Cryptochrome 2 and phototropin 2 regulate resistance protein-mediated viral defense by negatively regulating an E3 ubiquitin ligase

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Edited by Jonathan Dallas Jones, Sainsbury Laboratory, Norwich, United Kingdom, and accepted by the Editorial Board June 11, 2010 (received for review April 6, 2010)

Light harvested by plants is essential for the survival of most life forms. This light perception ability requires the activities of proteins termed photoreceptors. We report a function for photoreceptors in mediating resistance (R) protein-derived plant defense. The blue-light photoreceptors, cryptochrome (CRY) 2 and phototropin (PHOT) 2, are required for the stability of the R protein HRT, and thereby resistance to Turnip Crinkle virus (TCV). Exposure to darkness or blue-light induces degradation of CRY2, and in turn HRT, resulting in susceptibility. Overexpression of HRT can compensate for the absence of PHOT2 but not CRY2. HRT does not directly associate with either CRY2 or PHOT2 but does bind the CRY2-/PHOT2-interacting E3 ubiquitin ligase, COP1. Application of the proteasome inhibitor, MG132, prevents blue-light-dependent degradation of HRT, consequently these plants show resistance to TCV under blue-light. We propose that CRY2/PHOT2 negatively regulate the proteasome-mediated degradation of HRT, likely via COP1, and blue-light relieves this repression resulting in HRT degradation.

light | photoreceptors | plant defense | virus | resistance protein

Plants are dependent on light for their survival. The light-absorbing ability of plants is derived from the activities of three known classes of photoreceptors. These include phytochromes (PHY) that detect light in the red/far-red (600–700 nm) range, and cryptochromes (CRY) and phototropins (PHOT) that detect light in the blue and UVA (320–500 nm) range (reviewed in refs. 1–6). Photon-absorption activates the PHY proteins from their physiologically inactive to active far-red absorbing forms. Light also modulates the phosphorylation and nucleocytoplasmic translocation of PHY proteins, which is essential for their function in mediating light-responsive physiological changes in plants. CRY photoreceptors are flavoproteins that share sequence similarity to DNA-repair enzymes called photolyases. However, CRY proteins have no DNA-repair activity (5, 7). CRY proteins were first characterized in Arabidopsis, but are also widely distributed in bacteria and eukaryotes. These proteins usually contain an amino terminal photolyase-related region and a carboxy domain of variable size. Both isoforms of CRY (CRY1 and CRY2) in Arabidopsis undergo blue-light-dependent phosphorylation (8, 9), and CRY2, but not CRY1, is degraded in response to blue light (10, 11). Both CRY1 and CRY2 interact with constitutively photomorphogenic 1 (COP1), an E3 ubiquitin ligase (12, 13). It is thought that blue-light perception by CRY photoreceptors triggers the rapid inactivation of COP1 through their direct protein–protein interactions (12, 13), resulting in the abrogation of COP1-mediated degradation of the bZIP transcription factor HY5 and other COP1 substrates (14). Although CRY1 protein shuttles between the cytoplasm and nucleus, the CRY2 protein is mostly present in the nucleus (15). Because CRY2 also contributes to anion channel-mediated currents across the plasma membrane (16), it is possible that some CRY2 might also be present in the cytosol.

The PHOT proteins are plasma membrane localized protein kinases that comprise two light-oxygen-voltage (LOV) domains in the N terminus and a serine/threonine kinase domain at the C terminus (reviewed in refs. 3, 17). LOV1 and LOV2 are essential for the photoreceptor activity of PHOT proteins. In the dark, LOV2 binds the kinase domain and inhibits its phosphorylation activity. Light inhibits the binding between the kinase domain and LOV2, resulting in the activation of kinase activity (18). Although the *in planta* substrates of PHOT-derived phosphorylation are unknown, both PHOT1 and PHOT2 autophosphorylate to likely promote their own dissociation from the plasma membrane (19–22). Upon blue-light irradiation, PHOT1 moves rapidly to the cytoplasm (19), while a fraction of PHOT2 moves to the Golgi apparatus (20). The significance of this relocalization or autophosphorylation remains unclear.

Increasing evidence indicates that light is important for the proper induction of plant defense and for resistance to pathogens (reviewed in refs. 23, 24). However, the molecular and biochemical interaction between light and defense signaling pathways remains unclear. Genetic evidence supporting the role of light in defense was provided by studies on mutants that are defective in the perception of light. Mutations in either *phyA* or *phyB* compromise the ability to induce localized cell death at the site of pathogen entry (25). This phenomenon, termed the hypersensitive response (HR), is one of the earliest visible manifestations of induced defense signaling and resembles programmed cell death in animals (26). In addition to HR development, the *phyA* and *phyB* mutants are also repressed in the salicylic acid (SA)-induced expression of the pathogenesis-related (*PR*) gene. The more severe effect of the *phyA phyB* double mutant on the SA-mediated pathway suggests that light perception has a cumulative effect on SA signaling and plant defense (27). Recent analysis has suggested a major role for *PHYA PHYB* in systemic immunity and a rather minor role in local defense response (27).

Previously, we showed that light is required for resistance to Turnip Crinkle virus (TCV) in Arabidopsis (28). Resistance to TCV is dependent on the R protein HRT, which contains a coiled coil, nucleotide binding, and leucine-rich–repeat domain

Author contributions: R.-D.J., A.C.C.-S., S.R.B., A.K., and P.K. designed research; R.-D.J., A.C.C.-S., S.R.B., D.N., A.K., and P.K. performed research; R.-D.J. and P.K. contributed new reagents/analytic tools; R.-D.J., A.C.C.-S., S.R.B., D.N., D.F.K., A.K., and P.K. analyzed data; and A.K. and P.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. J.D.J. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1004529107/-/DCSupplemental.

