Col-0 leaves results in necrotic, semitranslucent lesions (Delledonne et al., 1998). When scoring macroscopic HR development 5 d after bacterial treatment, we found that tissue necrosis developed most prominently after morning inoculations and that macroscopic lesion intensity gradually decreased in the order of morning, midday, evening, and night inoculation, respectively (Fig. 3A). Finally, we assessed whether the stronger defense capacity following morning compared with evening inoculations would express itself in a higher plant resistance toward *Psm avrRpm1* by scoring bacterial growth in leaves at 3 d postinoculation (dpi) for each

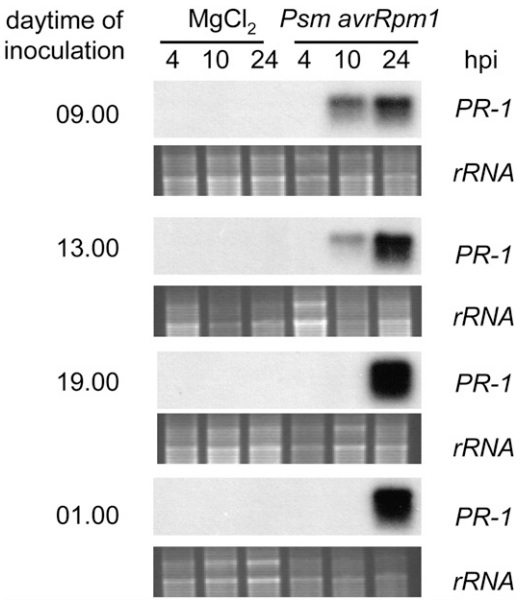


**Figure 2.** Expression of defense genes is dependent on inoculation daytime. *PR-1* expression in leaves inoculated with *Psm avrRpm1* (OD = 0.005) at different daytimes were assessed by northern-blot analysis. Plants were kept in the light/dark regime depicted in Figure 1A. Control samples were treated with 10 mM $MgCl_2$. Samples were taken at 4, 10, and 24 hpi.

case. Plants inoculated at 9 AM indeed were able to restrict bacterial growth more efficiently than plants inoculated at 7 PM, with a statistically significant, 3-fold lower multiplication of bacteria at 3 dpi (Fig. 3B). Together, these data demonstrate that, like SA accumulation and *PR-1* expression, HR lesion development and disease resistance in Arabidopsis leaves are markedly influenced by the daytime of *P. syringae* inoculation and are positively correlated with the length of the light period during the early plant-pathogen interaction.

To exclude that the observed differences in defense responses and resistance result from bacterial rather than plant performance, we used batches of bacteria originating from the same overnight culture for each daytime inoculation. We attempted to minimize relative aging effects of bacterial batches by growing the overnight culture already 5 d before the pathogen experiments were initiated, and we stored purified batches at 4°C before use. Moreover, permutation of the experimental starting point (e.g. comparing the inoculation series 9 AM, 1 PM, 7 PM, and 1 AM with the series 7 PM, 1 AM, 9 AM, and 1 PM) had no influence on the relative tendencies of defense responses (Figs. 1–3), indicating that light-mediated differences in plant performance were causative for the observed defense outcomes.

### Photoreceptor Signaling Only Moderately Affects Induction of Arabidopsis Defenses at Sites of *Psm* (± *avrRpm1*) Inoculation

Light could influence defense responses through photosynthetic means or by cross talk of photoreceptor-mediated light signaling with plant defense signaling. Light signaling is mediated by the blue/UV-A-absorbing cryptochromes and phototropins, and the red and far-red light-absorbing phytochromes (Gyula et al., 2003). To test whether light perception by these photoreceptors is required for *P. syringae*-induced defense responses and disease resistance, we examined the interactions of the following Arabidopsis double mutants impaired in either cryptochrome, phototropin, or phytochrome photoperception, with an avirulent (*Psm avrRpm1*) or a virulent strain (*Psm*) of *P. syringae* pv. *maculicola*: *cry1cry2* (*cry1-304 cry2-1*; Mockler et al., 1999), *phot1phot2* (*phot1-5 phot2-1*; Sakai et al., 2001), and *phyAphyB* (*phyA-211 phyB-9*; Cerdán and Chory, 2003). Common genetic background for all examined mutants is accession Col (Col-0 for *cry1cry2* and *phyAphyB*; Col-3 for *phot1phot2*), implicating that each line harbors the resistance gene *Rpm1* whose product recognizes the bacterial avirulence protein AvrRpm1. This recognition event is causative for the *Psm avrRpm1*-induced HR and early SA accumulation in wild-type Col (Bisgrove et al., 1994; Mishina et al., 2008).

