CHARACTERIZATION OF THE STABILITY OF PRR7, A CLOCK PROTEIN IN *ARABIDOPSIS THALIANA*

By

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ABSTRACT

CHARACTERIZATION OF THE STABILITY OF PRR7, A CLOCK PROTEIN IN *ARABIDOPSIS THALIANA*

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The circadian clock in *Arabidopsis* is comprised of a series of transcriptional/translational feedback loops. The pseudo-response regulators (PRRs) play a central role in the Arabidopsis circadian network. Members of the PRR family have been shown to be post-translationally regulated by light. PRR5 and TOC1 are targeted for degradation by ZTL, an F-box protein with a blue light absorbing LOV domain. Blue light has been shown to stabilize PRR5. PRR7 protein expression increases throughout the day and peaks near dusk before being degraded in darkness. Previous work has shown that this protein is stabilized by white light relative to darkness. In the present study, PRR7 protein stability was characterized under different light conditions. PRR7 degradation under white light, blue light, and darkness was similar during the subjective day and night. Under red light, the half-life of PRR7 protein was extended by almost an hour in the subjective evening as measured by Western blotting. PRR7 protein is stabilized by red light relative to darkness in the subjective evening. Blue light did not stabilize PRR7. ZTL does not affect PRR7 protein abundance; as blue light does not seem to affect PRR7 stability, it is unlikely that other members of the ZTL family regulate PRR7.

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ANOVA	Analysis of Variance
CCA1	CIRCADIAN CLOCK-ASSOCIATED 1
CCT	CONSTANS, CONSTANS-LIKE, TOC1
CHE	CCA1 HIKING EXPEDITION
CIB	CRYPTOCHROME-INTERACTING FACTOR
CO	CONSTANS
COP1	CONSTITUTIVE PHOTOMORPHOGENEIC 1
CRY	Cryptochrome
ELF3	EARLY FLOWERING 3
FAD	FLAVIN ADENINE DINUCLEOTIDE
FHY1	FAR-RED ELONGATED HYPOCOTYL1
FKF1	FLAVIN-BINDING KELCH REPEAT F-BOX1
FMN	FLAVIN MONONUCLEOTIDE
GI	GIGANTEA
HA	Hemagglutinin
HFR1	LONG HYPOCOTYL IN FAR-RED1
HRT	HYPERSENSITIVE RESPONSE TO TCV
HY5	ELONGATED HYPOCOTYL5
LAF1	LATE ELONGATED HYPOCOTYL
LD	Light/Dark
LHY	LONG AFTER FAR-RED LIGHT1
LKP2	LOVE KELCH PROTEIN2
LL	Constant Light
LUC	Luciferase
LUX	LUX ARRHYTHMO
PHY	Phytochrome
PIF	PHYTOCHROME-INTERACTING FACTOR
PR	PSEUDO-RECIEVER
PRR	PSEUDO-RESPONSE REGULATOR
RLU	Relative Light Units
SCF	Skp, Cullin, F-box
SEM	Standard Error for the Mean
SPA1	SUPPRESSOR OF PHYA-105
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, and PCF
TOC75	TRANSLOCON OUTER MEMBRANE COMPLEX
ZT	Zeitgiber
ZTL	ZEITLUPE

Introduction

Overview of Circadian Oscillators

The endogenous circadian oscillator generates rhythmic outputs of biological processes (Harmer, 2009). The circadian clocks of different organisms share several defining characteristics. First, they are endogenous and self-sustained. The biological rhythms can be observed under constant environmental conditions, such as constant light or temperature and have a period of about 24 hours. Second, they are entrainable; the oscillation of daily biological rhythms receives input from the surrounding environment, predominantly in the form of light/dark or thermal cycles (Millar, 2003; McClung & Salome, 2005). The phenomenon of entrainment allows the organism to adjust its daily rhythms to match the surrounding environment. Third, they are temperature compensated so that the period remains consistent over a range of physiologically relevant temperatures. In plants, different clock mutants display changes in temperature compensation (Salomé, et al., 2010). Alternative splicing has been proposed to be a mechanism by which temperature compensation is achieved in plants (James et al., 2012). This has also been demonstrated to be the case in *Drosophila* (Majercack, et al., 1999).

Circadian clocks have evolved independently among algae, plants, insects, and mammals (Bell-Pedersen, et al., 2005). In eukaryotes the molecular components of the oscillator are not conserved, although the structure is similar among different taxa. Eukaryotic clocks are based on a network of transcriptional and translational feedback loops (Robash, 2009). However, in eukaryotes there is also evidence to support non-

transcriptional oscillators. Circadian rhythms in Cyanobacteria are based on a transcriptional-less oscillator. Here the oscillator is comprised of three proteins, KaiA, KaiB, and KaiC. KaiC is an ATPase that also exhibits autokinase activity and its phosphorylation status oscillates with a period of 24 hours. KaiA activates the autokinase and ATPase activity of KaiC (Iwasaki, et al., 2002), while KaiB serves to inhibit the activity of KaiA (Kitayama, et al., 2003). This system has been reconstituted *in vitro* (Nakajima, et al., 2005). Rhythmic outputs have been observed in the absence of new transcription. Peroxiredoxin proteins in human red blood cells, as well as those in the unicellular algae *Osterococcus tauri*, undergo rhythmic oscillations in redox state with a period of 24 hours in the absence of transcription (O'Neill & Reddy, 2011; O'Neill, et al., 2011).

In plants, processes such as de-etiolation, flowering time, photosynthesis, immune responses, and light sensitivity are regulated by the circadian clock (Harmer 2009). Transcriptional regulation is a primary mechanism by which this is achieved in *Arabidopsis* (Doherty & Kay 2010). Upward of 30% of the *Arabidopsis* transcriptome is circadian regulated, with the organism showing oscillations in gene expression under constant environmental conditions (Harmer, et al., 2000; Covington, et al., 2008).

The circadian clock in *Arabidopsis thaliana*

Relative to other plants, the circadian clock of *Arabidopsis* is the best characterized to date. In *Arabidopsis*, the oscillator is comprised of at least three

transcriptional/translational feed-back loops (Figure 1). The first loop described consists of the morning expressed Myb transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) that repress transcription of the evening expressed *TOC1* (*TIMING OF CAB EXPRESSION*) gene during the day by binding a sequence in its promoter known as the evening element (AAATATCT) (Alabadí et al., 2001, Perales & Más, 2007) (Figure 1). TOC1 had initially been thought to promote expression of *CCA1* and *LHY* in the morning by antagonizing the binding of a TCP (TEOSINTE BRANCHED1, CYCLOIDEA, and PCF) transcriptional repressor CCA1 HIKING EXPEDITION 1 (CHE1) (Pruneda-Paz et al., 2009), although recent evidence suggests it functions as a repressor of morning genes, including *CCA1* and *LHY* during the night (Huang et al., 2012) (Figure 1).

TOC1 is a member of the small gene family in Arabidopsis known as the pseudo response regulators (*PRR*s) (Matsushika, et al., 2000). There are four other members of this family involved in the *Arabidopsis* circadian clock: *PRR3*, *PRR5*, *PRR7*, and *PRR9*. PRRs 5, 7 and 9, together with CCA1 and LHY form an additional regulatory loop (Farre et al., 2005; Nakamichi, et al, 2005) (Figure 1). During the day, these PRRs sequentially bind the promoters of *CCA1* and *LHY* to inhibit transcription (Nakamichi, et. al, 2005). PRR9 binds early in the day, followed by PRR7 through the afternoon, and PRR5 through the evening. In turn, CCA1 and LHY activate the transcription of *PRR7* and *PRR9* by directly binding their promoters (Farre et al., 2005).

A third transcriptional loop exists within the *Arabidopsis* circadian network. CCA1 and LHY bind the evening element in the promoter of *LUX ARRHYTHMO* (*LUX*) to prevent its transcription (Hazen et al., 2005); in turn, LUX specifically binds to the LUX binding site within its own promoter as well as the promoter of *PRR9* (Helfer et al., 2011). LUX binding to the *PRR9* promoter has been suggested to be a mechanism for indirect regulation of *CCA1* and *LHY* transcription by the evening-phased LUX. *EARLY FLOWERING 3* (*ELF3*) is another evening-expressed gene whose protein product associates with the *PRR9* promoter to repress its expression in a time-dependent manner (Dixon et al., 2011). LUX has been shown to be necessary for ELF3 binding to DNA (Chow et al., 2012).

Additional components of the oscillator include GIGANTEA (GI), which interacts with ZEITLUPE (ZTL), a component of the SCF-E3 ubiquitin ligase complex that targets TOC1 and PRR5 proteins for degradation during the night (Fujiwara et al., 2008) (Figure 1). The interaction between TOC1 and ZTL is inhibited by PRR3 (Para et al., 2007) promoting stabilization of the TOC1 protein at the beginning of the night.

The circadian regulated pseudo-response regulators

The *PRR* genes implicated in the clock mechanism also undergo circadian regulation. These genes are expressed sequentially throughout the day, beginning with *PRR9* in the morning, followed by *PRR7*, *PRR5*, *PRR3*, and ending with *PRR1* (*TOC1*) in the evening (Figure 2A, Matsushika et al., 2000). Peak protein expression of these

PRRs is delayed 4-8 hours relative to the maximum mRNA accumulation (Fujiwara et al., 2008). PRR 9, 7, and 5 each repress the transcription of *CCA1* and *LHY*. In *Arabidopsis*, mutations in either *PRR7* or *PRR9* cause period lengthening in constant light of up to two hours, although no effect on period length is observed in constant darkness. This result indicates that these genes play a role in transmitting light signals to the central oscillator (Farre, et al., 2005). *prr9* mutants show period lengthening defects under a broad range of red and blue light fluences, while the *prr7* mutant period length is more dramatically affected by red light. All *prr7* mutant lines are hyposensitive to red light, as determined by hypocotyl measurements (Nakamichi, et al., 2005). The *prr7-3 prr9-1* double mutant has a long period phenotype, which is stronger under red than blue light relative to the wild type. These mutant-specific light defects indicate specific functions of each of the PRRs , or may point to their differential regulation by light, in addition to their overlapping roles as transcriptional repressors within the circadian network.

The circadian-regulated pseudo-response regulators share a conserved domain structure. The C-terminus contains a CCT (CONSTANTS, CONSTANTS LIKE, TOC1) motif that is conserved among all circadian-regulated PRR proteins (Figure 2B). The CCT domain resembles the DNA binding motif of the HAP2/3/5 complex in the budding yeast *Saccharomyces cerevisiae* (Wenkel et al., 2006). Recently it has been shown that this domain is able to bind DNA directly *in vitro*, although the degree of sequence specificity remains to be demonstrated (Gendron et al., 2012).

At the N-terminus, the pseudo-receiver (PR) domain resembles the receiver domain found in bacterial and plant response regulators (Figure 2B; Mizuno & Nakamichi, 2005). The PR domain lacks the conserved aspartate that is phosphorylated in the signaling pathway mediated by response regulators (Mizuno & Nakamichi, 2005). The receiver domain of PRR5 has been shown to mediate the degradation of the protein, as well as its interaction with ZTL (Kiba, et al., 2007). The PR domain of PRR5 also interacts with TOC1 (Wang, et al., 2010). In some organisms, such as the cyanobacterium *Synechococcus elongatus*, the pseudo-reciever domain has been suggested to mediate protein-protein interactions important for regulating the pace of the oscillator (Gao, et al., 2007). The cyanobacterial protein lacks the CCT motif found in the circadian PRRs of *Arabidopsis*.