(29). However, *HRT* by itself is insufficient and requires the recessive allele of an as yet unidentified locus, *rrt*, to confer resistance (30, 31). Following TCV inoculation, *HRT rrt* plants develop HR, induce defense gene expression, and accumulate SA (30, 31). Plants lacking *HRT* fail to develop HR and allow systemic spread of the virus, resulting in a crinkled leaf/drooping bolt appearance, followed by death of the plant (31). The requirement for *rrt* can be overcome by increasing the levels of *HRT* transcript via exogenous application of SA (28, 30, 32). Strikingly, unlike resistance, TCV-induced HR and *PR-1* gene expression function independent of the SA pathway and *rrt*.

This study was undertaken to decipher the genetic and biochemical basis of dark conferred susceptibility using Arabidopsis-TCV as a model system. Our analysis shows that the blue-light photoreceptors, CRY2 and PHOT2, are specifically required for the stability of HRT, whereas, CRY1 and PHOT1 influence HRT-mediated resistance without affecting the stability of the R protein. We further show that HRT, as well as CRY2 and PHOT2, interact with the E3 ubiquitin ligase COP1, which is known to target proteins for 26S proteasome-mediated degradation. Conversely, inhibition of the 26S proteasome restores HRT levels and resistance to TCV under blue-light. We conclude that CRY2/PHOT2 negatively regulate COP1 activity, thereby maintaining the stability of the HRT protein.

Results and Discussion

HRT Is a Plasma Membrane Localized Protein That Is Degraded in the Dark. We previously showed that a critical period of illumination, immediately after TCV inoculation, is essential for HRT-mediated resistance to the virus (28). To monitor the effects of light versus dark on the R protein HRT, the epitope (FLAG)-tagged HRT protein was expressed via its native promoter in Dijon (Di)-17 (*HRT*/*rrt*, resistant) and Columbia (Col)-0 (*hrt*/*RRT*, susceptible) ecotypes (Fig. S1*A*). Inoculation of TCV on the Col-0 *HRT-FLAG* lines induced HR formation (Fig. S1*B*) and *PR-1* gene expression (Fig. S1*C*), similar to that in Di-17 or Di-17 *HRT-FLAG* plants indicating that the HRT-FLAG fusion protein was functional. Consistent with the requirement of a recessive locus *rrt* for TCV resistance (30–32), Col-0 *HRT-FLAG* lines remained susceptible to TCV (Fig. S1*D*). To test whether the dark-triggered susceptibility in Di-17 plants was associated with a change in HRT levels, we compared HRT-FLAG levels in Di-17 *HRT-FLAG* plants grown under 14-h light and 10-h dark (14 h L:10 h D) photocycles versus those kept continuously in the dark for 24, 48, or 72 h. As expected, dark-treatment caused susceptibility in Di-17 *HRT-FLAG* plants (Fig. S2 *A* and *B*). Western analysis showed that although HRT-FLAG was detectable in the dark-treated plants, these levels were greatly reduced compared with plants grown under a normal day and night cycle (Fig. 1*A*). Unlike HRT protein, dark treatment did not alter *HRT* transcript levels (Fig. 1*B* and Fig. S2*C*), suggesting that light was required for the posttranscriptional stability of the HRT protein. Together, these results suggest that lack of light promoted susceptibility in Di-17 *HRT-FLAG* plants by reducing the levels of HRT protein.

RPM1, a peripheral plasma membrane protein, which confers resistance to *Pseudomonas syringae* expressing *avrRpm1*, is specifically degraded in response to HR (33). However, unlike HRT, dark treatment did not result in degradation of the RPM1-MYC protein (Fig. 1*C*). Normal levels of HRT-FLAG during early and late stages of HR development, suggests that HRT was not degraded in response to HR (Fig. 1*A*). Similarly, no significant changes in HRT-FLAG levels were observed during HR to *P. syringae* expressing *avrRpt2* (Fig. 1*D*), even though HR to *P. syringae* covered the entire leaf. Together, these results suggest that HRT is not degraded during HR formation and that light is specifically required for the stability of HRT.

We next evaluated whether, in addition to altering HRT levels, absence of light also affected the localization of HRT. HRT-