At sites of *Psm avrRpm1* inoculation, loss of UV/blue-light perception by cryptochrome or phototropin in *cry1cry2* and *phot1phot2*, respectively, did not impede plants to mount light-dependent defense responses (Figs. 4 and 5). Whereas leaves of the *phot1phot2* double

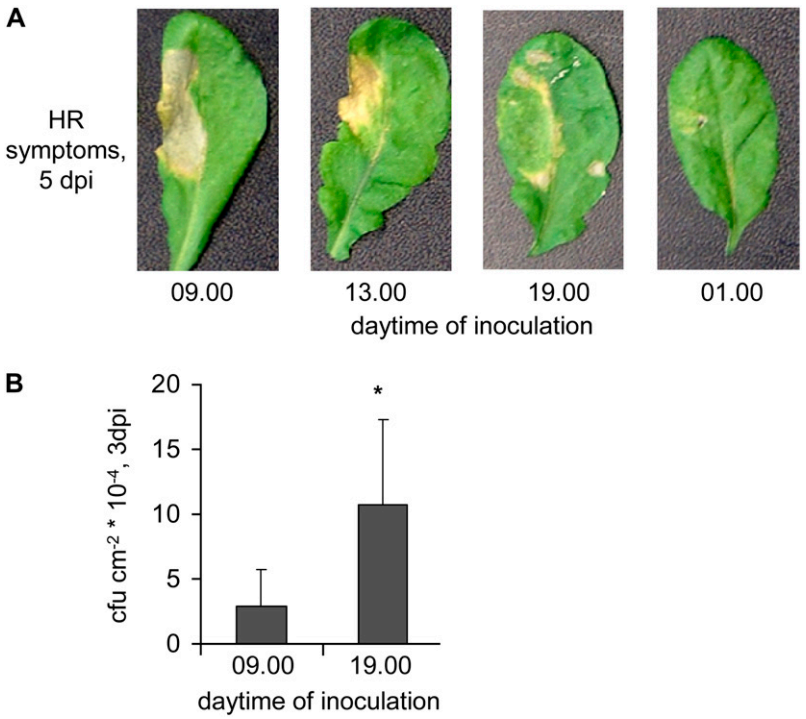


**Figure 3.** HR symptoms and disease resistance are dependent on inoculation daytime. A, Macroscopic HR symptoms of leaves 5 dpi treatment with *Psm avrRpm1* (OD = 0.005) at different daytimes, as described in Figure 1A. B, Bacterial growth quantification in Col-0 leaves 3 d after *Psm avrRpm* inoculation (OD = 0.002) at either 9 AM or 7 PM. Bars represent mean values (± SD) of colony forming units (cfu) per square centimeter from at least five parallel samples, each sample consisting of three leaf discs. Asterisk denotes statistically significant differences between 9 AM and 7 PM inoculations (*, $P < 0.006$; Student's *t* test). To ensure the uniformity of infiltrations, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for both inoculation times (data not shown).

mutant and the corresponding Col-3 wild type showed similar levels of total SA at 10 hpi, leaves of *cry1cry2* actually accumulated SA to significantly ($P = 0.04$) higher levels than Col-0 wild-type leaves (Fig. 4A). Trypan-blue staining at 24 hpi of leaves inoculated with the avirulent pathogen revealed that both UV/blue-light receptor mutants were able to execute a wild-type-like hypersensitive cell death response (Fig. 4B). Moreover, pathogen-induced expression of the light-dependent defense genes *PR-1* and *PAL1* occurred independently of either a functional cryptochrome or phototropin pathway (Fig. 5). Assessment of $H_2O_2$ production at inoculation sites through staining of leaves with 3,3′-diaminobenzidine (data not shown), and expression patterns of the ROS-inducible *GST1* gene further indicated that the oxidative burst is not affected in *cry1cry2* or *phot1phot2* (Fig. 5). Likewise, *Psm avrRpm1*-induced accumulation of JA and camalexin occurred to similar levels in *cry1cry2*, *phot1phot1*, and the respective wild-type leaves (data not shown).

Although phytochrome photoperception has been previously implicated with SA signaling (Genoud et al., 2002), *phyAphyB* plants appreciably induced SA biosynthesis and expression of the SA-responsive *PR-1* gene in *Psm avrRpm1*-inoculated leaves. Compared with the Col-0 wild type, however, accumulation of both free and glucosidic SA were modestly reduced in *phyAphyB* (Fig. 4A), and *PR-1* expression was marginally delayed (Fig. 5C). After trypan-blue staining of *Psm avrRpm1*-infiltrated leaves, we observed distinct blue-stained patches of dead cells in both *phyApyhB* and in Col-0 (Fig. 4B), indicating that *phyAphyB* plants are able to mount a wild-type-like HR. 3,3′-Diaminobenzidine staining, metabolite determination, and gene expression analyses further revealed that *phyAphyB* leaves induce an oxidative burst, JA biosynthesis, camalexin accumulation, and expression patterns of *GST1* and *PAL1* that are similar to the respective responses in Col-0 leaves (Fig. 5C; data not shown).

When comparing resistance toward the avirulent *Psm avrRpm1* strain in terms of bacterial multiplication at 3 dpi, we did not detect statistically significant differences between wild-type and photoreceptor mutant plants (Fig. 6A). In compatible interactions with the disease-causing, virulent *Psm* strain, bacterial growth differences between Col-0 and *phyAphyB* were more pronounced, and a significant, 3-fold higher multiplication of *Psm* in leaves of *phyAphyB* was detected compared with Col-0 leaves. In contrast to this moderate attenuation of basal resistance in the phytochrome mutants, no *Psm*-growth differences in the UV/blue-light receptor mutants and wild-type plants existed (Fig. 6B). Taken together, these data suggest a marginal cross talk between phytochrome-mediated light signaling and defense signaling at sites of pathogen attack, and indicate an even lesser influence of the cryptochrome and phototropin pathways on local defense and resistance.

