Life-times of Arabidopsis cryptochrome signaling states *in vivo*

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SUMMARY

One crucial component in light signaling is the amount of photoreceptor present in the active signaling state. The life-time of the signaling state of a photoreceptor is limited due to thermal or otherwise back reversion of the chromophore to the ground state and/or degradation of the photoreceptor in the light-activated state. It was previously shown that the lit state of plant cryptochromes contains flavin neutral semiquinone, and half-lives of the lit state in the range of 3-4 minutes were determined *in vitro*. However, it was unknown how long-lived the signaling states of plant cryptochromes are *in situ*. Based on loss of degradation of cry2 after prolonged dark incubation and loss of reversibility of photoactivated cry1 by a pulse of green light we estimate the *in vivo* half-lives of the signaling states of cry1 and cry2 to be in the range of 5 min and 16 min, respectively. Based on EPR-measurements, the life-time of the *Arabidopsis* cry1 lit state in insect cells was found to be ~ 6 min and thus very similar to the life-time of the signaling state *in planta.* Thus, the signaling state life-times of plant cryptochromes are not or only moderately stabilized *in planta*.

INTRODUCTION

Plants adapt to their light environment by utilizing a large number of photoreceptors. The UV-A/blue light region is monitored by cryptochromes (Liu *et al.*, 2010; Chaves *et al.*, 2011), phototropins and members of the Zeitlupe family (Briggs and Christie, 2002; Demarsy and Fankhauser, 2009); phytochromes operate in the red and far-red region of the light spectrum (Rockwell *et al.*, 2006). The WD-40/ß-propeller protein UVR8 has been identified as a specific UV-B receptor (Rizzini *et al.*, 2001; Christie *et*

al., 2012; Wu *et al.*, 2012). With the exception of the UV-B receptor all other photoreceptors are encoded by small gene families in *Arabidopsis thaliana.* Together, these photoreceptors enable the plant to utilize light in an optimal way for photosynthesis and to avoid light damage.

Cryptochromes including cry1 and cry2 from *Arabidopsis thaliana* are UV-A/blue light photoreceptors that regulate plant photomorphogenesis. The *cry1* mutant shows a severe phenotype in seedling development specifically under blue light, resulting in elongated hypocotyls (Ahmad and Cashmore, 1993) and strongly decreased formation of anthocyanins (Ahmad *et al.*, 1995; Jackson and Jenkins, 1995) as a result of lack of transcriptional induction of flavonoid and anthocyanin biosynthetic genes such as chalcone synthase (*CHS*) (Christie and Jenkins, 1996; Fuglevand *et al.*, 1996). cry2, the second cryptochrome in *Arabidopsis* has a prominent role in photoperiodic flowering under long-day conditions (Guo *et al.*, 1998; El-Assal *et al.*, 2001), but only a minor function in deetiolation (Lin *et al.*, 1998). In contrast to cry1, cry2 is light-labile at least during the transition of seedlings from darkness to blue light with strong and rapid degradation at fluence rates above 1 µmole m^{-2} s^{-1} (Ahmad *et al.*, 1998; Lin *et al.*, 1998; Yu *et al.*, 2007; Weidler *et al.*, 2012; Zuo *et al.*, 2012). A third cryptochrome-like protein (cry3) exists in *Arabidopsis* that belongs to the so called cryDASH family (Brudler *et al.*, 2003; Kleine *et al.*, 2003). cry3 has repair activity for UV-B lesions (cyclobutane pyrimidine dimers) in single-stranded DNA (Selby and Sancar, 2006) and loop structures of duplex DNA (Pokorny *et al.*, 2008). Cryptochromes exist in plants, animals, fungi and bacteria (Chaves *et al.*, 2011) and are related to DNA-photolyases that repair UV-lesions in DNA using photoexcited and fully reduced FADH^{-*} as catalytic chromophore (Sancar, 2003). Like DNA-photolyases, cryptochromes contain FAD as chromophore but normally lack DNA-repair activity. They behave in a similar way as DNA-photolyase

to photoreduce FAD. However, plant cryptochromes accumulate the neutral FAD semiquinone (FADH^o) instead of fully reduced FADH$^-$. This form of the photoreceptor is believed to represent the lit state (Chaves *et al.*, 2011). For animal cryptochromes, it has likewise been postulated that the oxidized flavin represents the ground state based on direct *in vivo* measurements of radical accumulation in response to blue light (Hoang *et al.*, 2008), whereas other workers postulate the photoexcited anionic radical (FAD$^{\cdot -}$*) represents the lit state (Ozturk *et al.*, 2011). Upon irradiation, phosphorylation of Arabidopsis cry1 and cry2 *in planta* has been detected within minutes. This phosphorylation *in planta* was only observed under blue light illumination and does not depend on the presence of phytochromes (Shalitin *et al.*, 2002; Shalitin *et al.*, 2003). The cryptochrome C-termini (CCT) are targets for phosphorylation and are constitutively phosphorylated when expressed in plants without the N-terminal photosensory domain (Shalitin *et al.*, 2002). CCT overexpression causes a constitutive photomorphogenic phenotype (Yang *et al.*, 2000). Thus, only the phosphorylated form of cryptochrome is biologically active. Moreover, structural changes in plant cryptochromes upon photoexcitation were monitored (Partch *et al.*, 2005; Kondoh *et al.*, 2011). Taken together, formation of the lit state is the prerequisite to form the signaling state which is distinguished from the lit state by further biochemical and structural alterations. In case of cry2, phosphorylation is probably also the initial signal for proteasome-mediated degradation which occurs in the nucleus (Shalitin *et al.*, 2002; Yu *et al.*, 2007; Yu *et al.*, 2009; Weidler *et al.*, 2012; Zuo *et al.*, 2012). When *Arabidopsis* plants are transferred from blue light to darkness, dephosphorylation of cry1 and cry2 is observed with essentially no phosphorylated photoreceptors left after about 15 minutes (Shalitin *et al.*, 2002; Shalitin *et al.*, 2003).

Cryptochromes interact with other proteins such as the E3 ubiquitin-ligase COP1 (Wang *et al.*, 2001; Yang *et al.*, 2001), the bHLH transcription factor CIB1 (Liu *et al.*, 2008) and the members of the COP1-related SPA family (Lian *et al.*, 2011; Liu *et al.*, 2011; Zuo *et al.*, 2011; Weidler *et al.*, 2012). Interaction of cry2 with CIB1 and of cry1 and cry2 with members of the SPA quartet is blue light-induced (Liu *et al.*, 2008; Lian *et al.*, 2011; Liu *et al.*, 2011; Zuo *et al.*, 2011). Initially, the interaction of COP1 with cryptochromes was thought to be independent of light. Recent data showed, however, that cry2 binds COP1 more strongly under blue light in the presence of SPA1, whereas the SPA1-COP1 interaction is alleviated by cry1 under blue light (Liu *et al.*, 2011; Zuo *et al.*, 2011). Since cry2 interacts with its photosensory N-terminal domain with CIB1 and SPA1 (Kennedy *et al.*, 2010; Zuo *et al.*, 2011), this domain is not only important for signal perception but also for signal transduction.