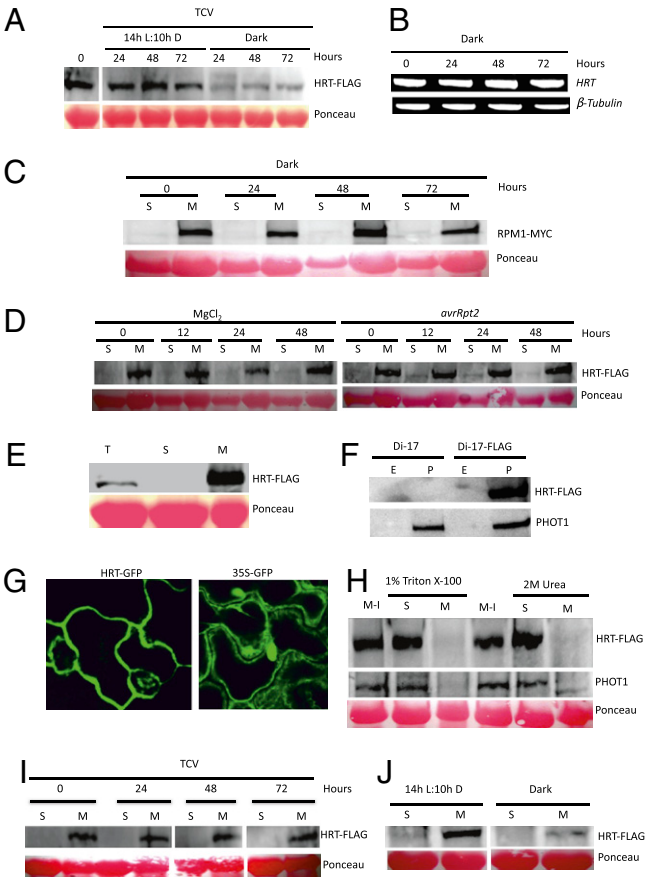

Fig. 1. HRT-FLAG protein is degraded in dark. (*A*) Western blot showing HRT-FLAG levels in plants kept under 14-h L: 10-h D photocycles or in dark after TCV inoculation for indicated hours. Total proteins were extracted and analyzed by immunoblotting. (*B*) RT-PCR analysis showing *HRT* transcript levels after 0–72 h of dark treatment. The level of *β-tubulin* was used as an internal control to normalize the amount of cDNA template. (*C*) RPM1-MYC levels in soluble (S) and microsomal (M) fractions extracted from plants kept in dark for 0–72 h. (*D*) HRT-FLAG levels in S and M fractions extracted from plants inoculated with *P. syringae* expressing *avrRpt2* at indicated hours postinoculation (hpi). (*E*) HRT-FLAG levels in total (T), S, and M fractions. (*F*) Subcellular localization of HRT-FLAG and PHOT1 proteins to the plasma membrane (P) fractions. E indicates endomembranes. (*G*) Confocal image showing the subcellular localization of GFP and HRT-GFP expressed under 35S or *HRT* promoters, respectively. (*H*) HRT-FLAG and PHOT1 levels in M or S fractions before (M-I; input) and after treatment with 2 M urea or 1% Triton X-100. (*I*) HRT-FLAG levels in S and M fractions extracted from TCV-inoculated plants at indicated hpi. (*J*) HRT-FLAG levels in S and M fractions extracted from plants kept under 14-h L: 10-h D photocycles or in dark for 48 h. Ponceau-S staining of the Western blots shown in *A*, *C*, *D*, *E*, *H*, *I*, and *J* were used as the loading control.

FLAG protein was detected in the membrane fraction of leaf extracts from healthy plants grown under 14-h L:10-h D photocycles, with no visible protein in the soluble fraction (Fig. 1*E*). Further subfractionation localized HRT-FLAG to the plasma membrane (Fig. 1*F*). This was confirmed using transgenic lines expressing *HRT-GFP* under the *HRT* promoter, where HRT-GFP was localized exclusively to the periphery of the cell, compared with GFP alone, which was distributed uniformly throughout the cell (Fig. 1*G*). The release of HRT into the soluble fraction by treatment with either Triton X-100 or urea argues that it is a peripheral plasma membrane protein (Fig. 1*H*). These treatments also released PHOT1, a plasma membrane-localized blue-light photoreceptor that relocalizes to the cytosol in the presence of blue light (19). These data confirmed the peripheral plasma membrane localization of both HRT and PHOT1. The possible TCV-

responsive release of HRT from plasma membranes was analyzed at 24, 48, and 72 h after TCV inoculation. HRT was detected only in the microsomal fractions at all time points (Fig. 1*I*), suggesting that HRT-mediated resistance signaling was likely not associated with the relocalization of this R protein into the soluble fraction. Similarly, HRT-FLAG protein was not found in soluble fractions of leaves subjected to dark treatment (Fig. 1*J*).

Mutation in Blue-Light Photoreceptors Compromises HRT-Mediated Resistance. Epistatic mutant analysis was next used to determine whether light-absorbing photoreceptors participated in HRT-mediated resistance. The *phyC*, *phyD*, *phyE*, *cry1*, *cry2*, *phot1*, *phot2* or *phyA phyB* mutations were crossed into the Di-17 background and F_2 plants were analyzed for defense phenotypes (Tables S1 and S2). All *hrt*/*hrt* F_2 progeny showed susceptibility to TCV. Approximately 75% (homo/heterozygous for *HRT*) of F_2 progeny from an *HRT rrt* × *hrt RRT* cross developed HR upon TCV infection. However, only 25% (homo/heterozygous for *HRT,* but homozygous for *rrt*) of these HR-developing progeny were able to resist TCV infection (Table S1). The resistance phenotype in *HRT phyC*, *HRT phyD, HRT phyE*, and *HRT phyA phyB* plants also showed expected Mendelian segregation (Table S1), suggesting that mutations in *PHYC*, *PHYD,* or *PHYE* do not affect HRT-mediated resistance. Furthermore, *phyA* and *phyB* single or *phyA phyB* double mutations did not alter HRT-mediated resistance. In contrast, mutations in *cry1, cry2, phot1,* or *phot2* abrogated *HRT*-mediated resistance; all plants containing *HRT* and mutant *cry1, cry2, phot1,* or *phot2* loci showed typical phenotypes associated with susceptible plants (Fig. 2*A* and Table S1). The appearance of disease symptoms also correlated with the presence of TCV transcript in the systemic uninoculated tissues (Fig. S3*A*). Together, these data suggest that blue-light photoreceptors are required for HRT-mediated resistance.