Post-translational Regulation of PRR Proteins

The circadian PRR proteins are post-translationally modified; each PRR protein becomes increasingly phophorylated through its daily expression cycle, reaching maximal phosphorylation prior to being degraded (Fujiwara et al., 2008). Neither the location of the phosphorylation site(s) or the kinase(s) responsible for the modification are known. In addition, the role of phosphorylation has not been fully described for the PRR proteins in Arabidopsis. PRR5 and TOC1 phosphorylation enhances their interaction with ZTL, a blue-light absorbing F-box protein that targets them for degradation by the proteasome (Fujiwara et al., 2008). This agrees with new insights suggesting that variation in phosphorylation state can induce conformational changes

generated by charge repulsion within a protein. These conformational changes mediate the protein's stability by affecting its ability to interact with other proteins as has been proposed for some mammalian clock proteins (Menet & Rosbash, 2011). Experiments with proteasomal inhibitors have shown that all circadian PRR proteins are degraded by the 26S proteasome (Farre & Kay, 2007; Fujiwara et al., 2008), although the regulatory partners for PRR3, PRR7, and PRR9 have not been identified. Phosphorylation status has also been shown to facilitate the interaction of TOC1 with PRR3 to prevent TOC1 interaction with ZTL and stabilize the protein, although the interaction of TOC1 with ZTL also depends on its phosphorylation state (Fujiwara et al., 2008).

Post-translational modifications might mediate the sub-cellular localization of the PRRs. TOC1 nuclear accumulation is mediated by PRR5 (Wang, et al., 2010). Nuclear localized TOC1 appears to be more highly phosphorylated than that which remains in the cytosol, although it is unclear whether this enhanced phosphorylation precedes nuclear import. It has been demonstrated that phosphorylation status regulates the sub-cellular distribution for clock proteins in other organisms (Diernfellner, et al., 2009).

Photoreceptors and Light Input to the Circadian Clock

In plants, the phytochromes, cryptochromes, and ZTL family of proteins have been implicated as the major light receptors for generating rhythmic oscillations (Somers, 1998, Baudry et al., 2010; Yanovsky, et al., 2000). These plant photoreceptors bind a chromophore that induces an activating conformational change within the protein

upon absorbing a photon of light.

There are five red/far-red sensing phytochromes (phyA-phyE) in *Arabidopsis*. Phytochromes can be divided in two classes: light labile (phyA) and light stable (phyB-phyE). The phytochromes are localized to the cytosol in their biologically inactive red light absorbing state, or Pr form. After irradiation with red light, they undergo a conformational change to their biologically active far-red light absorbing Pfr form.

The UV-A/blue light perceiving cryptochromes (CRY) were first identified in plants, and have since been identified in other multicellular eukaryotes (Lin & Shalitin, 2003). In mammals, the cryptochromes are molecular components of the central oscillator (Cashmore, 2003), though it has been shown that this is not true in *Arabidopsis* where they are involved in light input to the clock (Yanovsky, et al., 2000). The cryptochromes structurally resemble DNA photolyases; at the amino terminus is a photolyase-like domain, a central chromophore binding region, and a carboxy-terminal effector region known as the cryptochrome carboxy-terminal extension (Lin, et al., 2003). Only CRY3 has been shown to exhibit DNA repair activity in *Arabidopsis* organelles and does not appear to have any blue light signaling activity (Kleine, et al., 2003). Upon blue light perception, the FAD (Flavin Adenine Dinucleotide) cofactor of cryptochrome becomes reduced, and the protein is subsequently phosphorylated, a modification considered important for its signal transducing activity. Following phosphorylation, it is proposed that the proteins undergo a conformational change that results in dissociation of its amino and carboxy terminal domains to facilitate

subsequent signaling events (Yu et al., 2007). Phosphorylation has also been shown to be important for degradation of CRY proteins (Liu et al., 2011; Shalitin, et al, 2003).

ZTL, as well as FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) and LOV KELCH PROTEIN 2 (LKP2), are a family of F-box proteins that function predominantly in the circadian clock and confer time-specific changes in protein abundance (Baudry, et al., 2010). Members of this family contain a LIGHT OXYGEN VOLTAGE (LOV) domain at their amino terminus that perceives blue light. This domain binds flavin mononucleotide (FMN) non-covalently where it serves as a chromophore (Möglich, et al., 2010). These proteins also possess a Kelch repeat domain that seems to be important for mediating protein-protein interactions (Ito, et al., 2012).

The cryptochromes and phytochromes mediate circadian entrainment by light (Sommers, et al., 1998). The photoreceptors have specific and overlapping roles in transmitting light signals to the clock that in turn affect circadian period. phyA and phyB act as the main mediators of red light signaling to the clock. phyB has been demonstrated to be the principal high-fluence red light receptor for circadian light input (Somers, et al., 1998), and *phyB* mutants have long periods under high fluences of red light (Sommers, et al., 1998; Devlin & Kay, 2000). phyA is responsible for far-red response of the clock (Kolmos, et al., 2011), as well as low-fluence red light input (Sommers, et al., 1998; Devlin & Kay, 2000). *phyA* mutants exhibit long periods under low-fluence red light (Sommers, et al., 1998; Devlin & Kay, 2000) . phyA is also able to absorb blue light, and acts with CRY1 to transduce low fluence blue light to the clock

(Sommers, et al., 1998; Devlin & Kay, 2000). In accordance with its role in perceiving blue light, *phyA* mutants have long periods under low fluence blue light (Sommers, et al., 1998). CRY1 is also responsible for providing high-intensity blue light information to the oscillator (Sommers, et al., 1998). *cry1* mutants exhibit long periods under low fluences of blue light (Sommers, et al., 1998). *cry2* single mutants do not exhibit a fluence-dependent period phenotype relative to wild type in blue light (Devlin & Kay, 2000). The *cry1cry2* double mutant exhibits a long period phenotype over a range of blue light fluences that is longer than the period defects observed in the *cry1* single mutant. Thus, the cryptochromes may have partially redundant roles in mediating blue light entrainment (Devlin & Kay, 2000). Taken together, these results point to a complex and interwoven network of signaling from the photoreceptors to convey light information to the clock.

There are several mechanisms through which the photoreceptors transduce light signals to the clock. The transcription of some clock genes is activated in a manner dependent on phytochromes. *PRR9* transcription has been shown to be rapidly induced by light in a phytochrome-dependent manner (Ito, et al., 2007). *ELF4* is regulated by both red and far-red light in a phyB and phyA dependent manner respectively (Khanna et al.; Li, et al., 2011). FHY3, HY5, and FAR1 are all positive regulators of phyA signaling that bind the promoter of *ELF4* and activate its transcription during the day (Li, et al., 2011). In turn, ELF4 has been shown to mediate the transcriptional activation of *CCA1* and *LHY* in response to red light (Kikis, et al., 2005).

Light signaling to the clock is also regulated through post-transcriptional mechanisms. The translation of *LHY* mRNA is induced by light (Kim, et al., 2003). Recently it has been shown that some red-light irradiated phytochrome is retained in the cytosol where it can act to regulate translation (Paik, et al., 2012). This may be how *LHY* mRNA translation is regulated by light.

The post-translational regulation of several clock components by light is another mechanism by which light information is conveyed to the clock. PRR9 and PRR7 proteins are degraded in the dark by the proteosome (Ito, et al., 2007; Farre & Kay, 2007). How these proteins are targeted for degradation in the dark remains to be elucidated. PRR5 is stabilized by blue light and targeted for degradation in the dark by ZTL (Kiba, et al., 2007; Fujiwara, et al., 2008). ZTL, which perceives blue light through its LOV domain, is also stabilized by light in a manner that depends on GI (Kim, et al., 2007). In the dark, ZTL is able to interact with PRR5 and target it for degradation (Fujiwara, et al., 2008).

Photoreceptor Mediated Protein Stability

Phytochromes have been shown to regulate protein levels in a light-dependent manner, particularly proteins that act downstream in light signaling. phyA regulates FHY1 protein levels through direct interaction and causes phosphorylation by the phytochrome to regulate FHY1 protein stability (Shen, et al., 2009). FHY1 mediates phyA nuclear import (Genoud, et al., 2008). phyA regulation of FHY1 protein levels

serves as a mechanism through which phyA can refine red light transcriptional responses (Shen, et al., 2009). PIF3 protein levels have also been demonstrated to be regulated in a phytochrome-dependent manner, mainly by phyB (Soy, et al., 2012).

COP1 mediates the dark dependent degradation of several proteins. For example the red light signaling components LONG AFTER FAR-RED LIGHT 1 (LAF1) and LONG HYPOCOTYL IN FAR –RED (HFR1) are also regulated at the level of protein abundance by different qualities of light. The differential regulation of these proteins by light depends on the phytochromes. These proteins are targeted for degradation by the E3-ubiquitin protein ligase COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC1) (Jang, et al., 2007). The HFR1 protein is degraded in the dark by COP1 and stabilized by light, regardless of light quality (Yang, et al., 2005). LAF1, another positive regulator in phyA signaling, is similarly degraded by COP1 in the dark. In the light, COP1 becomes excluded from the nucleus, although residual COP1 is able to interact with and degrade LAF1 through its interaction with SPA1 (SUPPRESSOR OF PHYA-105) to attenuate light signaling (Seo, et al., 2003). LAF1 and HFR1 positively regulate different aspects of red light signaling; however, these two proteins interact to promote the stability of each other by preventing their interactions with COP1 (Jang, et al., 2001). phyA protein itself is regulated by light. The Pfr form interacts with the COP1/SPA1 complex. This interaction is dependent on the phosphorylation state of the protein, as hypophosphorylated phyA interacts preferentially with FHY1, presumably to prevent premature interaction with the COP1/SPA1 complex (Saijo, et al., 2008).

Cryptochromes also regulate protein stability at the post-transcriptional level in response to blue light. An example of this is the regulation of CO protein, a floral inducer, by CRY2. The interaction of CRY2 with SPA1 facilitates the interaction of CRY2 with COP1. CRY2 interaction with COP1 sequesters COP1 away from CO protein, which is then able induce flowering in long days. (Zuo, et al., 2011). CRY2 protein is phosphorylated, polyubiquitinated, and subsequently targeted for degradation by COP1 in response to blue light. The interaction of CRY2 with COP1, and thus its stability, is regulated by phyA and SPA1 (Weidler et al., 2012). CRY1 also interacts with the COP1/SPA complex to disrupt the interaction of COP1 with SPA1 (Lian, et al., 2011). CRY1 interaction with COP1 prevents the degradation of HY5 in response to blue light and allows HY5 to initiate light induced transcription (Liu, et al., 2011). The cryptochromes also mediate the stability of proteins involved in pathways other than light signaling. An example of this is the blue light stabilization of viral resistance R protein HRT (HYPERSENSITIVE RESPONSE TO TCV) mediated in part by CRY2. CRY2 promotes the stabilization of HRT in blue light by interacting with COP1 to prevent HRT degradation by the proteasome (Jeong, et al., 2010).