The life-time of a photoreceptor in its signaling state determines the pool size of active photoreceptor and thus the extent of a photoresponse. For phytochromes the fraction of physiologically active Pfr to Ptot depends on the fluence rate and the spectral composition of the light because of light-induced photoconversion of the inactive Pr-form that has peak absorption at around 666 nm to Pfr, and back conversion of Pfr to Pr by photoexcitation of Pfr with peak absorption at 730 nm (Rockwell *et al.*, 2006). Furthermore, thermal conversion of the 15E isomer of the phytochromobilin chromophore in the Pfr-state to the 15Z isomer in the Pr-state reduces the amount of phytochrome in its signaling state. This process is also known as dark-reversion with half-lives of the PfrPr heterodimer of 19 min for phyA and 3 min for phyB determined for the recombinant Arabidopsis receptors *in vitro* at 4°C (Eichenberg *et al.*, 2000). Notably, the half-lives of PfrPr heterodimers can differ strongly from those of the PfrPfr homodimers. For example, the dark-reversion of the

Arabidopsis phyB PfrPfr homodimer has a half-life of >12 h in contrast to the 3 min of the heterodimer (Eichenberg *et al.*, 2000). The signaling state of phyB is stabilized by interaction with the response regulator 4 (ARR4) (Sweere *et al.*, 2001). Therefore, the measured life-time of the lit state of a photoreceptor *in vitro* or in a heterologous expression system does not necessarily reflect its real life-time *in situ*. Furthermore, the concentration of a photoreceptor's signaling state can be regulated by protein degradation. Well-defined examples in plants are phyA with a half-life of about 22 h in dark-grown seedlings and of 30 min in red light-treated seedlings (Hennig *et al.*, 1999; Hennig *et al.*, 2000), and cry2 as mentioned above.

The half-life of the semireduced FADH^o in plant cryptochromes, which represents the lit state is in the range of 3-4 min as determined *in vitro* for insect cell-expressed cry1 and cry2 (Banerjee *et al.*, 2007; Bouly *et al.*, 2007; Müller and Ahmad, 2011). However, essentially no data exist on lifetimes of the signaling states of cryptochromes *in situ*. The only example is *Drosophila melanogaster* cry, which is rapidly degraded *in vivo* upon photoexcitation, like Arabidopsis cry2 (Koh *et al.*, 2006). Based on the kinetics of decay a half-life of ~ 27 min was calculated for its signaling state (Ozturk *et al.*, 2011). Physiological studies on UV-B-induced chalcone synthase (*CHS*) induction in parsley cell cultures showed that a single pulse of blue light enhances this process strongly. This effect of blue light was very long lasting for at least 20 h (Ohl *et al.*, 1989). These data suggest that either a stable signaling component is formed by the action of a cryptochrome-type photoreceptor that enhances UV-B signaling or that the signaling state of this cryptochrome photoreceptor is very stable *in situ*. To discriminate between these possibilities and to obtain reliable data on the life-span of the signaling states of plant cryptochromes *in situ* we studied the decay time of the signaling state of Arabidopsis cry2 by measuring the degradable fraction of photoexcited cry2 in living Arabidopsis cells

after various dark periods on ice, implying that these conditions allow normal photochemistry but blocked biochemistry. These studies revealed a signaling state life-time for cry2 of 16±5 min. Loss-of-reversibility of a pulse of blue light by a pulse of green light on the induction of *CHS* transcription, which is mediated by cry1, was observed for dark intervals between the two pulses with a half-life in the range of 5±1 min. A half-life of the lit state of *Arabidopsis* cry1 in insect cells in the range of ~ 6 min was determined by EPR spectroscopy. These findings led us to conclude that the signaling states of plant cryptochromes are short-lived, both *in vitro* and *in vivo*, with essentially no or only little stabilization *in planta*.

RESULTS

Life-time of the cryptochrome 2 signaling state in Arabidopsis cell culture

The *Arabidopsis* cry2 protein is rapidly degraded in etiolated seedlings upon exposure to blue light. There is compelling evidence that under blue fluence rates above 10 µmoles m^{-2} s^{-1} no other photoreceptor than cry2 is involved in this response (Yu *et al.*, 2007; Weidler *et al.*, 2012). Thus, the degradable fraction of cry2 represents roughly the fraction of cry2 in the signaling state. To determine the half-life of the cry2 signaling state *in vivo* we used an approach applied before to determine the life-time of the signaling state of Drosophila cry (Ozturk *et al.*, 2011), which also is light-labile. This approach is based on the assumption that the photochemistry of cry2 including dark reversion occurs normally on ice, whereas the enzymatic modifications such as proteolytic degradation is inhibited on ice. Instead of etiolated seedlings we used dark-adapted *Arabidopsis* cell cultures for these experiments to allow rapid cooling and warming of the suspension culture. Immunoblot analysis showed that under continuous blue light irradiation at 25°C essentially no cry2 protein was

detectable after 60 min (Figure 1b). Thus, the cell culture behaves similarly to etiolated seedlings regarding cry2 degradation (Lin *et al.*, 1998; Yu *et al.*, 2007; Weidler *et al.*, 2012; Zuo *et al.*, 2012). Next, precooled cell cultures were treated with a 15-min pulse of saturating blue light followed by dark incubation on ice for various periods of time. Cells were then shifted to 25°C for 60 min in darkness to allow complete degradation of this cry2 fraction, which remained in the signaling state after the dark period on ice (see Figure 1a for the experimental set up). These pulse experiments revealed that the prolongation of the dark period on ice after the blue light pulse led to a successive increase in the amount of cry2 and thus to a successive decrease in the amount of degradable cry2 protein as expected (Figure 1b). A first-order exponential fit of the quantitative immunoblot data revealed a half-life of the cry2 signaling state of 16 ± 5 min (Figure 1c).

To test our supposition that degradation of cry2 is not taking place when the cells are cooled on ice the level of cry2 protein was analyzed by Western blots. Cells were kept on ice or at 20°C and irradiated with continuous blue light (471 nm, 50 µmol m^{-2} s^{-1}) for up to 60 min. Figure S1 shows a rapid and strong decrease in the amount of cry2 protein for cells incubated at 20°C. In contrast, no decrease in the cry2 signal could be detected for cells kept on ice. Quantification and normalization of the cry2 signal against the tubulin signal revealed a constant cry2 level in the cells kept on ice whereas the cry2 level decreased to ~25% after 60 min of blue light irradiation at 20°C. We conclude from these data that the cooling of the cells to 0°C does prevent *de novo* synthesis and degradation of cry2.