CRY2 and PHOT2 Are Required for Posttranscriptional Stability of HRT. To determine whether mutations in the *CRY* or *PHOT* genes caused susceptibility by altering HRT levels, we introduced the *HRT-FLAG* transgene into *cry1*, *cry2*, *phot1*, or *phot2* mutant plants. Interestingly, HRT-FLAG levels were significantly reduced in *HRT cry2* and *HRT phot2* plants but not in *HRT cry1* or *HRT phot1* plants (Fig. 2*B*). Reduced levels of the HRT protein in *HRT cry2* or *HRT phot2* plants were not attributable to transcript instability because these plants contained wild-type levels of the *HRT* transcript (Fig. 2*C* and Fig. S3*B*). Together, these data suggested that CRY2 and PHOT2 were required for stability of the HRT protein. Furthermore, normal levels of HRT in *cry1* and *phot1* plants, suggest that CRY1 and PHOT1 were not required for stability of the HRT protein and likely functioned elsewhere in the HRT-mediated resistance signaling pathway.

To determine if *cry1* and *phot1* mutations and/or reduced levels of HRT protein in *HRT cry2* and *HRT phot2* plants impaired HRT-mediated downstream signaling, we analyzed SA levels, *PR-1* expression and HR phenotypes post-TCV inoculation. Interestingly, TCV-induced levels of SA did not correlate with the levels of HRT protein in the various mutant backgrounds. The *HRT cry1* (wild-type levels of HRT protein) and *HRT cry2* (low levels of HRT protein) plants accumulated marginally lower levels of SA, and similar levels of SAG, as Di-17 plants (Fig. S3 *C* and *D*). In contrast, *HRT phot1* (wild-type levels of HRT protein) and *HRT phot2* (low levels of HRT protein) plants accumulated significantly lower levels of both SA and SAG compared with Di-17 (Fig. S3 *C* and *D*). Normal increase of SA in TCV inoculated *HRT cry2* plants suggest that reduced HRT protein in these plants is sufficient to trigger a signaling response related to SA accumulation but not resistance. This was further evident upon evaluation of HR response in *HRT cry2* and *HRT phot2* plants; plants containing mutations in *PHY*, *CRY,* or *PHOT* genes showed normal visible and microscopic HR (Fig. 2 *D* and *E*) and induced

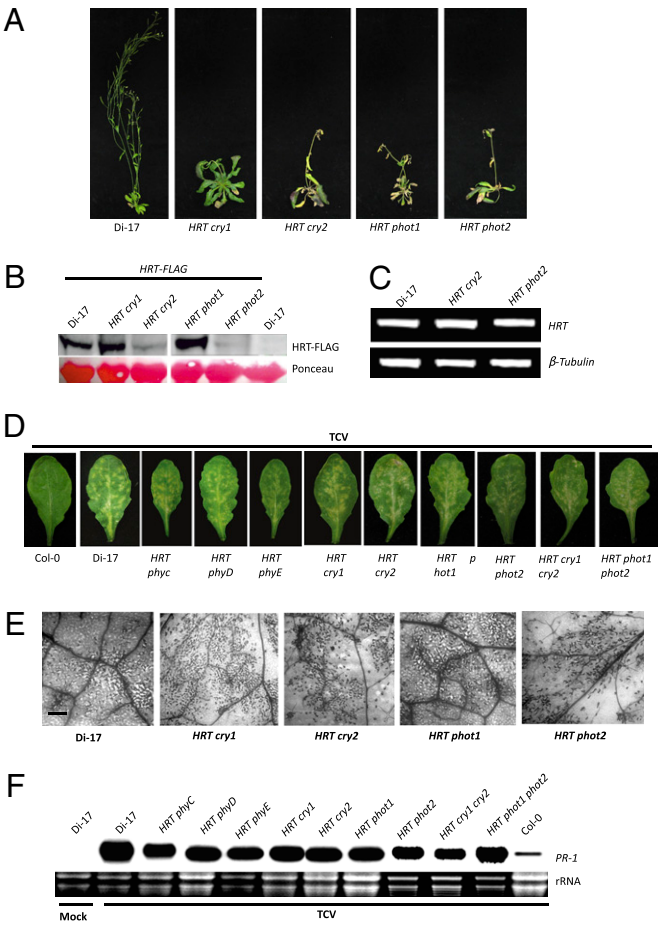

Fig. 2. Mutations in blue-light photoreceptors compromise HRT-mediated resistance. (*A*) Typical morphological phenotypes of TCV-inoculated genotypes. The susceptible plants showed stunted, crinkling phenotypes and accumulated virus in their systemic tissues (Fig. S3*A*). (*B*) Western blot showing HRT-FLAG levels in indicated genotypes. Wild-type Di-17 plant was used as a negative control (last lane). Total proteins were extracted and analyzed by immunoblotting. Ponceau-S staining of the Western blots was used as the loading control. (*C*) RT-PCR analysis showing *HRT* transcript levels in indicated genotypes. The level of *β-tubulin* was used as an internal control to normalize the amount of cDNA template. (*D*) Typical HR in TCV-inoculated leaves at 3 d postinoculation (dpi). The indicated genotypes were homozygous for the mutant loci and contain at least one copy of the *HRT* gene. (*E*) Microscopy of trypan blue-stained leaves from TCV-inoculated plants shown in B. (Scale bars: 270 μm.) (*F*) *PR-1* gene expression in mock- or TCV-inoculated plants. Leaves were sampled at 3 dpi. Ethidium bromide staining of rRNA was used as a loading control.

normal expression of *PR-1* gene (Fig. 2*F*). However, HR phenotype in these mutant backgrounds did not correlate with the fact that dark-treated plants showed compromised HR (28). One possibility is that multiple photoreceptors might be involved in regulating HR against TCV. However, normal HR and *PR-1* expression phenotypes were seen in double and triple mutant plants including, *HRT cry1 cry2*, *HRT phot1 phot2*, *HRT phyA phyB*, and *HRT phyC phyD phyE* plants (Fig. 2*D* and Fig. S3*E*). These data suggest that the PHOT and CRY photoreceptors might act in a redundant manner, and/or factors other than photoreceptors regulate the effect of light on HR to TCV.