The ZTL familiy of proteins mediates protein degradation in a manner that is regulated by blue light. For example, FKF1 targets the CO repressor CDF1 for degradation. Blue light induces the interaction of FKF1 with GIGANTEA (GI) through the LOV domain of FKF1 (Sawa, et al., 2007). The light-dependent interaction of FKF1 with GI allows FKF1 to interact with CDF1. This enables FKF1 to target CDF1 for

degradation (Sawa, et al., 2007). CDF1 degradation results in the expression of CO. CO is then able to go on to induce FT expression and cause subsequent flowering in long days. FKF1 also stabilizes CO protein through interaction with its LOV domain to promote flowering (Song, et al., 2012). ZTL interaction with GI under blue light stabilizes ZTL and confers oscillations in the relative abundance of ZTL protein, as ZTL mRNA is constitutively expressed (Kim, et al., 2007). The regulation of ZTL protein abundance by light in addition to the role of ZTL regulating other proteins is likely a mechanism for fine-tuning clock protein expression. ZTL targets PRR5 and TOC1 proteins for degradation in the dark (Kiba, et al., 2007b; Más, et al., 2003, Fujiwara et al). Blue light absorption by the LOV domain of ZTL prevents its interaction with these PRR5 and TOC1, and thus results in their stabilization in blue light (Kiba, et al., 2007b; Fujiwara et al., 2008). FKF1 and LKP2 share some of the targets of ZTL since TOC1 and PRR5 proteins are more stable in a *ztlfkf1lkp2* triple mutant than in the *ztl* single mutant background (Ito, et al., 2011).

Light regulated post-transcriptional control is known to be an important mechanism for regulating the pace of the circadian clock in all organisms (Kojima, et al, 2011). Protein modification and regulated proteolysis are processes that govern the peak of expression for circadian proteins. PRR7 protein levels have been shown to be post-transcriptionally regulated by light and the circadian clock (Farré & Kay, 2007). The protein expression peaks at the end of the light period and is degraded in the dark by the proteasome. This dark-mediated degradation occurs regardless of the time of day, although PRR7 protein is more stable earlier in the day in both light and dark than

during the subjective night. The question of how the protein is degraded remains unanswered, as it does not interact with ZTL (Kiba, et al., 2007). COP1 is another candidate E3 ligase mediating PRR7 protein degradation, although the interaction between PRR7 and COP1 remains to be tested. To further investigate the light-mediated stability of PRR7, I measured the protein levels in seedlings expressing PRR7 under control of its endogenous promoter fused to an amino-terminal HA tag under different light conditions. The 35S::PRR7-LUC+ line was also used to characterize PRR7 degradation during the subjective evening.

Results

Cycloheximide Treatment Optimization

PRR7 protein abundance has previously been demonstrated to be regulated post-translationally by light and the circadian clock (Farre & Kay, 2007). Other circadian PRR proteins have been shown to be regulated by specific qualities of light (Fujiwara, et al., 2008; Kiba, et al., 2007). As part of this study, an *Arabidopsis* line expressing the PRR7 coding region fused to Luciferase under the control of the constitutive 35S promoter in the *prr7-3* mutant background (*prr7-3* 35S::PRR7-LUC-HA) was used to further elucidate the qualities of light that mediate the stability of PRR7 protein. The relative abundance of this fusion protein cycles independently of the mRNA, as measured by Western blot (Farre & Kay, 2007), providing further evidence that PRR7 protein is subject to strong post-transcriptional regulation.

Cycloheximide can be used as a translational inhibitor in eukaryotes to study protein degradation to determine protein half-life in the absence of additional translation. The measurement of luciferase activity has been used by other groups to study the stability of proteins using Luciferase translational fusions (Shen, et al., 2005). To determine the stability of PRR7 in the *prr7-3 35S::PRR7-LUC-HA* line, seedlings were incubated in MS media supplemented with 500 μM cycloheximide and 0.01% Triton X-100. Other groups have used similar methods for performing cycloheximide treatments (Fujiwara, et al., 2008). A line expressing firefly Luciferase under the control of a light-inducible *PRR9* promoter (Para et al., 2007) was used to test the effectiveness

of this treatment. Seedlings were transferred in darkness to MS media supplemented with different concentrations of cycloheximide before putting them in the light for four hours. As shown in Figure 4, this treatment is able to inhibit translation over concentrations of cycloheximide ranging from 200 μM to 1 mM relative to no treatment. Free Luciferase protein is relatively stable in plants (Millar et al., 1992). Thus, luciferase activity measured from samples collected at the beginning of the treatment is presumed to be residual Luciferase from the previous day(s) light exposure.

This method of cycloheximide treatment was used to measure the degradation of the PRR7-LUC fusion protein under white and red light as well as darkness. In treatments beginning at ZT12, the stability of the protein in red light increased relative to darkness up to four hours (Figure 5). Despite this trend, these two points are not statistically significant as determined by the student's t-test. At ZT4, when the protein begins to accumulate there was no apparent difference in the degradation rates between the different light conditions and darkness (data not shown). Farre & Kay (2007) had previously reported PRR7 protein is stabilized by light relative to darkness. The luciferase activity in samples collected over one diurnal period does cycle similarly to the protein as detected by Western blotting (Figure 6, this study; Farre & Kay, 2007). However, the activity shows large variability, and this variability likely masks the differences in degradation between experimental conditions. Therefore I decided to analyze PRR7 stability using the *prr7-3 PRR7::HA-PRR7* line that had been previously used (Farre & Kay, 2007) and the protein levels were measured by Western blotting to confirm the Luciferase results.

Performing cycloheximide treatments using a method that relies on diffusion instead of vacuum infiltration poses a problem, particularly in samples transferred to darkness. Light mediates stomatal opening, and stomata close in the dark (Chen, et al., 2011), which would prevent the cycloheximide from quickly entering the cells and inhibiting translation at the start of the treatment. When measuring the relative abundance of PRR7 in the *prr7-3 PRR7::HA-PRR7* line in darkness, an increase in PRR7 protein abundance was observed up to two hours after beginning the treatment, indicating the treatment was not immediately inhibiting protein synthesis. This increase was also observed in some samples transferred to different light conditions, although to a lesser extent under white light. Pre-treating the samples with cycloheximide in white light for 10-30 minutes at room temperature prior to transfer to darkness was sufficient to allow cycloheximide to enter the cells and resulted in a decrease in HA-PRR7 protein abundance at two hours (Figure 7).

Degradation of PRR7 Under Different Light Conditions

To study the effect of different qualities of light on PRR7 protein stability, one week old seedlings were grown under 12:12 light:dark cycles. Seedlings were transferred to MS media supplemented with 2 mM cycloheximide and 0.01% Triton X-100 before being transferred to different light conditions (70 μmol/m^2/sec white and red, 30 μmol/m^2/sec for blue) or darkness at ZT4 or ZT12. Samples were collected 2, 4, and 7 or 8 hours after beginning the cycloheximide treatment. I determined HA-PRR7

protein abundance by Western blotting using anti-HA antibody. The signal at each time-point was normalized to Direct Blue 7.1 total protein staining of the membrane (Hong, et al., 2000), and to the amount of protein at the starting time of the experiment. A chloroplast import protein TOC75 was used as an additional loading control for several experiments and found to be similar to the results obtained using Direct Blue staining (Figure 8).

Under these experimental conditions, there was not a difference in the protein half-life at ZT4 relative to ZT12 in white light as had been reported in Farre & Kay (2007) (Figure 9, Table 1). Similar experiments were performed under red light (70 $\mu mol/m^{-2}/sec^{-1}$) and blue light (30 $\mu mol/m^{-2}/sec^{-1}$) as well as darkness at ZT4 and ZT12. Under blue light the rate of HA-PRR7 protein degradation was similar between ZT4 and ZT12 (Figure 9, Table 1). Under red light, the half-life of PRR7 is longer at ZT12 than ZT4 (Table 1). The relative abundance at each timepoint for both subjective day and night, however, are not statistically different (Figure 9).

At ZT4, there was not a difference in the degradation of PRR7 under red and blue light relative to each other or to white light. At ZT12, red light treatment appeared to stabilize the protein relative to blue light. The relative abundance four hours after the beginning of the treatment under red light is statistically higher than the abundance under blue light (student's t-test, $p = 0.0234$). Red light did have a statistically significant effect on the stability of PRR7 relative to darkness four hours after treatment with cycloheximide (Figure 10, student's t-test, $p = 0.047$). In similar experiments, the PRR7-

LUC line did show a tendency for this effect at ZT12, although the points are not statistically different (Figure 5). Red light did not have a statistically significant effect on the degradation relative to white light as measured by Western blotting.

Discussion

This study describes an attempt to determine if red or blue light promotes the stability of PRR7 protein. It has been previously shown that PRR7 protein is stabilized by white light (Farre & Kay, 2007). The mechanism for this stabilization has not yet been defined. In the previous study the *prr7-3 PRR7::HA-PRR7* line was used to determine the stability of the protein during the subjective day and night under white light and darkness. White light was reported to promote the stability of the protein relative to darkness regardless of the time of day although the PRR7 protein was more stable during the subjective day than during the subjective night (Farre & Kay, 2007). In accordance with these results the levels of a PRR7-LUCIFERASE translational fusion expressed under the control of the constitutive 35S promoter cycled under constant white light independently of the mRNA abundance (Farre & Kay, 2007).

In this study, I first used the *prr7-3 35S::PRR7-LUCIFERASE-HA Arabidopsis* line (PRR7-LUC) to determine the stability of the protein under different light conditions. Relative abundance of PRR7-LUC was measured by luciferase activity. The *in vitro* luciferase activity of this line oscillates over one diurnal cycle (Figure 6) similarly to the protein as measured by Western blot (Farre & Kay, 2007). This indicates that the expression of this fusion protein is similar to the expression of PRR7 under its native promoter. Thus, the luciferase activity was expected to reflect the relative abundance of PRR7 under different light conditions.

In experiments beginning at the start of the subjective night, PRR7-LUC under 70 μmol/m^2/sec^1 red light showed a tendency to be stabilized relative to darkness four hours after beginning the treatment (Figure 5), although the difference is not statistically different. In experiments beginning at ZT4, or early in the day when PRR7 protein abundance is lower, no difference between white light, red light and darkness was observed (data not shown). This results contrasts with previous experiments that showed that PRR7 expressed under its native promoter was stabilized by white light relative to darkness during the subjective day (Farre & Kay, 2007). The extractable luciferase activity of the *prr7-3 35S::PRR7-LUCIFERASE-HA* line oscillates over one diurnal cycle with a peak at ZT12, but the variability is large (Figure 6). This may explain why I am unable to distinguish between the protein abundance under different light conditions in treatments beginning at ZT4 using this line. These results prompted the use of the *prr7-3* PRR7::HA-PRR7 line that was used in the previous studies (Farre & Kay 2007).