### SAR Requires Functional Phytochrome Photoperception But Is Independent of Cryptochrome and Phototropin Signaling

Because our previous studies indicate an absolute requirement of light for biological induction of SAR

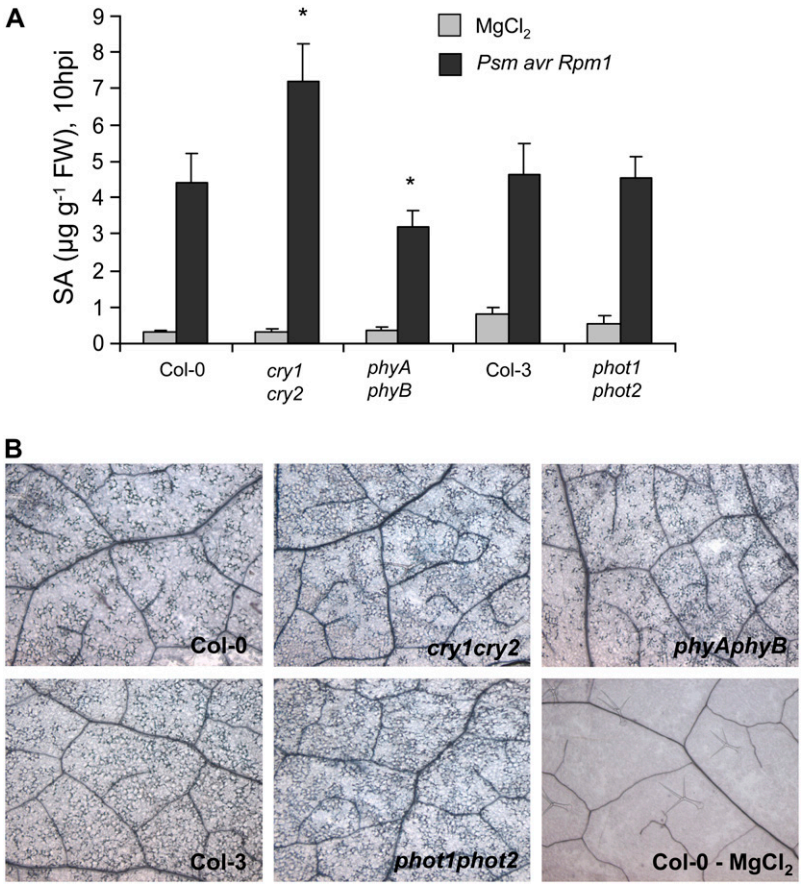


**Figure 4.** SA accumulation and HR development in leaves of Col-0, Col-3, *cry1cry2*, *phot1phot2*, and *phyAphyB* plants treated with *Psm avrRpm1* (OD = 0.005). Inoculations were performed at 10 AM within the light/dark regime depicted in Figure 1A. A, Total SA levels in leaves 10 h after *Psm avrRpm1* or $MgCl_2$ treatment. Bars represent mean values (± SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisk denotes value with statistically significant difference to the values of the respective wild type (*, $P < 0.05$; Student's *t* test). Light bars, $MgCl_2$-treatment; dark bars, *Psm avrRpm1* inoculation. B, Microscopic HR lesions of representative leaf samples at 24 hpi with *Psm avrRpm1*, as assessed by trypan-blue staining. For all lines under investigation, inoculated leaf areas harbor patches of blue-stained, dead cells clearly delimited from surrounding healthy (unstained) tissue (magnification 100-fold). For comparison, the staining outcome of an $MgCl_2$-treated Col-0 leaf is depicted (bottom right). Similar staining results were obtained for $MgCl_2$-treated leaves of the remainder lines (not shown).

(Zeier et al., 2004), we tested whether this light dependency would be mediated by photoreceptors. To examine a potential pathogen-induced enhancement of systemic resistance, three lower rosette leaves (here designated as ''primary leaves'') of a given plant were either infiltrated with 10 mM $MgCl_2$ in a control treatment, or inoculated with a suspension of *Psm* (optical density [OD] 0.01) for SAR induction (Mishina and Zeier, 2007). Two days later, three upper, previously nontreated leaves (systemic leaves) were either collected and analyzed for SA content and *PR* gene expression, or they were subject to a subsequent challenge infection with lower inoculi of *Psm* (OD 0.002). SAR was directly assessed by scoring bacterial growth in systemic leaves 3 d after the challenge infection.

Compared to $MgCl_2$-inoculated controls, *Psm*-pretreated Col-0, *cry1cry2*, Col-3, and *phot1phot2* plants significantly enhanced their resistance toward challenge infections by factors ranging from 6 to 14 (Fig. 7A). SA contents of systemic leaves were considerably elevated in these lines after *Psm* infection of primary leaves (Fig. 7B). Moreover, expression levels of the SAR genes *PR-1*, a typical SA-inducible defense gene, and of *PR-2*, whose up-regulation is SA independent (Nawrath and Métraux, 1999), were both elevated in systemic leaves after *Psm* treatment (Fig. 8, A and C). Thus, SAR developed in both Col lines as well as in the *cry1cry2* and *phot1phot2* receptor mutants. By contrast, the *phyAphyB* mutant completely failed to enhance whole plant resistance in response to a primary *Psm* infection (Fig. 7A), and systemic levels of SA did not significantly increase upon the normally SAR-inducing bacterial treatment (Fig. 7B). In addition, the SA-marker gene *PR-1* was not up-regulated in systemic leaves of *Psm*-preinfected *phyAphyB* mutants (Fig. 8B). These data demonstrate that a functional phytochrome pathway is required for biological induction of SAR and systemic elevation of SA-associated defenses. Interestingly, *phyAphyB* mutant plants are not fully compromised in mounting systemic defense reactions, because they still proved capable to increase systemic expression of the SA-independent *PR-2* gene upon *Psm* inoculation (Fig. 8B).