Overexpression of *HRT* Increases HRT Levels in *phot2* but Not in *cry2* Background. To determine if overexpression of *HRT-FLAG* was able to compensate for reduced stability of HRT-FLAG in the *cry2* and *phot2* backgrounds, the plants were treated with SA or its active analog benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH),

which induced *HRT* transcription by sixfold (Fig. S4 *A* and *B*). However, BTH application increased HRT protein levels only in *HRT cry1*, *HRT phot1,* or *HRT phot2* plants, but not in *HRT cry2* plants (Fig. 3*A* and Fig. S4*C*). Inability to accumulate HRT protein in *cry2* background was not related to SA responsiveness or transcription of *HRT* because BTH application induced wild-type–like levels of *HRT* transcript and *PR-1* expression in *HRT cry2* and *cry2* backgrounds, respectively (Fig. S4*D*). The reduced accumulation of HRT protein in *cry2* background was further confirmed using a bioassay involving HR formation. This bioassay is based on the rationale that plants overexpressing *HRT* do not produce visible HR upon TCV inoculation (30, 32). Thus, absence of HR after TCV inoculation of BTH pretreated plants would indicate the presence of increased levels of HRT in those plants. As expected, TCV inoculation did not produce visible HR in BTH pretreated *HRT cry1* (Fig. S4*E*) or *HRT phot1* plants. In contrast, TCV inoculation continued to induce HR lesions on *HRT cry2* plants even when they were pretreated with BTH. Interestingly, *HRT phot2* leaves showed an intermediate phenotype; visible HR formation on *HRT phot2* leaves was reduced but not abolished (Fig. S4*E*). HR phenotype correlated well with resistance. BTH treatment led to a significant increase in the number of resistant *HRT cry1*, *HRT phot1,* and *HRT phot2* plants (Fig. S4 *F* and *G*). In contrast, *HRT cry2* plants continued to show high levels of susceptibility; these plants showed typical morphology associated with susceptible plants and accumulated virus in their systemic tissues. The percentage of BTH-treated *HRT phot2* plants showing resistance to TCV was lower compared with *HRT cry1* or *HRT phot1* plants and was likely related to the reduced levels of HRT accumulating in response to BTH in these plants. Together, these data suggest that increased expression of *HRT* overcomes a requirement for CRY1, PHOT1, and PHOT2 to varying degrees, but not CRY2.