Life-time of the cryptochrome 1 signaling state in Arabidopsis seedlings

In contrast to cry2, the protein level of cry1 is essentially not affected by light (Ahmad *et al.*, 1998; Ahmad *et al.*, 2002). We confirmed this light stability of cry1 for the light conditions applied in the following experiments and observed the same cry1 level in darkness and samples that were treated for 8 h with continuous blue light or dichromatic blue plus green light. A very small and about 2-fold higher amount of cry1 protein was detected in seedlings which were irradiated with green light for 8 h (Figure S2).

We considered cry1-controlled gene induction as a suitable and relatively fast readout system to analyze the life-time of the cry1 signaling state. cry1 signaling is known to be attenuated by green light, most likely because the flavin neutral semiquinone absorbs green light followed by photoconversion to the short-lived and inactive fully reduced flavin state (Banerjee *et al.*, 2007; Bouly *et al.*, 2007; Sellaro *et al.*, 2010). Thus, we wanted to use green light reversibility of the cry1 response to detect the amount of cry1 remaining in the signaling state after photoexcitation followed by dark periods of various durations. To this end, we analyzed *CHS* transcript levels in Arabidopsis seedlings including three wild type accessions and the *cry1*, *cry1cry2*, *phyAphyB* and *cry1cry2phyA* mutants. These studies revealed a strong ~ 60-fold upregulation of *CHS* transcripts in the wild types compared to dark-grown seedlings, which is mainly controlled by cry1 (Figure S3). However, we also observed a strong dependency of this blue light response on phytochromes similar to results from Ahmad and Cashmore (1997) who demonstrated weak accumulation of anthocyanin under blue light in the *phyAphyB* mutant. Thus, we considered endogenous *CHS* expression as not suitable to get information about the life-time of the cry1 signaling state.

To avoid the complexity caused by the requirement of phytochrome for full action of cry1 on the induction of the endogenous *CHS* gene we decided to use another readout system for analyzing the life-time of the cry1 signaling state which is independent from phytochromes. This is particularly important because blue and green light establish different Pfr/Ptot ratios and thus complicate further the interpretation of data from studies where the effect of a pulse of blue light should be reverted by a pulse of green light. We have shown before that the blue light-induced expression of the reporter ß-glucuronidase under control of a ~ 1 kbp fragment of the white mustard (*Sinapis alba*) *CHS*-promoter (*CHS::GUS*) in Arabidopsis is controlled by cryptochrome independent from phyA and phyB (Batschauer *et al.*, 1996). To this end, we first studied the effect of green light on blue light-induced expression of this chimeric gene construct under continuous irradiations. Monochromatic blue light led to a significant induction of *CHS*-promoter driven GUS expression (Figure 2). Green light alone had only a very low inductive effect. Most importantly the up-regulation of *CHS::GUS* expression by continuous blue light was strongly reduced by simultaneously applied green light. This confirms the before described effect of green light on the lit state of cry1 (Banerjee *et al.*, 2007; Bouly *et al.*, 2007; Sellaro *et al.*, 2010), and allowed us to replace continuous irradiations by repetitive light pulse treatments. For these experiments, etiolated seedlings were treated every 30 min with a 5-min pulse of blue light, followed by a green light pulse of the same fluence rate and duration without or with intermitting dark phases between 5 min and 20 min. These pulse treatments were applied for 8 h. From these seedlings protein extracts were made and GUS activities determined (Figure 3a). A complete loss of reversibility by the green light pulse was observed when the dark intervals between the blue and green light pulse were longer than 15 min. Plotting reversibility against

the dark intervals allowed an first-order exponential fit (Figure 3b) from which the half-life of green light reversibility was calculated to be ~ 5±1 min.

Life-time of the Cryptochrome 1 lit state in insect cells

We have previously shown that the formation of the flavin radical state of cry1 can be monitored in insect cells by EPR (Bouly *et al.*, 2007). We made use of this system to determine the life-time of the cry1 lit state in an artificial *in vivo* system that could roughly resemble the natural redox potential environment of plant cells but lacks the plant-specific components required for photoreceptor phosphorylation and dephosphorylation as well as interacting proteins. Blue light treatment of dark adapted insect cells which overexpressed cry1 led to a rapid formation of the radical state with a time constant of 15 s (Figure 4). The exponential fit of the decrease of the EPR signal in darkness revealed a half-life of the radical state of 360±20 s (Figure 4). Thus, the life-time of the cry1 lit state in insect cells is very close to the value calculated from the loss-of-reversibility of the cry1 signal by green light in Arabidopsis seedlings.

DISCUSSION

This work was performed to obtain information about the life-times of the signaling states of Arabidopsis cry1 and cry2. In the following discussion we differentiate between the photoexcited state or lit state of the photoreceptor, which contains the neutral semiquinone radical of FAD (Chaves *et al.*, 2011) and the signaling state, which is obtained from the lit state by further biochemical reactions including phosphorylations (Shalitin *et al.*, 2002; Bouly *et al.*, 2003; Shalitin *et al.*, 2003) and structural changes (Partch *et al.*, 2005; Kondoh *et al.*, 2011).

Life-time of the signaling state of cry2

A very fast response of cry2 in its signaling state is its degradation, which is detectable within minutes after blue light exposure of etiolated Arabidopsis seedlings (Ahmad *et al.*, 1998; Yu *et al.*, 2007). We show here that dark-adapted Arabidopsis cell cultures have a cry2 degradation response very similar to etiolated Arabidopsis seedlings (Figure 1, Figure S1). The rapidity of cry2 degradation makes this response an ideal system to study the life-time of the cry2 signaling state. We adopted the experimental setup established by Ozturk *et al.* (2011), which is based on the probable assumption that at 0°C photochemical reactions in cryptochrome occur more or less normal, whereas the biochemical reactions are blocked. This block also includes *de novo* synthesis of the photoreceptor protein. Shifting the cells to room temperature allows the biochemical reactions to occur. We confirmed this proposition for Arabidopsis cells by comparing the degradation of cry2 in cells that were kept either on ice or at 20°C (Figure S1). With this setup a half-life of 27 min for the Drosophila cry signaling state was calculated (Ozturk *et al.,* 2011). In our studies we used cell cultures instead of seedlings because temperature shifts are more rapid and traceable in the suspension culture. With the 60-min incubation at 25°C an almost complete degradation of cry2 remaining in the signaling state was assured (Figure 1b, cB). Based on quantitative immunoblot data a half-life of 16±5 min for the signaling state of cry2 was calculated. This value may have some experimental uncertainties for the following reasons: Signaling state formation requires phosphorylation of the lit state. Since this reaction is likely temperature-dependent there is some delay during warming up the sample. It is essentially impossible to estimate the rate of transition from the lit state to the signaling state during the

temperature shift. Moreover, kinetics of cry2 phosphorylation in dark-to-light shifts showed a peak at around 15 min (Shalitin *et al.*, 2002). These studies were done using a fluence rate of blue light of 5 µmol m^{-2} s^{-1}, which probably does not result in complete conversion of the cry2 pool from the ground state to the lit state. We used a ten-fold higher fluence rate of blue light but applied it only for 15 min. This light treatment did not allow converting all cry2 molecules to the signaling state because even continuous blue light treatment does not result in complete phosphorylation of all cry2 molecules (Yu *et al.*, 2007; Zuo *et al.*, 2012). This also is seen in our study by the fact that only ~ 65% of cry2 was degraded when cells were shifted directly after the pulse of blue light to 25°C (Figure 1c). Kinetics of cry2 dephosphorylation in light-dark transitions is not analyzed in detail. Data published by Shalitin *et al.* (2002) showed rapid dephosphorylation of cry2 in darkness clearly visible after 10 min. A similar reversion rate of t$_{1/2}$ ~5.5 min was observed for the cry2/CIB1 interaction in mammalian cells (Kennedy *et al.*, 2010). Thus, the kinetics of cry2 dephosphorylation and reversion of interaction of cry2 with CIB1 in darkness fit quite well with the half-life of the signaling state determined here for cry2.