Using the *prr7-3 PRR7::HA-PRR7* line I observed differences in the ability of cychloheximide treatment to block translation, especially in the dark at ZT12 (Figures 10B, 11B). The relative abundance of HA-PRR7 would frequently increase two hours into the cycloheximide treatment before degradation was observed. This indicated that cycloheximide was unable to quickly enter the tissue in the dark. One explanation may be dark induced stomatal closure, which would prevent the cycloheximide from entering the tissue to inhibit translation. Stomata are closed in the dark, whereas both red and blue light regulate stomatal opening, as does the circadian clock (Chen, et al., 2012). At

ZT12, the plants may have closed their stomata as a result of entrainment, expecting the onset of darkness. Under white light at ZT12, cycloheximide seemed to act more quickly at inhibiting translation than under red or blue light as well as darkness at (Figures 10, 11). Therefore, I pretreated seedling in white light with cycloheximide to test if the reagent was able to enter the tissue faster before being transferred to darkness. Figure 7 shows that pre-incubating the seedlings with cycloheximide in the presence of Triton X-100 in the light results in a decrease in PRR7 protein two hours after beginning the treatment in the dark. These results may suggest that the degradation of PRR7 in the dark at ZT12 as measured in this study is artificially slower due to insufficient translational inhibition. Pre-treating samples with cycloheximide under a uniform white light regime before treatment under various light conditions might remove some of the variability from these measurements.

The results of the experiment using *PRR9::LUC* expressing seedlings (Figure 4) indicate that under white light, the cycloheximide treatment used should be sufficient to inhibit translation and prevent new protein synthesis. This permits the measurement of the degradation of existing protein under these conditions. Previously it had been shown that PRR7 protein is degraded faster during the subjective night than during the subjective day under white light (Farre & Kay, 2007). Table (1) shows the half-life of PRR7 calculated in this study. Under white light, I did not measure a significant difference in the half-life of PRR7 during the subjective day and night. Seedlings used in the previous study were older than those used presently, and so the differences in the stabilization may be an effect of the developmental stage of the seedlings. The half-life under blue light was also the same during the subjective day and night. In the dark, the

half-life of PRR7 was calculated to be 2.7 and 2.2 hours at ZT4 and ZT12, respectively. The half-life at ZT12 in the dark is similar to that previously reported of 2.4 hours (Farre & Kay, 2007). Red light in the subjective night extended the half-life by approximately one hour relative to the subjective day (4.2 hours and 3.4 hours, respectively). Therefore under the red light conditions used, PRR7 half-life was calculated to be nearly double the half-life in darkness at ZT12 (Figure 10B). Therefore my results suggest that PRR7 may be stabilized by red light in the evening prior to sun down. This effect could be a way to convey timing information to the oscillator.

PRR7 does not appear to be targeted for degradation by ZTL (Fujiwara, et al., 2008). Blue light stabilizes TOC1 and PRR5 by inhibiting their interaction with ZTL (Fujiwara et al., 2008; Kim, et al., 2003). Other members of the ZTL family include FKF1 and LKP2. These proteins have been shown to also contribute to the regulation of TOC1 and PRR5, particularly in the absence of ZTL (Baudry, et al., 2010). FKF1 also mediates blue light dependent protein interactions indicating that blue light might stabilize all the targets of this protein family. Since blue light did not stabilize PRR7 protein (Figure 11), it suggests neither ZTL or other members of the ZTL family are likely candidates for mediating the stability of PRR7. In contrast, the present study suggests it may instead be regulated by red light. The effect of red light on PRR7 stability could be mediated by the phytochromes.

The phytochromes have been shown to mediate the light-dependent stability of other proteins, particularly proteins that function in red light signaling. Positive regulators of light signaling, including HY5, HFR1, FHY1, and LAF1, are down regulated in the dark in a phytochrome-dependent manner (Osterlund, et al., 2000; Jang, et al., 2005; Shen, et al., 2005; Seo, et al., 2003). The exact mechanism for the phytochrome-dependent stabilization of proteins in the light is unknown. Each of these proteins is targeted for degradation by COP1, an E3-ubiquitin ligase that functions prominently in light signaling (Subramanian, et al., 2004). In the dark, COP1 is localized to the nucleus. Most of the COP1 pool is exported to the cytoplasm in the light (von Arnim, et al., 1998). Upon blue light absorption cryptochromes interact with COP1 in the nucleus and the sequestered COP1 is no longer able to target proteins for degradation (Zuo, et al., 2011; Liu, et al., 2011). The phytochromes may regulate protein stability in a similar manner by repressing COP1 activity in the light.

Since COP1 targets proteins for degradation in the dark it could be a potential PRR7 regulator. However, no interaction between COP1 protein and PRR7 protein has been demonstrated to date. PRR7 protein levels have also not been examined in *cop1* mutants. COP1 does not have an effect on PRR9 protein levels (Ito, et al., 2007); PRR7 and PRR9 are very similar proteins, and so COP1 may be an unlikely candidate in mediating PRR7 degradation. To test if COP1 is involved in the degradation of PRR7 protein would require a line expressing a tagged PRR7 protein in a *cop1* mutant background. There is currently no PRR7-specific antibody available, and thus a tagged protein is required for PRR7 protein detection. If COP1 were responsible for targeting

PRR7 for degradation, PRR7 would be expected to accumulate in the *cop1* mutant.

To confirm whether different qualities of light affect the stability and abundance of PRR7 protein, it would be informative to test how different photoreceptors affect protein oscillation. Photoreceptors mediate the stability of several proteins in a light-dependent manner (Kim, et al., 2003; Shen, et al., 2005; Shen, et al., 2007; Shen, et al., 2008). Under constant white light, PRR7 protein cycles robustly under the control of the native promoter as well as the constitutive 35S promoter. I have crossed the *prr7-3 35S::PRR7-LUC* line with phytochrome and cryptochrome mutants and homozygous lines are currently being selected. These lines can then be used to test if the PRR7-LUC protein oscillations change in different photoreceptor mutant backgrounds by determining protein levels by Western blot. It may also be possible to use these lines to measure the stability of the PRR7-LUC protein by luciferase activity by further refining the luciferase assay to improve reproducibility.

Post-transcriptional regulation of the circadian clock contributes to the robustness of the oscillator by defining the peak of the expression of clock proteins. Studying how these proteins are regulated is important for understanding how the clock functions and regulates subsequent output processes. Unlike PRR5 and TOC1, PRR7 does not appear to be regulated by blue light. Instead, it appears that red light may play a specific role in mediating PRR7 stability. Additional experiments to further characterize the effect of red light on PRR7 protein levels will need to be performed. These include

identifying which specific photoreceptor(s) play a role in regulating PRR7 abundance and how PRR7 is targeted for degradation by the proteasome.

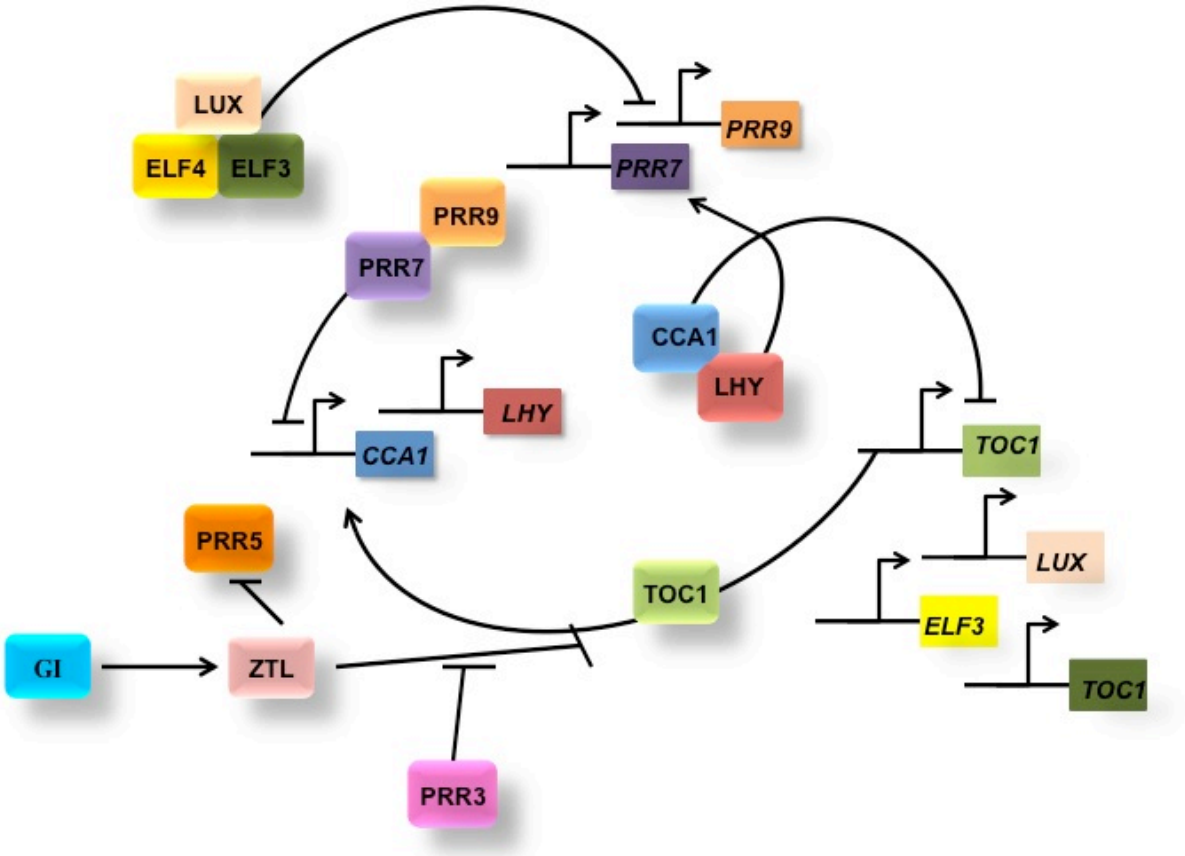
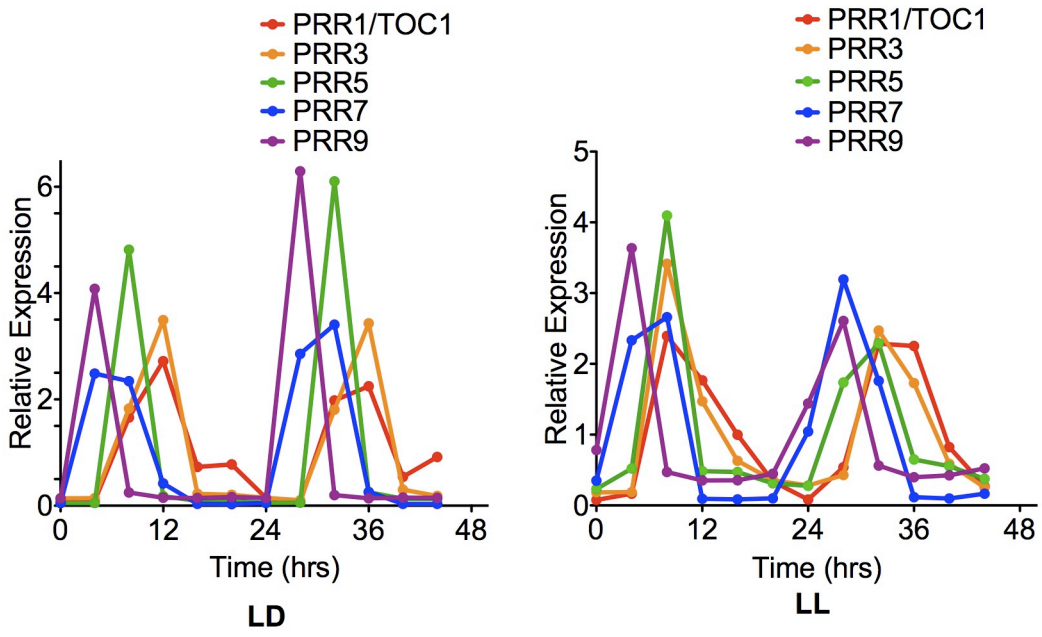