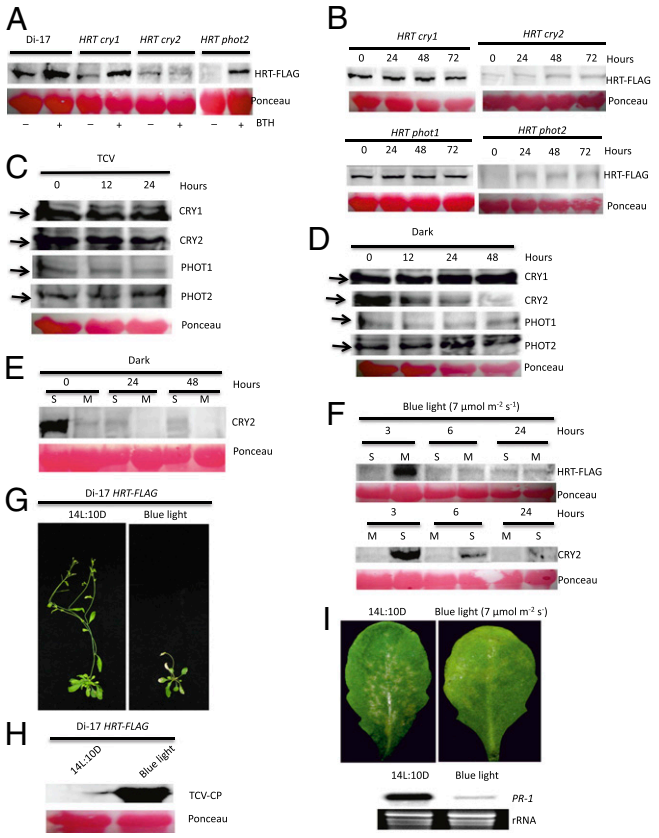

Fig. 3. Blue-light causes degradation of HRT-FLAG and overexpression of *HRT* is unable to increase HRT-FLAG levels in *cry2* plants. (*A*) Western blot showing HRT-FLAG levels in indicated genotypes treated with water or BTH for 48 h before sampling. Total proteins were extracted and analyzed by immunoblotting. HRT-FLAG levels in BTH-treated *HRT phot1* plants were similar to those observed in BTH-treated Di-17 plants (Fig. S4*C*). (*B*) HRT-FLAG levels in *HRT cry1*, *HRT cry2*, *HRT phot1*, and *HRT phot2* plants before (0 h) and after TCV inoculation. (*C*) CRY1, CRY2, PHOT1, and PHOT2 levels in total proteins extracted from Di-17 plants before (0 h) and after TCV inoculation. Arrows indicate respective proteins. (*D*) Levels of CRY1, CRY2, PHOT1, and PHOT2 at various time points after dark treatment. Arrows indicate respective proteins. (*E*) CRY2 levels in proteins extracted from soluble (S) and membrane (M) fractions before (0 h) and after dark treatments. (*F*) Immunoblot showing HRT-FLAG and CRY2 levels in total proteins extracted from Di-17 *HRT-FLAG* plants exposed to blue light (7 µmol m^{-1} s^{-1}) for 3–24 h. (*G*) Typical morphological phenotypes of TCV inoculated Di-17 *HRT-FLAG* plants kept in 14-h L:10-hr D photocycles or under blue light for 48 h postinoculation. (*H*) Immunoblot showing levels of TCV coat protein (CP) in total proteins extracted from systemic tissues of plants shown in G. (*I*) Typical HR (*Upper*) and *PR-1* expression (*Lower*) phenotypes of TCV inoculated Di-17 *HRT-FLAG* plants kept under 14-h L:10-h D photocycles or under blue light for 72 h. Ponceau-S staining of the Western blots shown in *A*, *B*, *C*, *D*, *E*, *F*, and *H* were used as the loading control.

Blue-Light Treatment Causes Degradation of HRT. Next, a time-course study was carried out to determine if resistance was associated with changes in CRY or PHOT proteins and if HRT levels in *cry* or *phot* backgrounds changed in response to TCV inoculation. TCV inoculation did not alter HRT levels significantly in *cry1*, *cry2*, *phot1*, or *phot2* backgrounds (Fig. 3*B*). TCV inoculation also did not alter levels of CRY or PHOT proteins in *HRT* plants (Fig. 3*C*). However, lack of light, which lowered HRT levels (Fig. 1 *A* and *J*), caused a gradual decline in CRY2, but not CRY1 or either PHOT proteins (Fig. 3*D*). A role for CRY2 in anion channel-mediated currents across plasma membrane (15), and the fact that HRT is a plasma membrane protein, prompted us to determine if the dark-triggered decrease in CRY2 was associated with relocalization of CRY2 into the membranous fraction. Absence of CRY2 in dark-treated plants was not associated with the relocalization of this protein to the membrane (Fig. 3*E*). The dark-induced changes in the levels of CRY2 were consistent with diurnal rhythms and maximal transcript seen during the light phase (34) as well as a previous report showing reduction in CRY2 levels in dark-treated seedlings (11). On the other hand the CRY2 protein has also been shown to exhibit a short-day specific diurnal rhythm, with increased accumulation of CRY2 at the end of the dark period (35). The fact that this study was carried out using continuous short-day photocycles versus a shift from 14-h L:10-h D photocycles to dark, could account for this discrepancy. Nonetheless, in our study, reduction in HRT levels did correlate with a decrease in CRY2 levels. However, at this point it is unclear whether photoactivated form of CRY2 is required for HRT stability, because most, if not all of CRY2, is thought to be inactive in dark.

Because CRY2 protein is degraded in the presence of blue-light (10, 11), we next exposed the Di-17 *HRT-FLAG* plants to blue-light. The blue-light-mediated disappearance of CRY2 correlated with degradation of HRT-FLAG; the levels of both CRY2 and HRT-FLAG were reduced significantly 6 h after blue-light treatment (Fig. 3*F*). Notably, reduction in HRT levels was quicker (within 6 h) than that in CRY2, which reduced more gradually. This suggested that a certain threshold level of CRY2 might be required for the stability of HRT. Consistent with the degradation of HRT, the Di-17 *HRT-FLAG* plants exposed to blue-light showed susceptibility to TCV (Fig. 3*G*) and supported the systemic movement of the virus (Fig. 3*H*). Interestingly, treatment with blue-light also compromised HR to TCV (Fig. 3*I Upper*) and these plants showed marked reduction in *PR-1* expression (Fig. 3*I Lower*).

HRT Interacts with COP1 and Is Degraded in a 26S Proteasome Dependent Manner. To determine if CRY2 and/or PHOT2 contributed to the stability of HRT by direct association with the R protein, we tested interactions between HRT and CRY2/PHOT2 using bi-

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molecular fluorescence complementation assays (BiFC) (Fig. 4*A* and Fig. S5*B*). Interaction between CRY2 and CIB1 (36) and HRT and CRT1 (37) were used as positive controls. No interaction was detected between CRY2 and HRT or PHOT2 and HRT, suggesting that CRY2 or PHOT2 do not affect stability of HRT via direct interactions with the R protein (Fig. 4*A*). As expected, CRY2 interacted with CIB1 in the nucleus (36; Fig. 4*A*). Similarly, HRT interacted with CRT1 and this interaction occurred in the endosomes, the site of CRT1 localization (38; Fig. 4*A*). To determine if the indirect association between HRT and its cognate avirulence factor CP is mediated via CRY2 or PHOT2, we evaluated interaction between CP and CRY2/PHOT2 proteins. The fact that CP was present in both soluble and membranous fractions of TCV infected cells (Fig. S5*A*) further supported a possibility for its interaction with the soluble CRY2 and the membranous PHOT2 proteins (8, 20). In addition to these, we also tested interaction between HRT and CP. However, CP did not interact with HRT, CRY2, or PHOT2 in the BiFC assays (Fig. 4*A* and Fig. S5*B*). All proteins were expressed at detectable levels indicating that the lack of interactions in BiFC assays was not due to insufficient protein expression.