Life-time of the signaling state of cry1

Cry1 is a light-stable photoreceptor at least in Arabidopsis (Ahmad *et al.*, 1998). Thus, the assay used here to determine the life-time of the signaling state of cry2 could not be applied for cry1. Instead we have chosen cry1-controlled *CHS* induction as relatively rapid response (Feinbaum and Ausubel, 1988; Feinbaum *et al.*, 1991; Christie and Jenkins, 1996; Fuglevand *et al.*, 1996; Hartmann *et al.*, 1998) to determine how long the cry1 signal lasts when blue light is switched-off. The experimental set-up we applied included repetitive pulses of blue light every 30 min

followed by pulses of green light with intermitting dark periods of increasing duration. This approach ensured that all samples had received identical doses of blue light and green light. Green light was used to inactivate the fraction of cry1 remaining in the signaling state (Banerjee *et al.*, 2007; Bouly *et al.*, 2007; Sellaro *et al.*, 2010).

The initial analysis of expression of the endogenous *CHS* revealed that its induction by blue light was clearly reduced in the *phyAphyB* mutant (Figure S3). Thus, cry1 requires phytochrome for full induction of the endogenous *CHS*. Ahmad and Cashmore (1997) described the same dependency of cry1 on phytochromes in particular on phyB for anthocyanin accumulation under blue light. CHS is the first enzyme in the pathway specific for flavonoids and anthocyanins (Martin, 1993). Thus, coaction of cry1 with phyB for this pathway may occur at the level of transcriptional control of *CHS* and possibly other genes in this pathway. This dependency of cry1 on phytochrome complicates green light reversibility studies because the calculated Pfr/Ptot ratios for the applied light conditions were 45% for blue light and 73% for the dichromatic blue and green light treatments. Considering the importance of phytochromes in particular of phyB for cry1-mediated *CHS* expression (Figure S3) and anthocyanin formation (Ahmad and Cashmore, 1997), the higher concentration of Pfr in the blue plus green light condition could counteract the decreased level of cry1 in its signaling state. This would particularly be important if the calculated 45% Pfr/Ptot under blue light would be insufficient for full action of phytochrome.

Considering these complications it is obvious that an experimental system is needed where the cry1 response does not depend on phytochrome. Indeed, the transgenic Arabidopsis line carrying the white mustard *CHS*-promoter::*GUS* gene fusion fulfills this criterion because it showed the same induction by blue light for wild type and the *phyAphyB* mutant (Batschauer *et al.*, 1996). Moreover, we show here

that green light reduces significantly the blue light-induced GUS expression in this line even under continuous dichromatic illumination (Figure 2). We conclude from these data that the lack of effect by green light on endogenous *CHS* expression is probably the result of counteraction between decreased levels of cry1 signaling state and elevated Pfr-levels. Why the expression of the *CHS::GUS* construct in blue light is uncoupled from phytochrome is not clarified.

The data presented in Figure 3 show loss of reversibility of *CHS::GUS* expression by a pulse of green light given after a pulse of blue light with intermitting dark intervals between the light pulses. The half-life for green light reversibility was calculated to be 5±1 min. Loss of reversibility has been applied before to study phytochrome responses (*e.g.* Lange *et al.*, 1971). Loss of reversibility includes two components: Dark reversion of the signaling state of the photoreceptor which is no longer accessible to the reverting light; and coupling of the light signal to downstream components which are likewise not amenable to the reverting light. If the decay of the signaling state in darkness would be much faster than the coupling of the light signal to the downstream component then the kinetics of loss-of-reversibility would approximately reflect the kinetics of dark-reversion. Since the decay of the signaling state or the lit state of cry1 cannot be measured by spectroscopic techniques in the plant, we made use of insect cells overexpressing cry1. In these cells the level of cry1 is high enough to detect its lit state formation and decay by EPR spectroscopy. The data presented in Figure 4 show that the normalized EPR signal reaches a plateau after a saturating pulse of blue light with a time constant of 15 s reflecting fast lit state formation. The decay of the lit state in darkness has a time constant of ~ 6 min. This value is very similar to the one calculated for the half-life for loss-of-reversibility *in situ*. We conclude from these data that lit state and signaling state decay is much faster than the time required for docking the cry1 signal to

downstream components. The nearly identical values determined for the decay of the lit state and the signaling state indicate that the biochemical reactions occurring on cry1 during light-to-dark transition are very fast. Indeed, Shalitin *et al.* (2003) showed that essentially all phosphorylated cry1 disappeared within 15 min after plants were shifted from blue light to darkness.

Analysis of dark reversion of the Pfr-state of various phytochromes expressed in yeast cells revealed half-lives in the range of 6-10 min at $4^{o}C$ for light stable Arabidopsis phytochromes, whereas Pfr dark reversion half-life of Arabidopsis phyA is slightly (30 min) and of rice phyA (>12 h) much slower than of light-stable phytochromes (Eichenberg *et al.*, 1999; Eichenberg *et al.*, 2000). Thus, the kinetics of dark reversion of cry1 and cry2 is in the same range as determined for light-stable phytochromes. However, it is important to note that the half-lives of Pfr-states determined for isolated recombinant phytochromes or the ones expressed and present in yeast cells do not necessarily reflect the situation *in planta*. Indeed there are examples for acceleration (Vierstra and Quail, 1985) as well as deceleration (Sweere *et al.*, 2001) of phytochromes dark reversion *in planta*. This may include interaction with plant specific factors. One example is the interaction of phyB Pfr with the pseudo response regulator ARR4 which stabilizes the signaling state of phyB (Sweere *et al.*, 2001). Comparing the kinetics of dark reversion of the lit states (neutral flavin semiquione) of cryptochromes determined *in vitro* with the values calculated here for the life-times of the signaling states *in vivo* indicates that these values are in the same range for cry1 with 2.6 min *in vitro* (Burney *et al.*, 2009) and 5 ± 1 min *in planta* (Figure 3). Thus, the signaling state of cry1 is not or only moderately stabilized *in planta*. This stabilization does not necessarily require the interaction with partner proteins. In case of cryptochromes it is well-known that reduced concentrations of oxygen (Müller and Ahmad, 2011) and addition of ATP

(Immeln *et al.*, 2007; Burney *et al.*, 2009) stabilize the lit state. The somewhat slower decay of the cry1 lit state in insect cells compared to the isolated protein could be caused by the presence of ATP in the cells and/or lower oxygen concentration within the cells compared to the solution. Indeed the minor differences observed in the EPR spectra of isolated cry1 and cry1 within the insect cells (Figure S5) could result from ATP-binding and or autophosphorylation (Bouly *et al.*, 2003; Shalitin *et al.*, 2003) in the cells which does not occur *in vitro* in the absence of ATP.