Figure 1. Overview of the circadian oscillator in *A. thaliana*. The central loop is comprised of the MYB-domain transcription factors CCA1 and LHY that activate transcription of *PRR9* and *PRR7*. PRR9 and PRR7, as well as PRR5, PRR3, and TOC1 sequentially repress the transcription of *CCA1* and *LHY*. CCA1 and LHY repress the transcription of *TOC1* and other evening genes such as *ELF3* and *LUX* during the day. ZTL, an F-box protein that functions as part of the SCF-E3-ubiquitin ligase, targets PRR5 and TOC1 for degradation. PRR3 prevents ZTL binding to TOC1 and stabilizes TOC1 protein. ZTL protein stability is dependent on GI. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

Figure 2. A, B

A



B

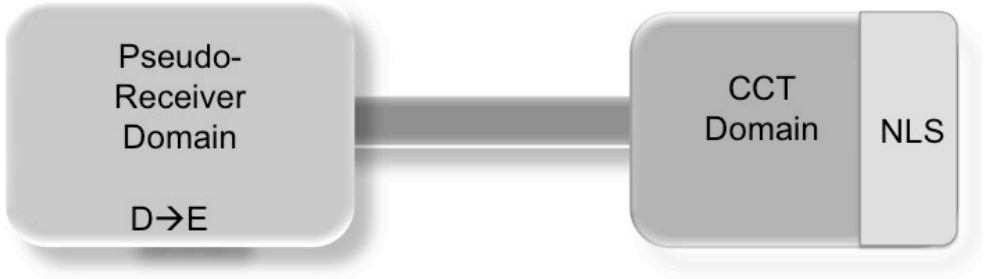


Figure 2. A, Expression of the circadian PRRs. The expression of the circadian PRR quintet cycles with a period of 24 hours under light/dark cycles (LD) as well as constant light (LL). Data were obtained from the Diurnal Project (www.diurnal.mockerlab.org), (Mockler, et al., 2007, Michael, et al., 2008). *PRR9* expression peaks early in the morning, followed by *PRR7*, *PRR5*, *PRR3*, and *TOC1*/*PRR1* in the evening. **B, The conserved domains of the circadian PRR proteins**. At the amino terminus, the pseudo-receiver domain resembles the receiver domain from canonical two-component response regulator proteins. The pseudo-receiver domain lacks the conserved aspartate residue that functions in the phospho-relay system of the response regulators. The carboxy-terminal CONSTANTS/CONSTANTSLIKE/TOC1 CCT motif is conserved among the PRRs, as well as the floral regulatory protein CONSTANTS. The carboxy-terminus also contains a putative nuclear localiztion signal (NLS).

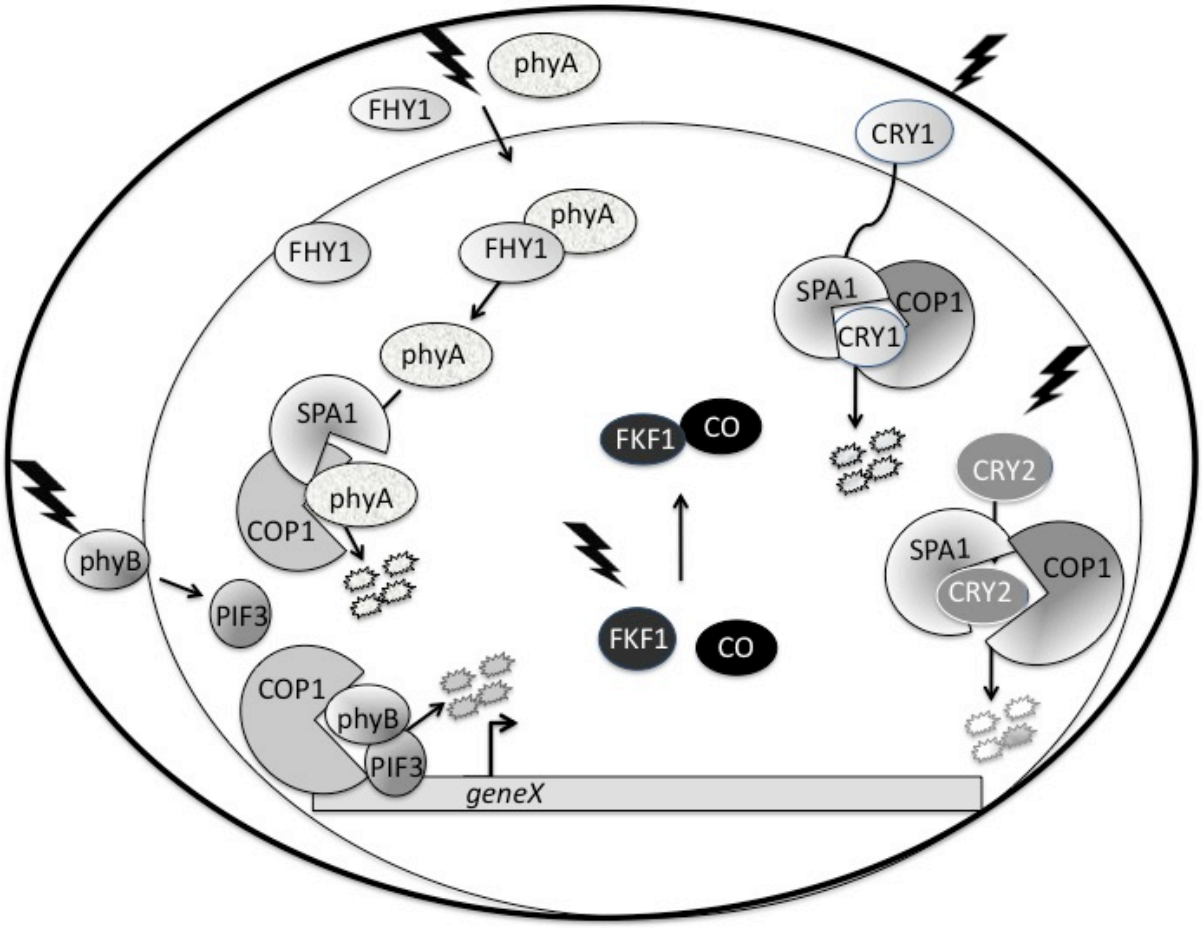


Figure 3. Examples of light-regulated proteolysis in *A. thaliana*. The photoreceptors phytochromes and cryptochromes are activated by red and blue light, respectively. Activated photoreceptors undergo a conformational change before being translocated into the nucleus, except CRY2, which is constitutively nuclear. phyA requires FHY1 and related proteins to enable it to enter the nucleus. phyB seems to require PIF3 for nuclear import. In the nucleus, activated photoreceptors interact with COP1, an E3-ubiquitin ligase that targets proteins for protesomal degradation. The interaction of photoreceptors with complexes of COP1 stabilize other proteins that function

downstream in light signaling. For example cryptochromes interact with COP1 to help stabilize CO protein and induce flowering in response to blue light. This process is also governed by FKF1, which interacts with CO in a blue light-dependent manner to stabilize the protein. Activated phytochromes and cryptochromes are targeted for degradadation by COP1.

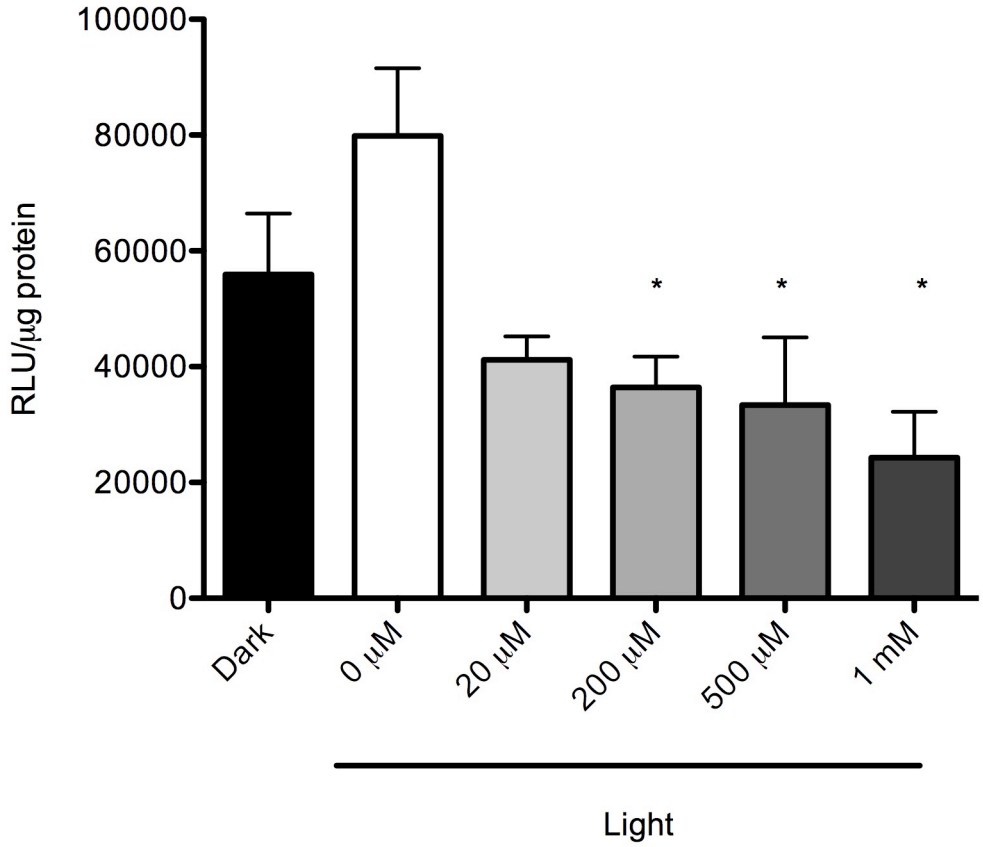


Figure 4. Optimization of cycloheximide treatment in the light. The *PRR9::LUC* line was used to test the effectiveness of different cycloheximide treatments for inhibiting translation in the light. The *PRR9* promoter is light-inducible. Seedlings were grown for one week in 12:12 light/dark cycles. At ZT0, seedlings were transferred to media with Triton X-100 and varying concentrations of cycloheximide in darkness before being transferred to light for four hours. Luciferase activity was compared to samples not treated with cycloheximide. * Indicates statistically significant by the student's t-test

($p = 0.0212, 0.0476, 0.0311$ for 200 μM, 500 μM, and 1 mM respectively). Each time point represents the average of three biological replicates and error bars represent the standard error for the mean (SEM).