We have previously shown that the FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) is an essential component for *P. syringae*-induced SAR in Arabidopsis (Mishina and Zeier, 2006). *FMO1* is up-regulated in both inoculated and systemic leaves, and *fmo1* mutant plants, although capable of mounting defense reactions at inoculation sites, completely lack induction of SAR and systemic defense responses. Notably, all SAR-defective defense mutants investigated so far fail to up-regulate *FMO1* in distant (but not necessarily in inoculated) leaves, indicating that

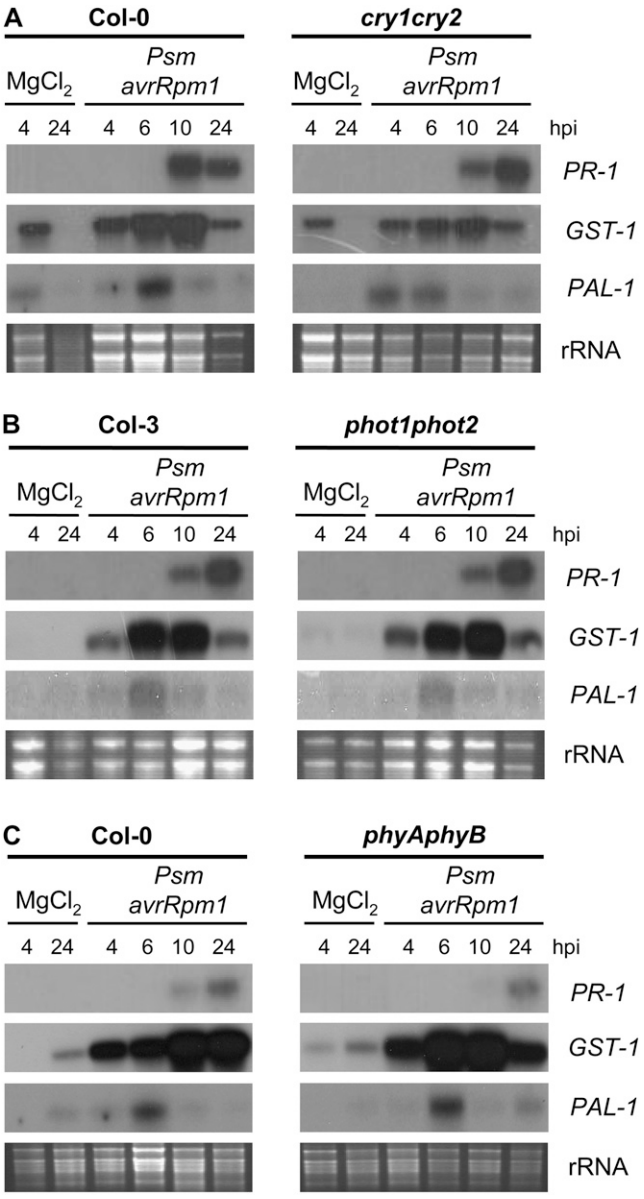


**Figure 5.** Expression of defense-related genes in leaves of wild-type Col and mutants impaired in light perception at sites of *Psm avrRpm1* (OD = 0.005) inoculation, as assessed by northern-blot analysis. Control samples were treated with 10 mM $MgCl_2$. Numbers indicate hpi. A, Comparison of Col-0 and *cry1cry2*. B, Comparison of Col-3 and *phot1phot2*. C, Comparison of Col-0 and *phyAphyB*.

systemic expression of *FMO1* is a prerequisite for the SAR-induced state. We examined expression of *FMO1* in noninoculated leaves of *Psm*-treated wild-type and photoreceptor mutant plants. Whereas the Col wild-type lines and the SAR-competent *cry1cry2* and *phot1-phot2* plants increased expression of *FMO1* in systemic leaves 2 d after *Psm* treatment, the SAR-defective *phyAphyB* mutants did not (Fig. 8B). These findings support our previous hypothesis that FMO1 is required in systemic leaves for SAR to be realized, and indicates that phytochrome-mediated light signaling is required upstream of FMO1 during SAR establishment.

## DISCUSSION

### Daytime Dependency of Resistance Responses

In this article we show that, within a fixed light/dark cycle, resistance responses of Arabidopsis plants toward the incompatible *P. syringae* strain *Psm avrRpm1* depend on the time of the day when pathogen contact takes place. Within the light/dark cycle, the length of the light period during the early plant-pathogen interaction correlates with the magnitude of SA production, *PR-1* accumulation, and macroscopic HR lesion development (Figs. 1–3). Moreover, a stronger activation of defenses observed after morning in comparison with evening inoculations entails a higher degree of resistance against *Psm avrRpm1* (Fig. 3B).