For cry2 the decay of the lit state *in vitro* with a time constant of 250±40 sec has been published (Banerjee *et al.*, 2007). This is about 4-times faster than the 16±5 min determined here for the half-life of the signaling state (Figure 1). The signaling state of cry2 *in vivo* could also be stabilized by ATP and by lower oxygen concentration. Moreover, the assay used to determine the half-life of the cry2 signaling state included warming up the samples from 0°C to 25°C to let the biochemical reactions occur. This could also have caused some delay in the dephosphorylation of cry2 resulting in the lengthening of the signaling state life-time.

Finally, cryptochromes form dimers (Sang *et al.*, 2005; Rosenfeldt *et al.*, 2008). For dicot phytochromes it is known that the thermal stability of the PfrPfr homodimer is much higher than that of the PfrPr heterodimer (Eichenberg *et al.*, 1999; Eichenberg *et al.*, 2000). Like phytochromes the cryptochromes dimerize already in darkness. Thus, the concentrations of ground state/ground state, ground state/signaling state and signaling state/signaling state dimers depend on the wavelength and the fluence rate of the applied light. Up to now it is completely unknown whether in case of cryptochromes only signaling state homodimers or even heterodimers are biologically active, whether cry1 and cry2 behave in this regard the same and whether dark reversion is affected by the concentration of signaling state

homodimers and heterodimers. These questions remain important topics for future research.

EXPERIMENTAL PROCEDURES

Plant materials, growth conditions and light treatments

Arabidopsis thaliana wild types (Landsberg *erecta* (L*er*), Columbia (Col-0), Wassilewskija (WS)), photoreceptor mutants (*cry1, cry1cry2, phyAphyB* in Ler background and *cry1cry2phyA* in Col-0 background) were used as well as a transgenic line carrying a *CHS::GUS* fusion construct in WS background (Kaiser and Batschauer, 1995). *Arabidopsis thaliana* seeds were surface-sterilized by treatment with 70% ethanol for 2 min followed by incubation in 5% sodium hypochlorite, 0.05% Tween 20 for 15 min. Seeds were rinsed five times in sterile distilled water and dispersed on filter paper (Bio-Rad, www3.bio-rad.com). After stratification for 3 d at 4°C in darkness, germination was induced by exposing seeds to white light for 4 h at 22°C. For analyzing GUS expression, plates were kept for 38 h in darkness at 22°C and exposed afterwards to continuous monochromatic blue, green or dichromatic blue and green light for 8 h. For quantitative real time PCR measurements wild type and mutant seedlings were treated the same way as for the GUS assay. Light treatments were performed in light chambers (CLF Plant Climatics, www.plantclimatics.de) equipped with LEDs having emission maxima at λ_{max} 471 nm (blue light) and λ_{max} 570 nm (green light) (for emission spectra of LEDs see Figure S4). Fluence rates were measured with an Optometer P-2000 spectroradiometer (Gigahertz Optik, www.gigahertz-optik.de) and set to fluence rates as indicated. Plant material was harvested under very dim red light and immediately shock-frozen in liquid nitrogen.

For analyzing cry2 degradation, a green Arabidopsis cell suspension culture (background Col-0) was used, which has been grown in 250 ml Erlenmeyer flasks under a 16 h white light 8 h dark regime at 25°C on a shaker set for 100 rpm. The volume of the medium in each flask was 50 ml. Subculturing and medium (MS medium with 2 X B5 vitamins) were essentially as described (Mathur and Koncz, 1998) except that the medium contained the following hormones: 0.1 mg l^{-1} naphtalene acetic acid (Sigma-Aldrich, www.sigmaaldrich.com) and 0.5 mg l^{-1} kinetin (Sigma-Aldrich, www.sigmaaldrich.com). Flasks were transferred to darkness for 3 d (4 d after subculturing) on a shaker set for 100 rpm. Afterwards, flasks were preincubated for 2 h in complete darkness on ice and temperature was controlled in the medium to have reached 0°C. In experiments presented in Figure S1, a second sample was kept at 20°C under otherwise identical conditions. Cells were transferred to Petri dishes under dim red light and kept continuously on ice or at room temperature as indicated. Afterwards cells were exposed to blue light (50 µmol m^{-2} s^{-1}) for 15 minutes on ice in the light chamber. After this blue light pulse samples were kept for various times on ice in darkness before transferring them to 25°C for 60 min in darkness. For comparison of cry2 degradation in cells kept at 0°C or 20°C, cells were exposed to blue (50 µmol m^{-2} s^{-1}) for up to 60 min. 2 ml of each sample was centrifuged (5 min at 20 000 g) and the pellet was immediately frozen in liquid nitrogen.

Protein extracts, gel separation and immunoblot analysis

Frozen cells from the cell culture were ruptured using a MM200 cell mill (Retsch, www.retsch.de) in 50 µl 2.5x SDS sample buffer (0.5% w/v SDS, 0.05% w/v bromphenol blue, 50 mM EDTA, 25% v/v glycerol) and afterwards boiled for 25 min.

Samples were centrifuged (5 min at 20 000 g) and supernatants used for SDS-PAGE followed by immunoblots. Protein concentrations were determined by the amido black protein assay (Schaffner and Weissman, 1973). Equal amounts (40 µg) of protein extracts were subjected to 10% reducing Laemmli mini-SDS-PAGE (Laemmli, 1970). Proteins were blotted to Parablot NCP nitrocellulose membranes (Macherey-Nagel, www.mn-net.com). For immunodetection polyclonal rabbit anti-CCT2 antibody (against cry2) and monoclonal mouse anti-α-tubulin antibody (Sigma-Aldrich, www.sigmaaldrich.com) in 1:1000 dilutions were applied. After three 10-min washing steps in TBS-T buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween 20) membranes were incubated with the secondary fluorophore-labeled antibodies (IRDye 680 goat-anti-mouse IgG and IRDye 800CW goat-anti-rabbit IgG; LI-COR, www.licor.com) diluted 1:10 000 in TBS-T. The membranes were washed three times in TBS-T and scanned using the Odyssey infrared imaging system (LI-COR, www.licor.com). Signal intensities of cry2 were calculated by the Odyssey software. The cry2 signal was normalized against the α-tubulin signal in the same lane. The level of cry2 in the dark sample of each genotype was set as 100%. For cry1 immunodetection 25 µg of total protein was used and the membrane was incubated with anti-CCT1 (against cry1) primary antibody as described above for cry2.