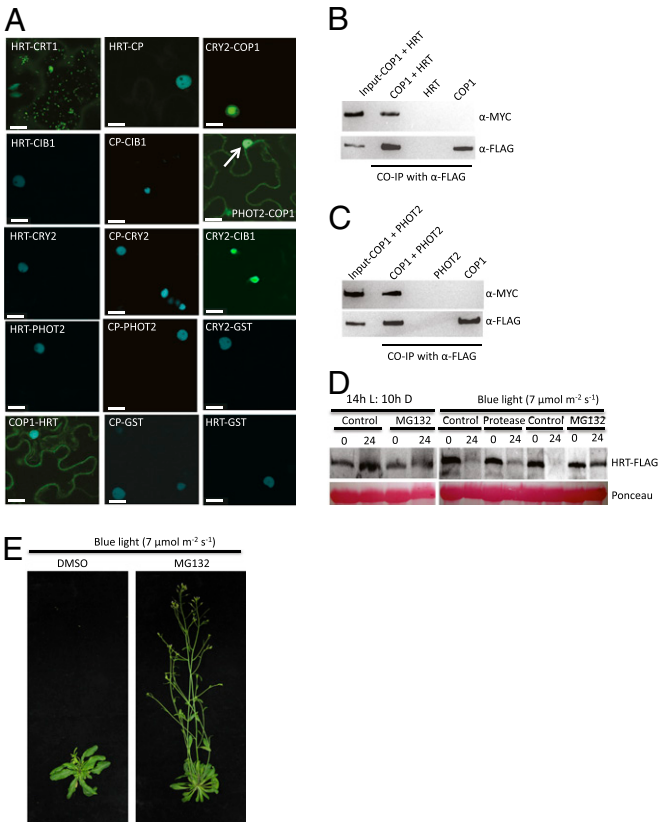

Fig. 4. HRT interacts with COP1 and is degraded via 26S proteasome pathway. (*A*) Confocal micrographs showing bimolecular fluorescence complementation for indicated proteins. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B or wild-type plants (Fig. S5*B*). The micrographs shown are CFP and YFP overlay images. The arrow indicates nucleus. (Scale bars: 10 μM.) (*B*) Coimmunoprecipitation of HRT-MYC with α-FLAG. *Nicotiana benthamiana* plants were agroinfiltrated with COP1-FLAG or HRT-MYC or both. Total extract (input) and immunoprecipitated proteins were analyzed using immunoblotting with α-MYC and reprobed with α-FLAG. (*C*) Coimmunoprecipitation of PHOT2-MYC with α-FLAG. *N. benthamiana* plants were Agroinfiltrated with COP1-FLAG or PHOT2-MYC or both. Total extract (input) and immunoprecipitated proteins were analyzed using immunoblotting with α-MYC and reprobed with α-FLAG. (*D*) Immunoblot showing HRT-FLAG levels in total proteins extracted from Di-17 *HRT-FLAG* plants infiltrated with mock solutions (0.1% DMSO, control), plant protease inhibitor mixture (protease), or the 26S proteasome specific inhibitor (MG132), before normal or blue-light treatments (7 μmol m^{-1} s^{-1}) for 24 h. (*E*) Typical morphological phenotypes of TCV inoculated Di-17 plants kept under blue light for 48 h after treatment with DMSO or MG132.

We next tested interaction of HRT with CIB1 and COP1 proteins, because both CIB1 and COP1 are known to interact with CRY2 (12, 36). Consistent with earlier results (38), CRY2 interacted with COP1 in the nucleus (Fig. 4*A*). Interestingly, HRT interacted with COP1 but not with CIB1 (Fig. 4*A*). Consistent with the plasma membrane localization of HRT, fluorescence was seen in the periphery of the cell. This is in contrast to the HRT-CRT1 interaction, which occurs primarily in the endosomes. Thus, although HRT normally localizes to the plasma membrane, it associates with proteins present in different cellular compartments.

The interaction between HRT and COP1 was further confirmed by coimmunoprecipitation (Fig. 4*B*). The COP1 protein also interacted with PHOT2 (Fig. 4 *A* and *C*), which is consistent with fact that COP1 serves as a negatively regulator of PHOT2-mediated signaling (39). The interaction between PHOT2 and COP1 was further confirmed by coimmunoprecipitation (Fig. 4*C*). Interestingly, the COP1-PHOT2 interaction was detected both inside and outside the nucleus, suggesting that, besides golgi apparatus (20), PHOT2 also relocalizes to the nucleus. However, at this point we are unable to discount the possibility that the nuclear localization of PHOT2 may be due to its increased expression.

COP1 is an E3 ubiquitin ligase that tags proteins with ubiquitin thereby subjecting them to degradation via 26S proteasome. We therefore examined the effects of the 26S proteasome-specific inhibitor MG132 on blue-light-dependent HRT degradation. The Di-17 *HRT-FLAG* leaves pretreated with MG132 accumulated 85% of HRT-FLAG protein after 24 h of blue-light treatment, compared with <15% HRT-FLAG in leaves treated with a protease inhibitor mixture and <10% in control plants (Fig. 4*D*). Consistent with this result, plants exposed to blue-light showed resistance to TCV when they were pretreated with MG132 (Fig. 4*E*). It is possible that HRT levels are maintained because of interactions of CRY2 or PHOT2 with COP1, both of which are thought to repress COP1 activity (12, 39). Under dark or blue-light conditions, CRY2 degradation and possible conformational changes in PHOT2 might relieve their repression of COP1 activity, enabling COP1 to interact with HRT, thereby targeting HRT for degradation. The slow kinetics of nuclear and cytoplasmic relocalization of COP1 in dark and light, respectively (40), suggests that sufficient amount of COP1 might be present in the cytoplasm in the dark to interact with HRT. Although our data clearly show that blue-light induces the degradation of HRT in a 26S proteasome-specific manner, a direct role for COP1 in this degradation remains unclear. Likewise, the exact role of COP1 in CRY1 and CRY2 stability remains unresolved. Notably, although both CRY1 and CRY2 interact with COP1, only CRY2 undergoes blue-light-dependent degradation (8–13).