The plant circadian clock runs with a period close to 24 h and controls several aspects of plant biochemistry and physiology. One of the consequences of circadian control is that stimuli of equal strength applied at

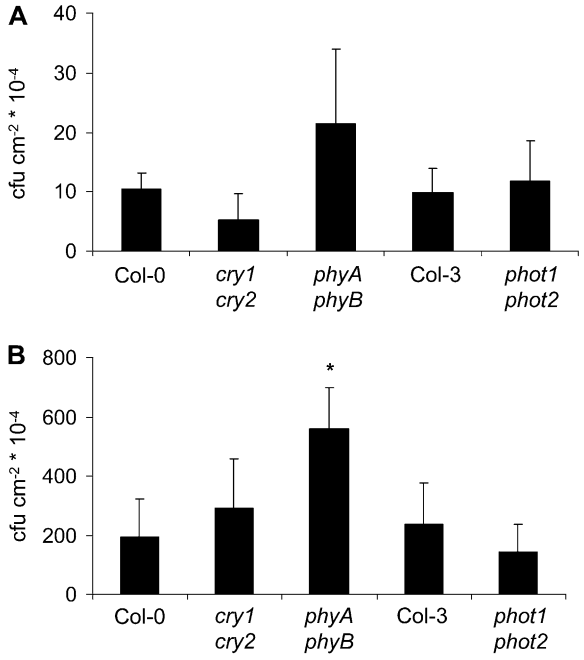


**Figure 6.** Specific and basal disease resistance of wild-type and photoreceptor mutant plants. A and B, Bacterial growth quantification of *Psm avrRpm1*-inoculated (A; OD = 0.002) and *Psm*-inoculated (B; OD = 0.002) leaves of wild-type and photoreceptor mutants 3 dpi. Bars represent mean values (± SD) of colony forming units (cfu) per square centimeter from at least five parallel samples, each sample consisting of three leaf discs. Asterisk denotes value with statistically significant differences to the value of the respective wild type (*, $P < 0.05$; Student's *t* test). To ensure the uniformity of infiltrations, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for leaves of different lines (data not shown).

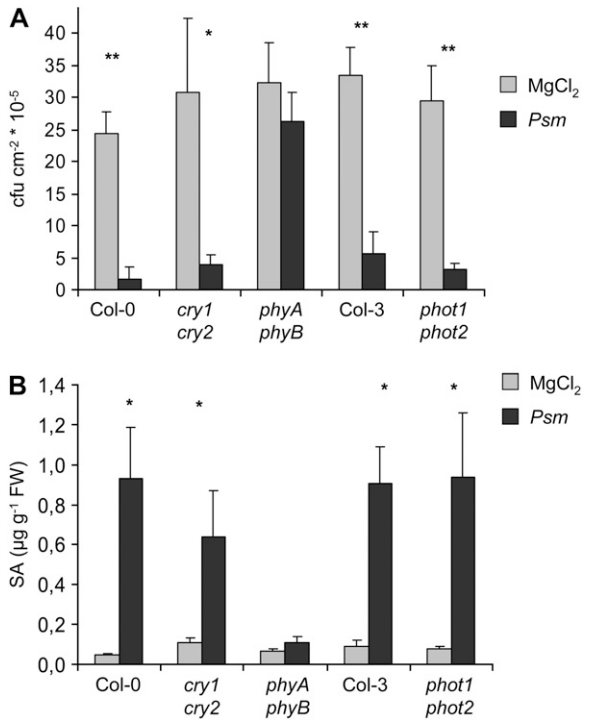


**Figure 7.** SAR is compromised in *phyAphyB*. A, Bacterial growth quantification to directly assess SAR. Wild-type and photoreceptor mutant plants were pretreated with 10 mM $MgCl_2$ or *Psm* (OD = 0.01) in three primary leaves (primary treatment); 2 d later three systemic leaves located directly above the primary leaves were inoculated with *Psm* (OD = 0.002). Bacterial growth in systemic leaves was assessed 3 dpi after the secondary inoculation. Bars represent mean values (± SD) of colony forming units (cfu) per square centimeter from at least five parallel samples consisting each of three leaf discs. Asterisks denote statistically significant differences in systemic growth between *Psm*-and $MgCl_2$-pretreated plants of a particular line (*, $P < 0.05$, **, $P < 0.001$; Student's *t* test). B, Systemic accumulation of free SA. Primary treatments were performed as described in A. Untreated upper leaves were harvested 2 d later for SA analysis. Bars represent mean values (± SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisks denote pathogen treatment with statistically significant differences to the respective $MgCl_2$ control (*, $P < 0.05$; Student's *t* test). Light bars, $MgCl_2$ pretreatment; dark bars, *Psm* preinoculation.

different times of the day can lead to different intensities of a particular plant response, a phenomenon designated as gating (Hotta et al., 2007). It would thus be conceivable that the observed daytime-dependent differences in *P. syringae*-induced plant defenses result from the circadian rhythm. On the basis that some genes implicated in plant defense follow a circadian expression pattern, a link between defense and circadian signaling has been established previously (Sauerbrunn and Schlaich, 2004). Examples for such genes are Arabidopsis *PCC1* (pathogen and circadian controlled 1) and *PAL1* (Sauerbrunn and Schlaich, 2004; Rogers et al., 2005). The plant circadian clock maintains a relatively constant period, even in the absence of environmental cues such as light (Hotta et al., 2007). To discriminate between circadian control and light effects, we have therefore conducted the daytime experiment both in continuous darkness and in continuous light (Fig. 1, C and D). In contrast to the light/dark cycle situation, the 7 PM inoculation did not result in diminished SA production when compared with the 9 AM inoculation under continuous light or darkness. This indicates that the circadian rhythm does not account for the daytime-dependent differences in plant defense activation under light/dark cycle conditions.