Gene expression analysis

RNA was extracted from *Arabidopsis* seedlings using the Plant RNeasy Plant Mini Kit (Qiagen, www.qiagen.com). 1 µg of RNA was used for digestion of remaining genomic DNA and reverse transcription using the QuantiTect Rev. Transcription Kit (Qiagen, www.qiagen.com). Control PCR reactions were performed to exclude genomic DNA contaminations. For real-time PCR the SensiFAST SYBR No-ROX Kit

(Bioline, www.bioline.com) was used following the instructions of the supplier. As reference gene actin (*ACT*) was used. Primers used were as follows: *CHS*-forward: 5'-GCA TGT GCG ACA AGT CGA C-3'; *CHS*-reverse: 5'-CCA GAG AAG GAG CCA TGT AAG C-3'; *ACT*-forward: 5'-ACC CTG TTC TTC TTA CCG AGG CTC C-3'; *ACT*-reverse: 5'-GAC ACA CCA TCA CCA GAA TCC AGC-3'. The cycling parameters used were 95°C for 5 min followed by 40 cycles of 95°C for 42 sec, 59°C for 35 sec and 72°C for 30 sec followed by final extension for 10 min at 72°C.

Glucuronidase assays

Fluorimetric GUS assays were performed essentially as described by Jefferson *et al.* (1987). In brief, frozen seedlings were ruptured using a MM200 cell mill (Retsch) in 300 µl extraction buffer (0.1 M potassium phosphate buffer pH 7.8, 1 mM DTT) and the lysate cleared by centrifugation (15 min, 20 000 g at 4°C). 50 µl of the protein extract was mixed with 500 µl of substrate solution (1 mg ml^{-1} 4-methyl-umbelliferyl-β-D-glucuronide in extraction buffer) and incubated at 37°C for 30 minutes. 100 µl of each sample was thereafter mixed with 900 µl stop buffer (0.2 M Na$_2$CO$_3$). Fluorescence was measured with an RF-5301 PC spectrophotofluorimeter (Shimadzu, www.shimadzu.de)   with λ_{Ex} = 365 nm, λ_{Em} = 455 nm. Values were normalized against the protein content of each sample and a calibration standard of 4-methyl-umbelliferone.

Insect cell expression of cry1 and EPR studies

Full-length Arabidopsis cry1 cDNA cloned in vector pBakPak9 (Clontech, www.clontech.com) was expressed in Sf21 insect cells and purified as described

previously (Bouly *et al.*, 2003; Bouly *et al.*, 2007). EPR spectroscopy on living insect cells expressing cry1 and on purified recombinant cry1 was performed as described by Hoang *et al.* (2008).

Calculation of half-times

The half-times ($t_{1/2}$) of cry2 protein degradability and of the green light reversibility of the blue light-induced GUS activity were calculated as $t_{1/2} = \tau \times \ln 2$ from the time constants (τ) of the mono-exponential decay fits of the data presented in Fig. 1c and Fig. 3b, respectively. Fits followed the formula Y-axis value = $y_0 + A \times e-(\text{X-axis value}/\tau)$ where the other two parameters y_0 and A are the offset (asymptote) and the amplitude of the decay (the difference in Y-axis values for zero and infinity X-axis values), respectively.

Calculation of Pfr/Ptot ratios

Pfr/Ptot ratios (φ_λ) were calculated using the formula and constants described by Mancinelli (1994).

GenBank accession numbers

GenBank accession numbers for the genes analyzed in this study are: *Arabidopsis thaliana CRY1* (AT1G04400), *CRY2* (AT4G08920), *CHS* (AT5G13930), *ACT2* (AT3G18780); *Sinapis alba CHS* (X16437).

ACKNOWLEDGMENTS

SUPPORTING INFORMATION

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

Figure S1. Protein levels of cry2 in cells kept on ice or at room temperature.

Figure S2. Cry1 protein levels in Arabidopsis seedlings under different light conditions.

Figure S3. *CHS* transcript levels in Arabidopsis wild type and photoreceptor mutant seedlings.

Figure S4. Emission spectra of blue and green light LEDs used in this study.

Figure S5. ENDOR spectra of Arabidopsis cry1 purified from insect cells and of insect cells overexpressing cry1.

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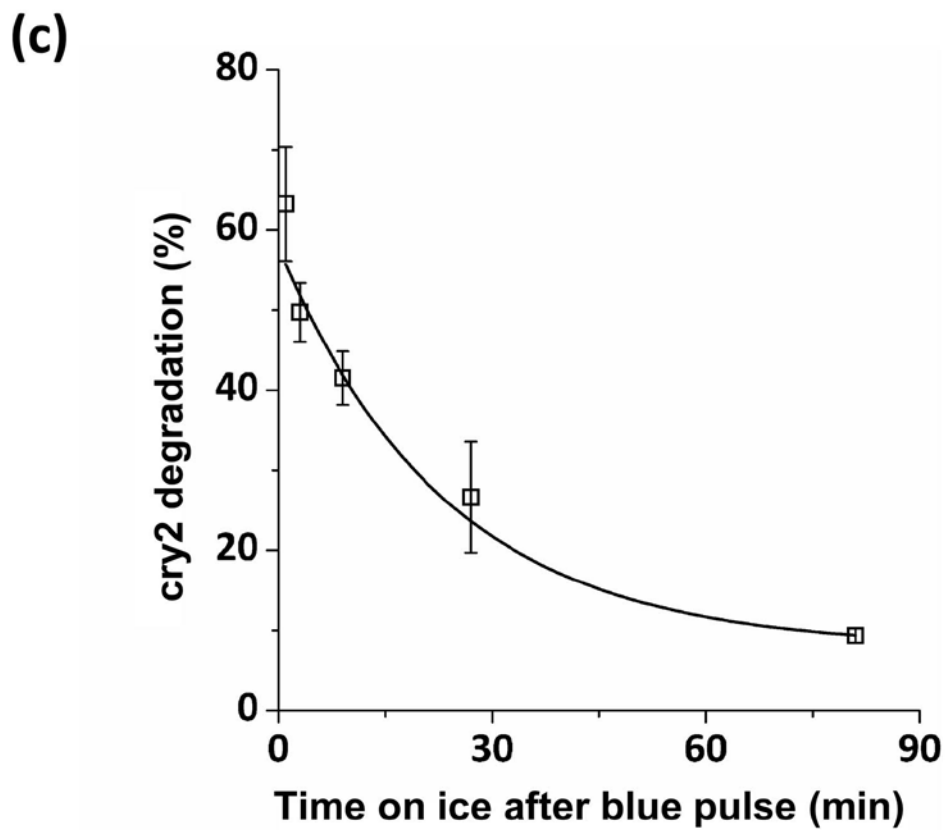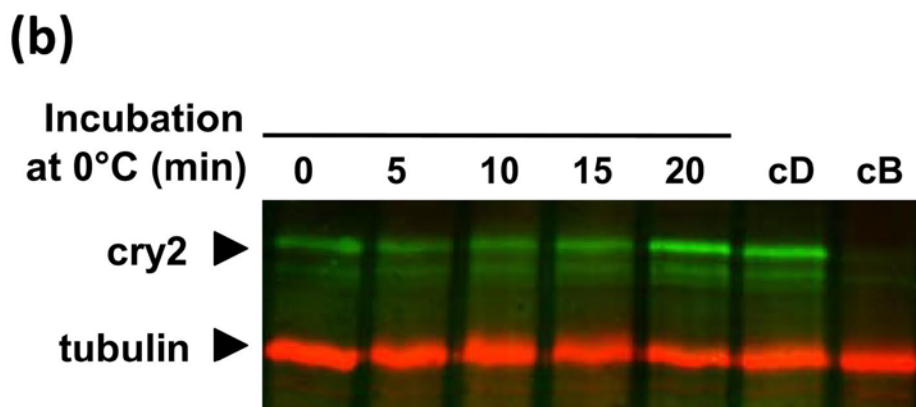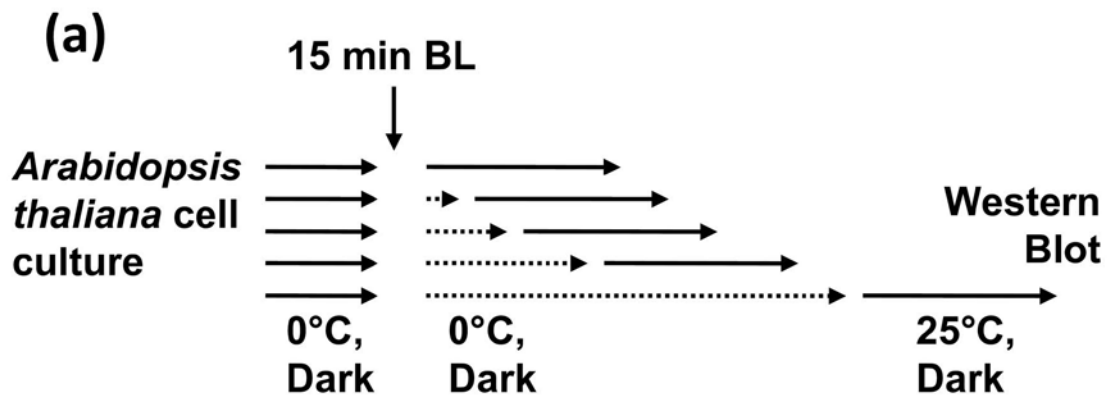
(a)