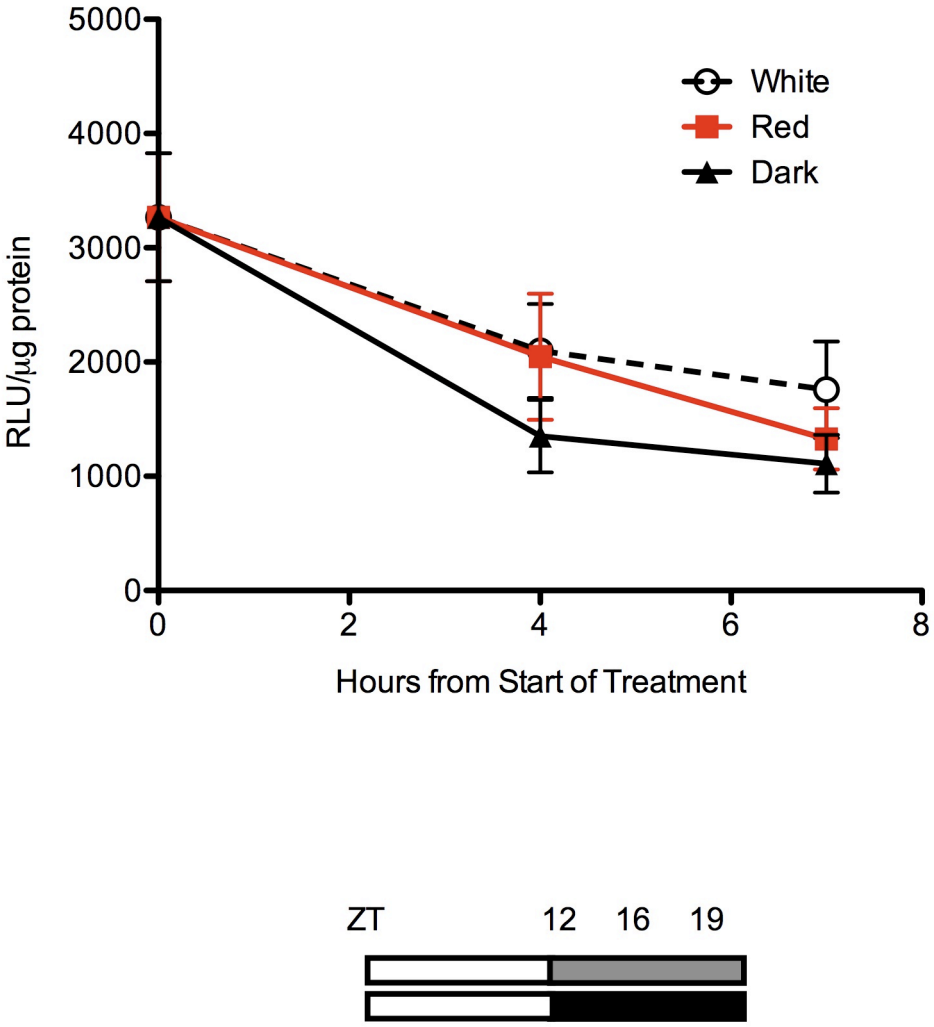


Figure 5. PRR7-LUC protein stability under different light conditions. *35S::PRR7-LUC-HA* seedlings were treated with 500 μM cycloheximide and 0.01% Triton X-100 and transferred to white light (70 μmol/m^2/sec^1), red light (70 μmol/m^2/sec^1) and darkness at ZT 12 for the times indicated. The black bar represents darkness and the gray bar represents white or red light. Time indicates hours since the start of the

cycloheximide treatment at the indicated ZT time. Luciferase activity was measured using the Bright Glo kit from Promega. Relative light units were normalized to protein concentration as determined by Bradford Assay. The data represent the average of 5 experiments under each light conditions and the error bars represent the standard error for the mean (SEM).

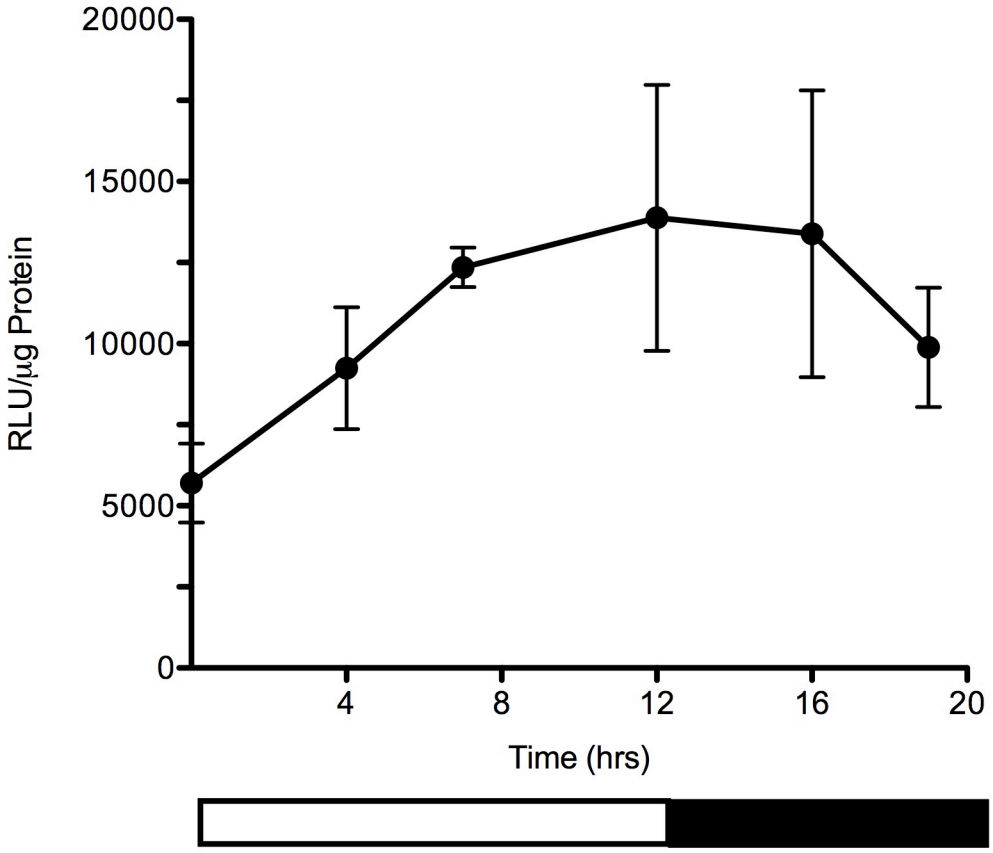


Figure 6. Luciferase activity of the *prr7-3 35S::PRR7-LUC-HA* line. Seedlings were grown for one week under 12:12 light/dark cycles. Samples were collected in triplicate at each time point. Day and night are represented by the white and black bars, respectively. Luciferase activity was assayed using the Bright Glo kit from Promega. Relative Light Units (RLU) were normalized to the protein concentration in each sample as determined by Bradford Assay. Error bars represent the standard error for the mean (SEM).

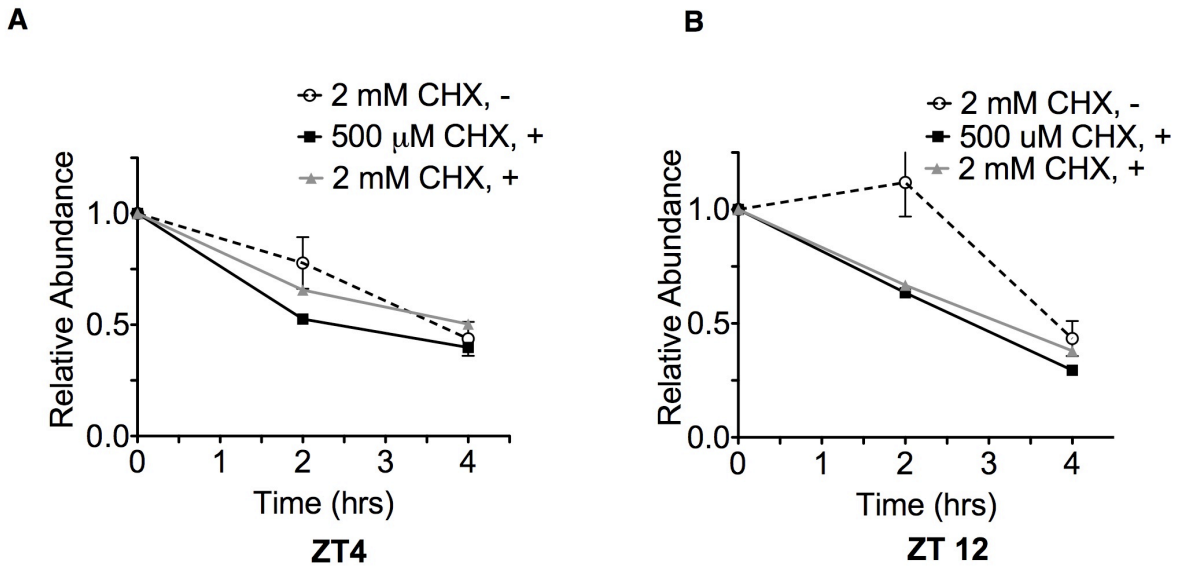
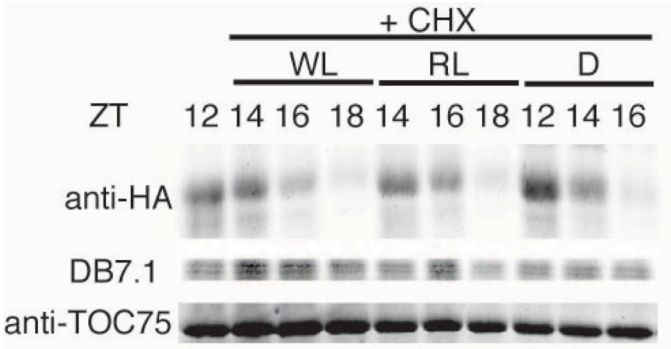


Figure 7. Optimization of cycloheximide treatment in darkness. The *PRR7::HA-PRR7* line was used to test if treating seedlings briefly in light before transfer to darkness affected the ability of cycloheximide to inhibit translation due to stomata closing. Seedlings were transferred to media with Triton X-100 and cycloheximide and directly transferred to darkness (-) or subjected to a 15 minute light treatment prior to being transferred to darkness (+). Samples were taken at 2 and 4 hours after the beginning of the treatment to evaluate the ability of cycloheximide to enter tissue and inhibit translation in the dark. Panel A and B show degradation at ZT4 and ZT12, respectively. For comparison, samples not pre-treated in the light ((-), n = 8) are shown (dashed line). Error bars represent the standard error for the mean (SEM). The experiment with the light pre-treatment was performed once.

A



B

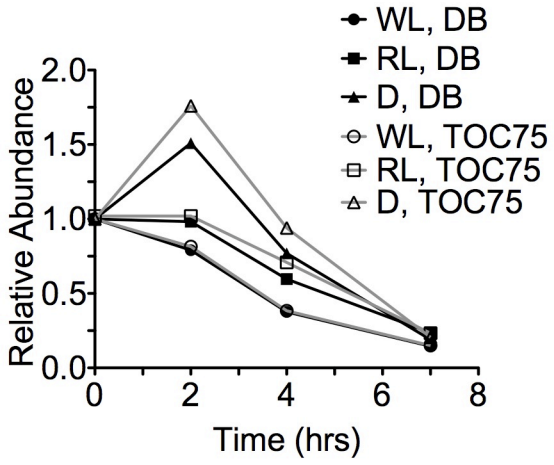


Figure 8. Comparison of DB7.1 staining and TOC75 detection as loading controls.