The correlation between the magnitude of defense activation and the number of available light hours after *P. syringae* inoculation rather suggests that the daytime dependency of defense responses in Arabidopsis is based on the direct influence of light on inducible plant defenses (Zeier et al., 2004; Roberts and Paul, 2006). A light period of a certain length after pathogen contact also has been reported as a prerequisite for optimal defense in other pathosystems. In the interaction between an incompatible *Xanthomonas oryzae* strain and rice, for instance, a minimum of 8 h of light after bacterial inoculation was required for proper development of HR cell death, lignin deposition at inoculation sites, and effective restriction of bacterial multiplication (Guo et al., 1993). Similarly, in the incompatible interaction of Arabidopsis accession Di-17 and *Turnip crinkle virus*, an HR and strong *PR-1* gene expression failed to occur when the initial light period after infection was less than 6 h (Chandra-Shekara et al., 2006). Together, these data suggest that light availability is important particularly during the early phases of plant defense activation. The absence of light during the early plant-pathogen interaction upon evening or night inoculations negatively affects development of the HR at later stages of the interaction, because the HR is determined during the first few hours after pathogen attack following specific recognition of avirulence factors (Fig. 3A). Responses like SA accumulation or *PR-1* gene expression, by contrast, are more continuously activated after recognition of both specific and general elicitors, and their magnitude at later infection stages is independent of the inoculation daytime (Fig. 2). However, the absence of light during the early interaction period entails a delayed and thus less efficient SA-associated defense mobilization (Figs. 1B and 2).

Inoculation daytime and light conditions do influence plant defenses and the outcome of a particular plant-pathogen interaction under laboratory conditions. To obtain reproducible results, researches should therefore aim to start comparative experiments at a fixed daytime rather than in a randomized fashion. A more effective activation of inducible plant defenses under light influence could be relevant also in naturally occurring plant-pathogen interactions. An attenuated plant defense capacity at night might influence the infection strategy of pathogens, i.e. favor an attack during the dark hours. There is evidence that germination of spores from certain pathogenic fungi is

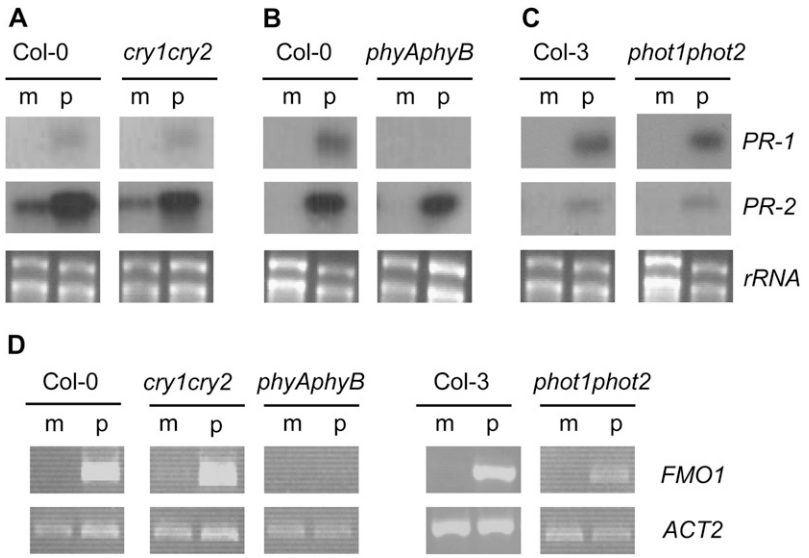


**Figure 8.** Systemic expression of defense-related genes in wild-type and photoreceptor mutant plants. Primary leaves were treated as described in the legend of Figure 7. Untreated distant leaves were harvested for analysis. A to C, Systemic expression of *PR-1* and *PR-2* as assessed by northern-blot analysis. D, Expression of the *FMO1* gene, as assessed by reverse-transcription PCR. m, $MgCl_2$ pretreatment; p, *Psm* pretreatment.

inhibited by light, and plants are probably subject to an overall greater pathogen challenge at night than during the day (Roberts and Paul, 2006). For pathogenic bacteria, however, besides a light-dependent effectiveness of plant defenses, a number of other factors can contribute to determine the timing of pathogen attack and the final outcome of a particular plant-pathogen interaction in natural habitats (Colhoun, 1973). These include the necessity for bacteria to enter through open stomata, temperature influences on bacterial virulence, and humidity effects (van Dijk et al., 1999; Underwood et al., 2007).

### Cross Talk of Photoreceptor Signaling and Plant Defense

A light-dependent nature of distinct plant defense responses has been established by several laboratories (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Bechtold et al., 2005; Chandra-Shekara et al., 2006). In *Psm avrRpm1*-inoculated Col-0 leaves, we have observed that SA accumulation, expression of *PAL1* and *PR-1*, as well as HR cell death are compromised in continuous darkness, whereas camalexin production, JA accumulation, and expression of *GST1* are not negatively affected. Moreover, local resistance against the avirulent *Psm avrRpm1* strain is diminished and SAR fully abrogated in darkened plants (Zeier et al., 2004). Two general mechanisms are conceivable by which light can regulate plant defense responses: (1) through photosynthesis and its consequences for energy status, reduction equivalents, and biochemical activity related with defense metabolism, or (2) through cross talk of photoreceptor signaling with components of plant defense activation.