15 min BL

Arabidopsis thaliana cell culture

0°C, Dark

0°C, Dark

Western Blot

25°C, Dark

(b)

Incubation at 0°C (min)

| | 0 | 5 | 10 | 15 | 20 | cD | cB |

cry2 ▶

tubulin ▶

(c)

cry2 degradation (%) vs Time on ice after blue pulse (min)

Figure 1. Decay of the cry2 signaling state *in vivo* determined by the degradable fraction of cry2 remaining after dark periods of various duration. Precooled dark-adapted *Arabidopsis thaliana* cell culture cells were illuminated with blue light (471 nm, 50 µmol m^{-2} s^{-1}) for 15 min at 0°C. After illumination, samples were kept for various time periods on ice in darkness before transfer to 25°C for 60 min in darkness. Protein samples were analyzed by western blotting using cry2-specific antibodies and antibodies against α-tubulin. (a) Experimental setup. Dotted arrows indicate dark incubation of various durations on ice after the blue light pulse, the continuous arrows incubation at 25°C for 60 min in darkness to allow cry2 degradation. (b) Results of a representative immunoblot. Samples cD (continuous darkness) and cB (continuous blue light) were kept on ice for 35 min in darkness or blue light, and then shifted to 25°C for 60 min in darkness or blue light, respectively. (c) Quantitative data of cry2 degradation. The cry2 levels were normalized against the α-tubulin signal in the same blot and lane using fluorophore-labelled secondary antibodies and the Li-COR Odyssey infrared imaging system. Given are mean values ± SE from three biological replicates.