A, blot for PRR7 degradation at ZT12, comparison of DB7.1 staining and TOC75 detection for protein quantitation. Membranes probed with anti-HA antibody were stripped and re-probed with anti-TOC75 antibody. The same membranes were stained with DB7.1 stain for total protein. **B**,panel, quantification of western blot signals using Metamorph image analysis software. Similar results were obtained in other experiments.

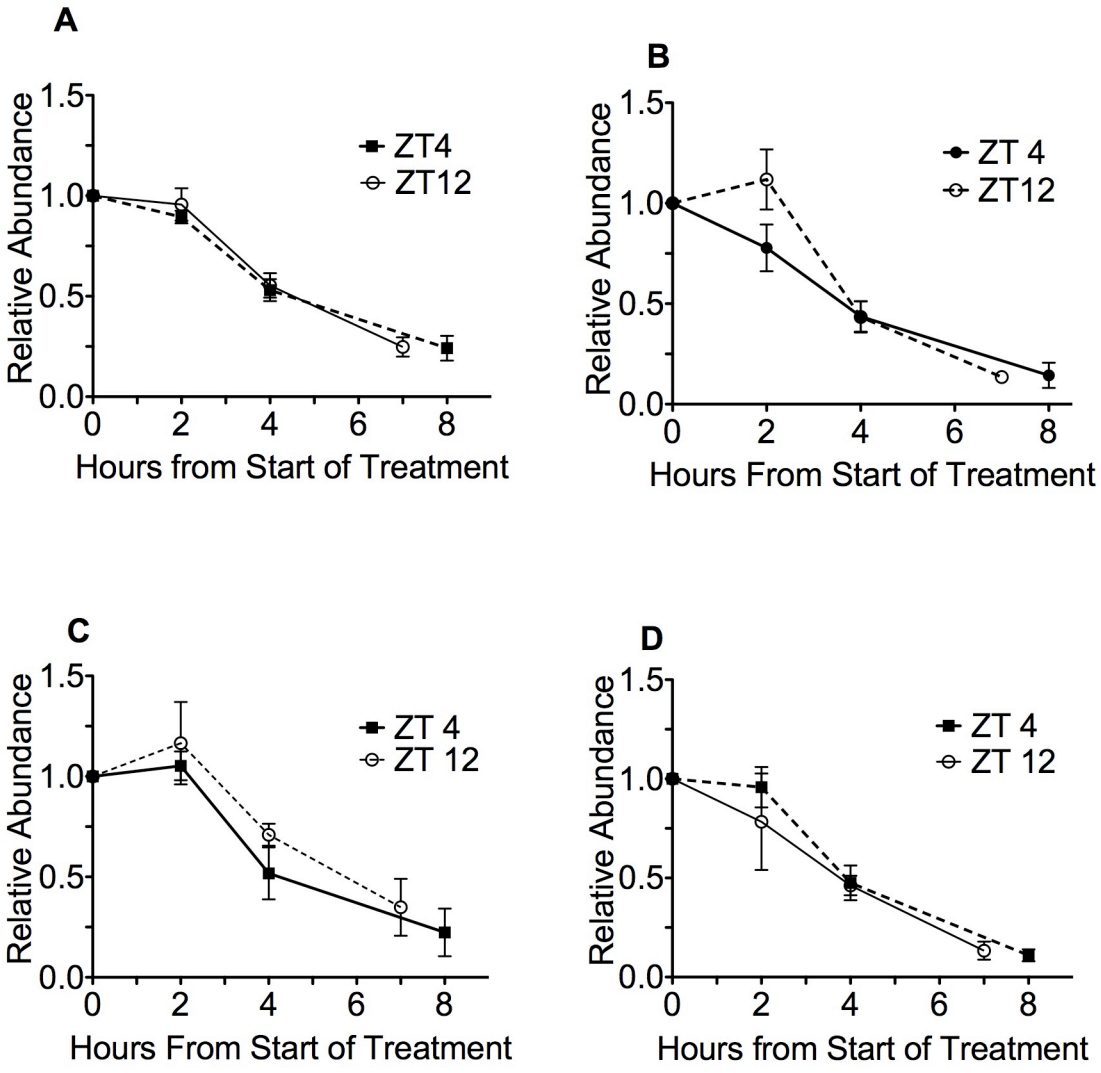


Figure 9. Comparisons of HA-PRR7 protein degradation rates during the subjective day and subjective night. **A**, White light; **B**, dark; **C**, red light; **D**, blue light. Data are from Figures 10 and 11. The values represent the average of 8 for dark, 9 for white light, 4 for red light, 3 for blue light independent experiments Error bars represent the standard error for the mean (SEM).

Figure 10. A, B

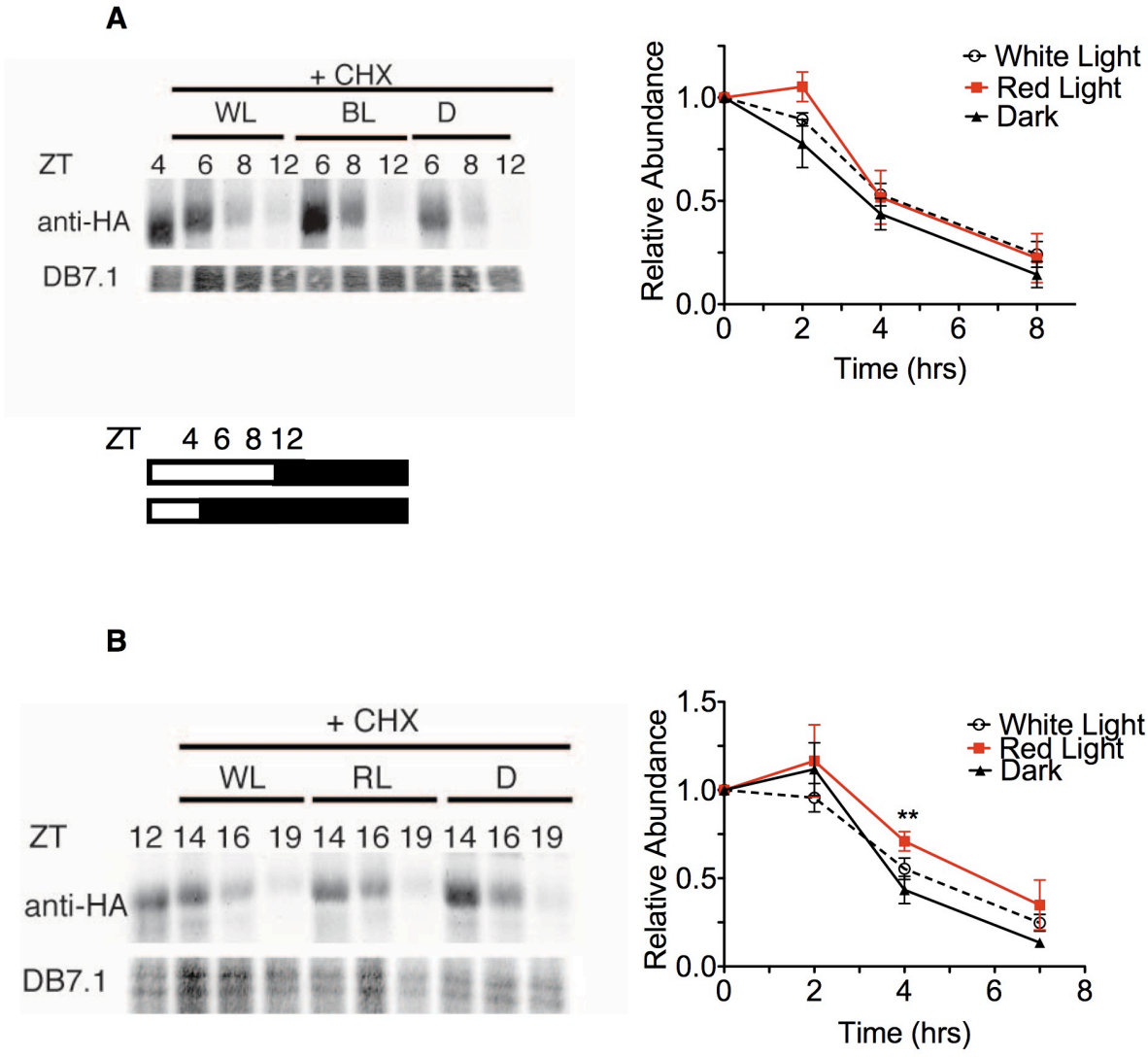
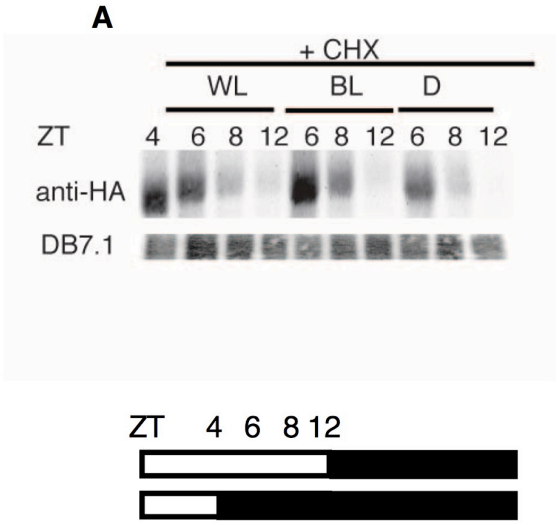

A
+ CHX
WL
BL
D
ZT 4 6 8 12 6 8 12 6 8 12
anti-HA
DB7.1
ZT 4 6 8 12
Relative Abundance
White Light
Red Light
Dark
Time (hrs)
B
+ CHX
WL
RL
D
ZT 12 14 16 19 14 16 19 14 16 19
anti-HA
DB7.1
ZT 12 14 16 19
Relative Abundance
White Light
Red Light
Dark
**
Time (hrs)

Figure 10. **Analysis of the stability of HA-PRR7 protein evaluated by Western blot under red light**. One week-old *prr7-3 PRR7::HA-PRR7* seedlings entrained under 12:12 light/dark cycles were transferred to MS media with 2 mM cycloheximide and 0.01% Triton X-100 before being transferred to white light (70 μmol/m^2/sec^1), red light (70 μmol/m^2/sec^1) or darkness at ZT4 (A) or ZT12 (B). Time indicates hours since the start of the cycloheximide treatment at the indicated ZT time. Protein levels were quantified by western blot using an anti-HA antibody. Graphs represent the average of 4 for red light, 9 for dark, and 10 for white light independent experiments and error bars represent the standard error for the mean (SEM). * Indicates statistically significant (student's t-test, p = 0.047). The blots are from one representative experiment.