In this work, we have addressed the latter issue by examining a possible requirement of light signaling pathways initiated by one of the three characterized photoreceptor systems, cryptochrome, phytochrome, and phototropin (Gyula et al., 2003), for the establishment of local and systemic resistance responses. Each photoreceptor double mutant used for these studies lacks physiological responses that are characteristically mediated by the respective light perception system. Seedlings of the *cry1cry2* mutant, for instance, are defective in the blue-light-induced but not the red-light-induced hypocotyl inhibition response (Mockler et al., 1999). Unlike *cry1cry2*, the *phot1phot2* mutant is blocked in the phototropin-dependent chloroplast, stomatal, and phototropic movements and lacks blue-light induction of calcium currents in mesophyll cells (Kinoshita et al., 2001; Sakai et al., 2001; Stoelzle et al., 2003). The *phyAphyB* double mutant is impaired in hypocotoyl length inhibition under both red and far-red light and shows an early-flowering phenotype (Cerdán and Chory, 2003). The phytochromes C, D, and E, which are still functional in *phyAphyB*, generally fulfill their physiological functions in combination with either PHYA or PHYB (Schepens et al., 2004).

Our data show that signaling events mediated by the blue-light receptors cryptochrome and phototropin are dispensible for local resistance responses of inoculated Arabidopsis leaves, i.e. SA accumulation, defense gene expression, the HR, and basal or specific resistance toward *P. syringae*. Moreover, many defense reactions triggered by *Psm avrRpm1*, including expression of *PAL1* and HR development, occur without functional phytochrome signaling (Figs. 4–7). The phytochrome independency of pathogen-induced *PAL1* expression in leaves was not necessarily expected, because light-dependent activation of the phenylpropanoid pathway in roots occurs in a phytochrome-dependent manner (Hemm et al., 2004). A slight attenuation of SA production and early *PR-1* gene expression is evident in the *phyAphyB* mutant, together with a modest decrease in specific and basal resistance. This indicates that the phytochrome pathway to a limited scale affects the SA resistance pathway at

infection sites, which qualitatively parallels earlier findings in Arabidopsis (Genoud et al., 2002). Quantitatively, however, Genoud et al. (2002) report a larger dependency of local resistance on phytochrome signaling, including a requirement of the system for HR development. These discrepancies might arise from the different experimental systems used in both studies. Genoud et al. (2002) infected accession ecotype Landsberg *erecta* of Arabidopsis (L*er*) and mutants in the L*er* background with the incompatible strain *Pseudomonas syringae* pv. *tomato* DC3000 harboring *avrRpt2*, which activates defense signaling pathways through the Rps2 resistance protein. By contrast, we studied Rpm1-mediated specific resistance as well as basal resistance in accession Col with *P. syringae* pv. *maculicola* ES4326 (± *avrRpm1*) strains. However, our data are both qualitatively and quantitatively comparable to the findings of Chandra-Shekara et al. (2006), who report that the light-dependent HR, *PR-1* expression, and resistance of Arabidopsis accession Di-17 toward *Turnip crinkle virus* are phytochrome independent.

According to these findings, cross talk with photoreceptor signaling is not causative for the strong light dependency of SA production, *PAL1* expression, upregulation of *PR-1*, and HR development in *Psm avrRpm1*-inoculated leaves (Zeier et al., 2004), leaving a possible direct or indirect role of photosynthesis to enable these defenses. SA biosynthesis proceeds through the shikimate pathway, which requires erythrose-4-P and phosphoenolpyruvate as metabolic precursors. Through the pentose phosphate pathway and glycolysis, respectively, availability of both metabolites is connected to the plant's carbohydrate status. Light might thus positively influence SA levels through photosynthesis and increased production of biosynthetic carbon precursors. Metabolizable sugars have been shown to positively influence secondary metabolism and defense gene expression in Arabidopsis, because they promote lignification in dark grown roots and induce *PR* transcript levels in seedlings (Thibaud et al., 2004; Rogers et al., 2005). As SA biosynthesis via isochorismate synthase occurs in plastids (Strawn et al., 2007), photosynthetic activity might be required to supply reducing equivalents and energy for SA accumulation. At least for HR execution, intact chloroplasts and associated ROS production seem to play an important role (Genoud et al., 2002; Liu et al., 2007). The impact of carbohydrate status and chloroplast function on pathogen-induced defense activation, however, requires further attention.

Although phytochrome signaling only moderately influences defense responses at inoculation sites, these data clearly demonstrate that activation of whole plant resistance during SAR depends on phytochrome photoperception. This finding provides a mechanistic explanation for the previously observed light dependency of SAR (Zeier et al., 2004). Phytochrome signaling seems to specifically control SA-associated systemic defenses such as SA accumulation and *PR-1*-expression, but not SA-independent systemic defenses such as *PR-2* expression. This is interesting because it suggests that at least two independent systemic signaling pathways are activated after a local pathogen inoculation; thereof, only the SA pathway provides protection against a *P. syringae* challenge infection. Considering the broad-spectrum character of SAR (Dean and Kuć, 1985), this does not necessarily exclude a contribution of SA-independent pathways to an enhanced resistance response against other microbial pathogens. Our data also show that intact phytochrome signaling is required for pathogen-induced expression of *FMO1* in noninoculated leaves. FMO1 is required for SAR in Arabidopsis, its overexpression confers increased plant resistance, and mutant plants unable to express the gene in distant tissue after a local infection, including *phyAphyB*, are all SAR deficient (Bartsch et al., 2006; Koch et al., 2006; Mishina and Zeier, 2006). During the SAR process, long-distance signal(s) generated in inoculated leaves are thought to travel through the plant and trigger resistance in distant tissue (Grant and Lamb, 2006; Park et al., 2007). In comparison to a local infection event, however, these long-distance signals are relatively low-defense stimuli, and for a sufficiently strong resistance response to occur in systemic leaves, they must be amplified. We have recently proposed an amplification mechanism to occur in systemic leaves in which FMO1 and other SAR regulators are involved to boost incoming SAR signals (Mishina and Zeier, 2006). In an extended model that is consistent with our previous and current findings, phytochrome photoperception regulates signal amplification of such weak defense stimuli and is therefore especially required for low-stimuli responses such as SAR, whereas it gets almost dispensable when stronger stimuli at infection sites trigger more massive local defense responses.