Mutations in Blue-Light Photoreceptors Do Not Alter Resistance to Bacterial Pathogen. To determine if blue-light photoreceptors play a specific or generalized role in R protein-mediated resistance, we evaluated *RPS2*- and *RPS4*-mediated resistance to the bacterial pathogen *P. syringae* expressing *avrRpt2* (Fig. S6*A*) or *avrRps4* (Fig. S6*B*), respectively. Evaluating *RPS2*-mediated resistance was particularly relevant because PHOT2 was recently shown to coimmunoprecipitate with RPS2 (41). Notably, *cry1*, *cry2*, *phot1*, *phot2* mutations did not alter *RPS2*- or *RPS4*-mediated resistance to *P. syringae* expressing *avrRpt2* or *avrRps4*, respectively (Fig. S6 *A* and *B*) or *PR-1* expression induced in response to *P. syringae* expressing *avrRpt2* (Fig. S6*C*). Furthermore, *cry1 cry2* and *phot1 phot2* double mutants also showed normal resistance to *P. syringae* expressing *avrRpt2*. These data suggest that RPS2- and RPS4-mediated resistance likely do not require the CRY or PHOT blue-light photoreceptors. These results, however, disagree with a recent report where CRY1 was shown to positively regulate

RPS2-mediated resistance (42). One possible explanation is that Wu and Yang (42) studied resistance response under continuous light, whereas we have used 10-h L:14-h D photocycles for bacterial infections. It is quite likely that photoreceptors function differently under different light conditions and/or that they play redundant roles in mediating bacterial resistance.

Conclusions

In conclusion, our results provide definitive evidence for a mechanistic role for blue-light photoreceptors in R protein-mediated resistance to TCV. These blue-light photoreceptors appear to affect multiple steps in the resistance signaling pathway including their indirect roles in maintaining R protein stability by repressing COP1 activity as well as by regulating pathogen-responsive accumulation of SA and downstream resistance signaling.

Materials and Methods

Plant Growth Conditions, Genetic Analysis, and Generation of Transgenic Plants. Plants were grown in MTPS 144 Conviron walk-in chambers at 22 °C, 65% relative humidity, and 14-hr photoperiod. The chamber was equipped with cool white fluorescent bulbs (FO96/841/XP/ECO; Sylvania). The photon flux density (PFD) of the day period was 106.9 μmol m^{-2} s^{-1} (measured using a digital light meter; Phytotronic Inc). Fluence rates were measured using LI-1400 data logger fitted with remote quantum sensor (Li-COR). Light spectra were measured using multi spectroradiometer (EPP 2000-VIS-200; StellarNet Inc.). For blue-light treatments, light was filtered through a blue Roscolene filter (Vincent Lighting Systems) and the spectra of the filtered light was measured using spectroradiometer. The efficiency of blue-light treatments was also verified by comparing the hypocotyl growth of Col-0 and *cry2* plants (Fig. S7). All of the genotypes and crosses analyzed in this work, and their genetic backgrounds are listed in Table S2. *HRT* or various mutant alleles were genotyped using primers listed in Table S3.

TCV and Bacterial Inoculations. Transcripts synthesized in vitro from a cloned cDNA of TCV using T7 RNA polymerase were used for viral inoculations. Viral transcripts at a concentration of 0.05 μg/μL were used to inoculate 4-wk-old plants as described earlier (31). All TCV inoculations were carried out during morning hours and plants kept under 14-h L:10-h D photoperiod received at least 8–10 h of light before the start of dark phase. The bacterial strain expressing *avrRpt2* and *avrRpt4* were grown overnight in King's B medium containing kanamycin and rifampicin (Sigma). The bacterial cells were harvested, washed, and suspended in 10 mM $MgCl_2$. The cells were infiltrated at a final density of 10^5 CFU/mL (A_{600}) into the abaxial surface of Arabidopsis leaves using a needle-less syringe. Plants inoculated with bacterial pathogens were transferred to a Conviron growth chamber maintained at 22 °C, 65% relative humidity, and 10-h L:14-h D photocycles.

Detailed materials and methods are included in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Peter Quail (University of California, Berkeley), Anthony Cashmore (University of Pennsylvania), Chentao Lin (University of California, Los Angeles), Ken-ichiro Shimazaki (Kyushu University), John Christie (University of Glasgow), Michael Goodin (University of Kentucky), Winslow Briggs (Carnegie Institution for Science), Haiyang Wang (Yale University), Gary Whitelam (University of Leicester), Jack Morris (University of Nebraska), and Jeff Dangl (University of North Carolina) for seeds and antibodies. We thank Joanne Holden for help with SA estimations, Elena Prior and Joseph Omielan for help with light measurements, and Michael Goodin for transgenic *Nicotiana* plants expressing CFP-H2B and pSITE-MYC vector. We thank David Smith, Bruce Downie, John Carr, Keshun Yu, and anonymous reviewers for useful suggestions, Ludmila Lapchyk and Amy Crume for technical help, and Enamul Huq for useful information about light filters. This work was supported by grants from the National Science Foundation (IOS-0641576) and the Kentucky Science and Engineering Foundation (1214-RDE-009).

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