Figure 11. A, B


A
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4 6 8 12 6 8 12 6 8 12
anti-HA
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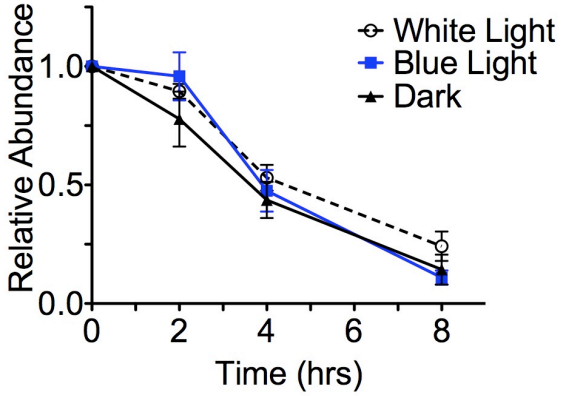
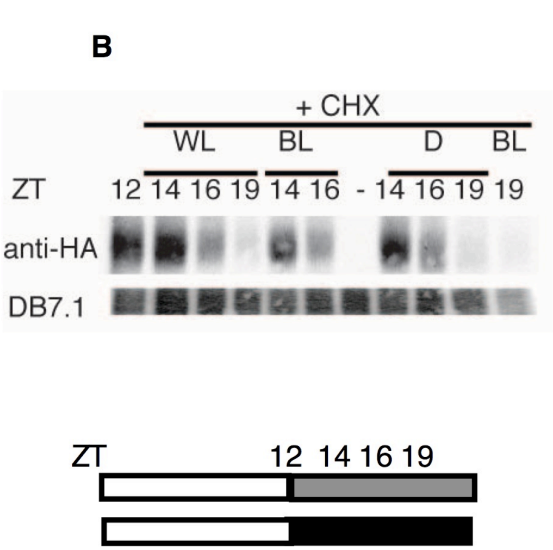

White Light
Blue Light
Dark
Relative Abundance
1.0
0.5
0.0
0 2 4 6 8
Time (hrs)


B
+ CHX
WL
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12 14 16 19 14 16 - 14 16 19 19
anti-HA
DB7.1
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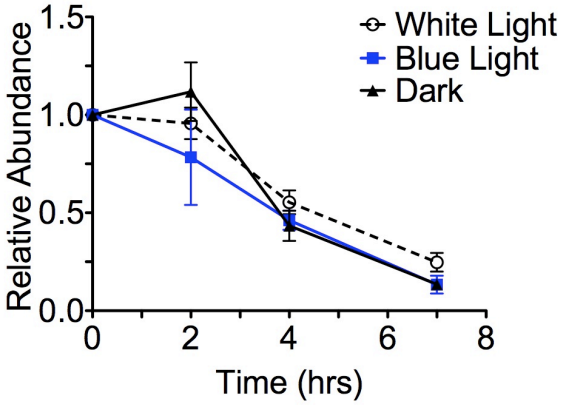
White Light
Blue Light
Dark
Relative Abundance
1.5
1.0
0.5
0.0
0 2 4 6 8
Time (hrs)

Figure 11. Analysis of the stability of HA-PRR7 protein evaluated by Western blot under blue light . Western blot of seedlings treated with white light (70 μmol/m^2/sec^1), blue light (30 μmol/m^2/sec^1), and darkness. One week-old *prr7-3 PRR7::HA-PRR7* seedlings entrained under 12:12 light/dark cycles were transferred to MS media with 2 mM cycloheximide and 0.01% Triton X-100 before being transferred to different light conditions at ZT4 (A) or ZT12 (B). Time indicates hours since the start of the cycloheximide treatment at the indicated ZT time. Graphs represent the average of 3 for blue light, 10 for white light, and 9 for dark independent experiments. – Indicates a misloaded negative control; the four hour time-point for blue light is the last lane labeled "19". Error bars represent the standard error for the mean (SEM).

	White Light	Red Light	Blue Light	Darkness
ZT4	3.5 hrs	3.4 hrs	2.4 hrs	2.8 hrs
ZT12	3.1 hrs	4.2 hrs	2.4 hrs	2.2 hrs

Table 1. **Half-life of PRR7 Under Different Light Conditions**. Half-life (in hours) of PRR7 under white light (70 μmol/m^2/sec^1), red light (70 μmol/m^2/sec^1), blue light (30μ mol/m^2/sec^1), and darkness. Degradation curves in Figures 10 and 11 were fit with the first order decay function. Half-life was calculated from the decay constant *k* using ln(*k*)/2 = t ½.

Materials and Methods

Plant material

The *Arabidopsis prr7-3* T-DNA line in the Col-0 accession has been described previously (Farre et al., 2005). The lines *PRR7::HAPRR7* and *35S::PRR7LUC-HA* in the *prr7-3* background have been described previously (Farre & Kay, 2007). The *PRR9::LUC* construct has been described previously (Para et al., 2007). Plants were grown on Murashige and Skoog media (Murashige & Skoog, 1962) supplemented with 0.8% agar and 2% sucrose. Seedlings were sterilized by gas sterilization and stratified at 4°C in darkness for 3-7 days before being grown for 7-9 days under 12:12 white light (70 μmol m^{-2} sec^{-2})/dark cycles at 22°C in Percival Environmental Chambers (Percival Scientific).

Cycloheximide Treatment Optimization

For determining the effectiveness of the cycloheximide treatment in white light, PRR9*::LUC* seedlings were grown as described above. Murashige and Skoog media supplemented with 0.01% Triton X-100 and either no cycloheximide, 20 μM, 200 μM, 500 μM, and 1 mM cycloheximide were prepared and aliquoted into Costar 24 well culture dishes (Fisher Scientific). Seven-day old *PRR9::LUC* seedlings grown under 12L:12D cycles were transferred to the media in the dark at ZT0 and immediately transferred to white light (70 μmol m^{-2} sec^{-2}). Seedlings were collected at ZT4, patted

dry, and flash frozen in liquid nitrogen. Luciferase activity was measured in these samples using the Bright Glo Luciferase Assay Kit from Promega as described below.

To test the effectiveness of cycloheximide treatment in the dark, seven-day-old *PRR7::HAPRR7* seedlings were transferred at ZT4 and ZT12 to Murashige and Skoog media supplemented with 0.01% Triton X-100 and 500 μM or 2 mM cycloheximide. Seedlings were incubated in the light for 10-15 minutes in white light before being transferred to darkness. Effectiveness of the treatment was determined by quantifying relative HA-PRR7 protein levels in the dark two and four hours after transfer (see below for extraction and blotting procedure).

Cycloheximide Treatment Under Different Light Conditions

Seedlings were transferred to Murashige and Skoog liquid media supplemented with 2 mM cycloheximide (Sigma) and 0.01% Triton X-100 (Sigma) in Costar 6-well culture dishes (Fisher Scientific) before being transferred to their respective light conditions. Light intensity for red and blue light was determined using a LI-COR 250A light meter (LI-COR Biosciences). 397 Pale Grey Roscolux filters (Premier Lighting) were used to adjust light intensity (Mazzoni, et al., 2005). Seedlings (15-20) treated with cycloheximide under different light conditions were collected at 2 hours, 4 hours, and 7 or 8 hours after the beginning of the light treatment. Plant material was patted dry and flash frozen in liquid nitrogen.

Protein Analysis by Western Blot

To prepare samples for analysis by Western blotting, frozen plant tissue was ground for 2.5 minutes at 30 Hz using a ball mill (Qiagen). Plant extracts were prepared by adding 50-75 µL of extraction buffer [100 mM sodium phosphate buffer, 150 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 0.1% Triton X-100, 10% glycerol, 3 µM dithithreitol, 50 uM MG132, Roche Protease Inhibitor, 5 mM Benzamidine, 1 mM PMSF, 0.5% Deoxycholate, pH 8.0, 0.1% sodiumdodecylsulfate] to each sample and immediately vortexing. Extracts were clarified by centrifugation at 14,000 x g, transferring the supernatant to a new tube, and repeating the centrifugation. Protein concentrations were determined using the BioRad Bradford Assay (BioRad). Protein standards were prepared in a 1:10 dilution of extraction buffer to account for the background signal caused by reducing agents in the extraction buffer in the samples. All extracts were diluted 1:10 in water before determining their protein concentration. Samples were mixed with sodium dodecyl sulfate loading buffer (final concentrations 25 mM Tris-HCl, pH 6.8, 2% SDS, 0.12% Bromophenolblue, 10% Glycerol) before incubating at 37°C for 2 minutes prior to loading. 80-150 µg of protein were loaded per lane on a 7% acrylamide/bisacrylamide gel run in the BioRad Mini-PROTEAN Tetra Cell System (Biorad).

Acrylamide gels were transferred to 0.22 µm NitroPure nitrocellulose membrane (GE Healthsciences, WP2HY00010) at 18 V overnight using the BioRad Criterion Blotter. Membranes were blocked with 5% dry milk dissolved in TBST [50 mM Tris, pH

7.5, 150 mM sodium chloride, 0.05% Tween-20 (Sigma)] for 30 minutes at room temperature, then treated with a 1:1,000 α-HA-HRP antibody (Anti-HA-Peroxidase from Mouse IgG, Roche) diluted in 5% milk in TBST at room temperature for one hour. Membranes were washed briefly 3 times, then three times for 5 minutes, and twice for 10 minutes with TBST. Peroxidase activity was detected using SuperSignal West Femto Chemiluminescent Substrate or SuperSignal West Pico Chemiluminescent Substrate (Pierce) at different ratios as needed for adequate detection. Blots were visualized using the BioRad VersaDoc 4000 MP (BioRad) Chemiluminescent Ultra Sensitivity in the Quantity One image acquisition software. Direct-Blue 71 (Sigma) staining was used for normalization to loading controls (Hong, et al., 2000). Images were quantified using the Metamorph image analysis software (Molecular Devices, LLC.). The HA-PRR7 signal was normalized to the corresponding region on the DB7.1 stained membrane before normalizing all time-points to the signal measured at the beginning of the treatment.

TOC75 detection

Membranes previously probed with α-HA-HRP antibody were first stripped using the Restore Western Stripping Buffer (Thermo Scientific) and subsequently blocked with 5% BSA dissolved in TBST. The membranes were probed with rabbit α-TOC75 (1:2,500) in TBST-BSA for 1 hour at room temperature or overnight at 4°C. Membranes were then washed with TBST and blocked with dry milk in TBST as described above. Goat α-Rabbit-HRP (Roche) was diluted 1:10,000 in TBST -5% dry milk and membranes were incubated at room temperature for one hour. Blots were visualized as described above

Analysis of Luciferase Activity

For Luciferase assays, frozen seedlings were ground as described for Western Blotting. 1X Cell Culture Lysis Reagent (CCLR, Promega) was prepared at room temperature supplemented with 3 μM dithiothreitol, 50 μM MG132, Roche Protease Inhibitor, 5 mM Benzamidine, 1 mM PMSF, 180 nM Epoxomycin, and 1X Phosphatase Inhibitor Cocktails I and III (Sigma). 150 μL of modified CCLR was added to each ground sample and vortexed. Samples were centrifuged for 10 minutes at 14,000 X g, the supernatant transferred, and centrifuged a second time. Clarified extracts were diluted in modified 1X CCLR. 10 μL of diluted extracts were transferred to a 96-well plate. Room temperature Bright Glo Assay Buffer (Promega) was added to each well and luminescence was measured for 2-8 seconds. Relative Light Units (RLU) were normalized to the protein content in the diluted extract as determined by Bradford Assay (Biorad) using an identical dilution of modified CCLR for diluting the standards to account for buffer background signal.

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