Although the extent of induced defense reactions in a single inoculated leaf is generally higher than in a single systemic leaf (Mishina et al., 2008), the sum of systemic defenses might well exceed defense reactions at infection sites. In fact, the SAR-induced state can entail considerable costs due to the allocation of resources from primary metabolism (van Hulten et al., 2006; Walters and Heil, 2007), and these costs might be procured by light-driven photosynthetic metabolism. The phytochrome system might monitor light availability and accommodate photosynthetic resources to the relatively costly increase in whole plant resistance.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used for the daytime experiments. To investigate the role of photoreceptors in plant defense, the following double mutants were used: *cry1cry2* (*cry1-304 cry2-1*; Mockler et al., 1999), *phot1phot2* (*phot1-5 phot2-1*; Sakai et al., 2001), and *phyAphyB* (*phyA-211 phyB-9*; Cerdán and Chory, 2003). Col-0 is the genetic background for both *cry1cry2* and *phyAphyB*, and Col-3 (*gl1*) is the background for *phot1phot2*.

The *phyAphyB* plants were put on Murashige and Skoog medium containing 3% Suc for germination, and seedlings were transferred to soil mixture

(see below) after 10 d. All other lines were already sown and grown on an autoclaved mixture of soil (Beetpflanzensubstrat Type RHP 16; Klasmann-Deilmann), vermiculite, and sand (10:0.5:0.5). Plants were kept in a controlled environmental chamber (J–66LQ4; Percival) with a 9-h day period from 9 AM to 6 PM (photon flux density 70 $\mu$mol $m^{-2}$ $s^{-1}$; temperature 21°C) and a 15-h night period (temperature 18°C). For experiments, 6-week-old, naive, and unstressed plants showing a uniform appearance were used. If not otherwise indicated, pathogen treatments were performed at 10 AM.

## Growth of Plant Pathogens and Inoculation

*Pseudomonas syringae* pv. *maculicola* ES4326 lacking (*Psm*) or harboring (*Psm avrRpm1*) the avrRpm1 avirulence gene were grown at 28°C in King's B medium containing the appropriate antibiotics (Zeier et al., 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to a final OD of 0.01 (SAR induction), 0.005 (determination of local gene expression and metabolite levels), or 0.002 (bacterial growth assays). The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1-mL syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed 3 d after infiltration (OD 0.002) by homogenizing discs originated from infiltrated areas of three different leaves in 1 mL of 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 d. All pathogen experiments depicted in the figures were repeated at least twice with similar results.

## Daytime Experiments

Arabidopsis plants were infiltrated with bacteria at different daytimes (9 AM, 1 PM, 7 PM, and 1 AM), and resistance responses were scored at constant times after inoculation. Batches of bacteria resulting from the same overnight culture were used for each inoculation series. To minimize relative aging effects of bacteria, overnight cultures were prepared 5 d before the inoculation experiment was started. Purified bacterial batches were stored at 4°C until use. Inoculation series were repeated with permutated starting times.

## Characterization of SAR

Three lower leaves of a given plant were first infiltrated with a suspension of *Psm* (OD 0.01), or with 10 mM $MgCl_2$ as a control. Two days after this primary inoculation, nontreated upper leaves were harvested for SA determination and gene expression analysis, or plants were inoculated on three upper leaves with *Psm* (OD 0.002). Growth of *Psm* in upper leaves was assessed 3 d later.

## Analysis of Gene Expression

Analysis of gene expression was performed as described by Mishina and Zeier (2006). Expression levels of *PR-1* (At2g14610), *PR-2* (At3g57260), *PAL1* (At2g37040), and *GST1* (At1g02930) were determined by northern blot analysis, and *FMO1* (At1g19250) expression was analyzed by reverse-transcription PCR. The following primers were used for PCR: 5′-CTTCTACTCTCCTCAGTGGCAAA-3′ (FMO1-forward), 5′-CTAATGTCGTCCCATCTTCAAAC-3′ (FMO1-reverse). Hereby, the actin2 gene (At3g18780) was amplified as a control with the primers 5′-TCGCCATCCAAGCTGTTCTCT-3′ (ACT2-forward), 5′-CCTGGACCTGCCTCATACTC-3′ (ACT2-reverse).

## Determination of Defense Metabolites

Determination of free SA, glycosidic SA, JA, and camalexin levels in leaves was realized by a modified vapor-phase extraction method and subsequent gas chromatographic/mass spectrometric analysis according to Mishina and Zeier (2006). Total SA contents were calculated by summing up free and glycosidic SA levels.

## Quantification of Microscopic HR Lesions and Assessment of $H_2O_2$ Production

The extent of microscopic HR lesion formation and $H_2O_2$ production were assessed by the trypan-blue and diaminobenzidine staining procedures, respectively, which are described in Zeier et al. (2004).

## ACKNOWLEDGMENTS

We thank Roman Ulm, Petra Dietrich, and Rosalia Deeken for the donation of photoreceptor mutant seeds, and Felix Mauch for proofreading of the manuscript.

Received March 25, 2008; accepted April 16, 2008; published April 23, 2008.

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