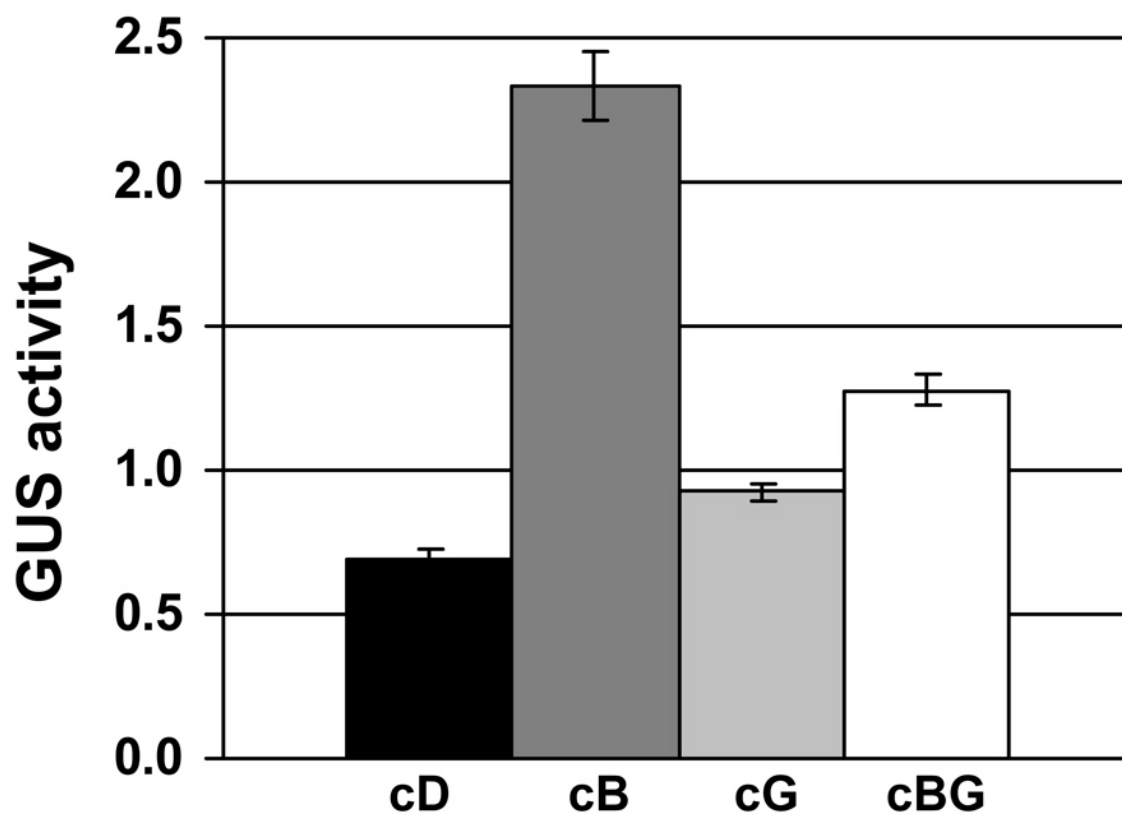
Figure 2. β-glucuronidase activity in transgenic *CHS::GUS Arabidopsis* seedlings grown under continuous irradiation. Transgenic seedlings carrying a white mustard chalcone synthase (*CHS*) promoter::β-glucuronidase (*GUS*) reporter fusion were kept for 38 h at 25°C in darkness followed by irradiation for 8 hours with LEDs emitting blue (cB), green light (cG), blue plus green light (cBG) each 10 μmol m^{-2} s^{-1}, or kept in darkness (cD). Spectra of the LEDs are shown in Figure S4. GUS activity is presented as formed pmol 4-methyl-umbilliferone (MU) mg^{-1} protein min^{-1}.
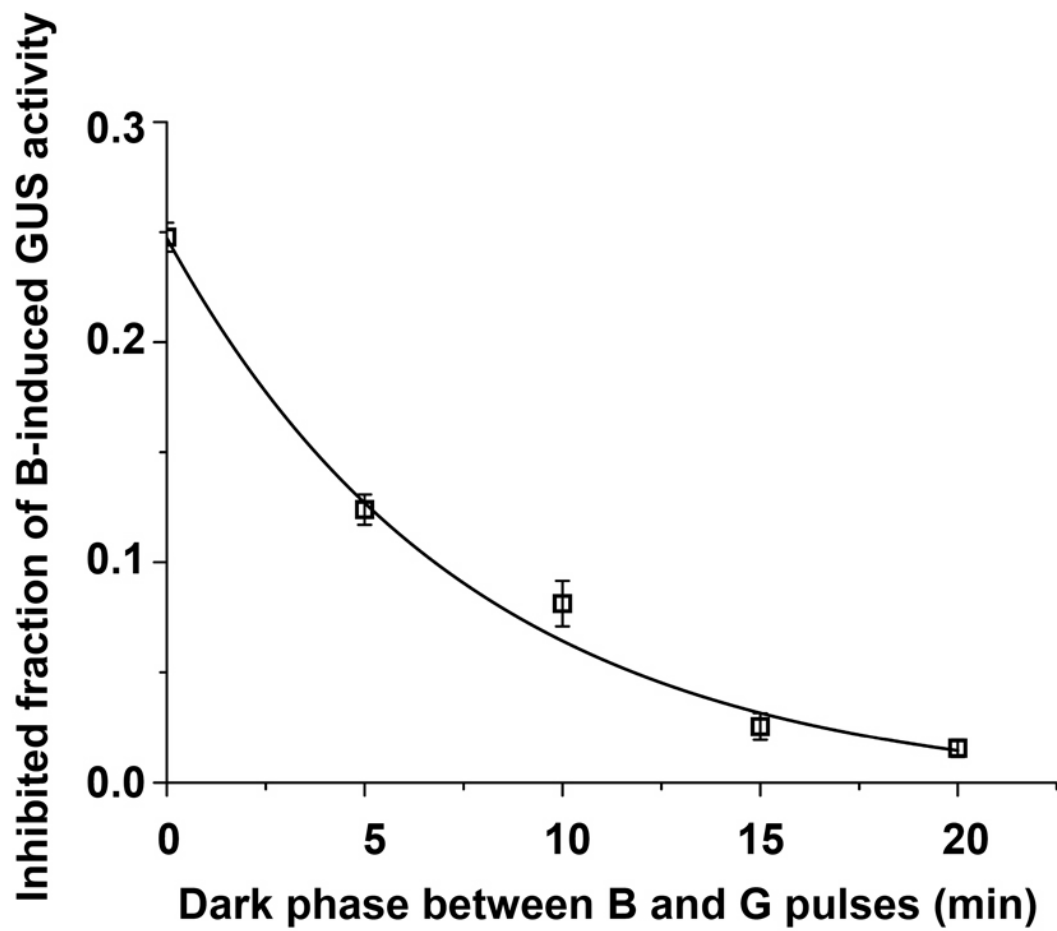
(a)
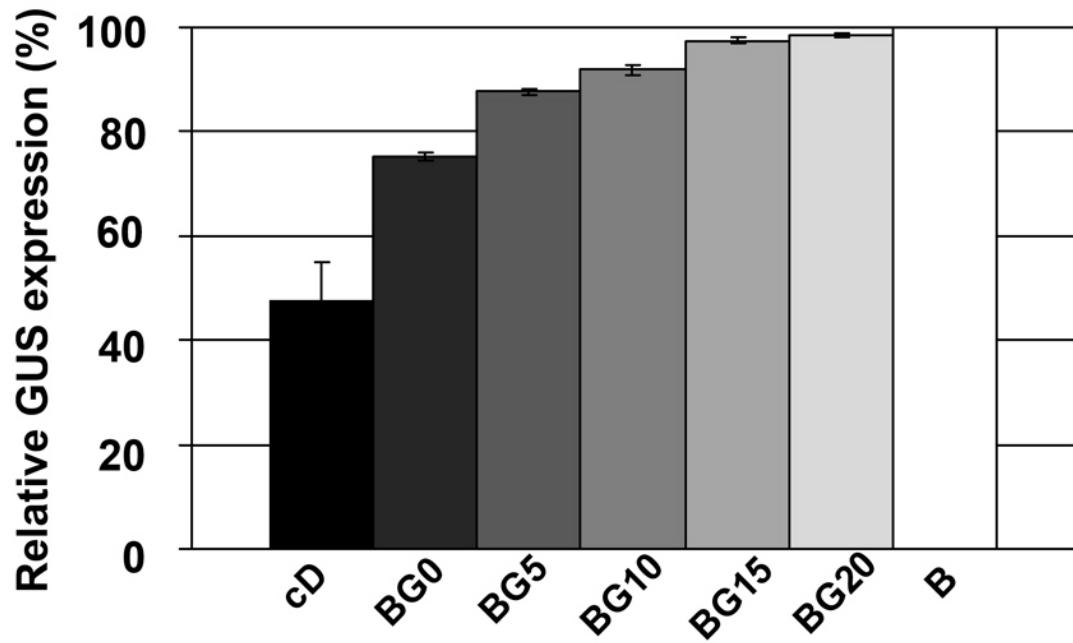
(b)

Figure 3. Escape from green light reversibility of blue light-induced *CHS::GUS* expression. The same material of transgenic *CHS::GUS* seedlings and grown as described in Fig. 3 were treated over a period of 8 h with 5-min blue light pulses (λ_{max} 471 nm, 10 µmol m^{-2} s^{-1}) given every 30 min. (a) Relative GUS expression normalized against the sample, which received only blue light pulses (B) and which was set as 100%. The other probes were either kept in constant darkness (cD), or received a 5-min green light pulse (λ_{max} 570 nm, 10 µmol m^{-2} s^{-1}) directly after the blue pulse (BG0) or after dark intervals of 5 min (BG5), 10 min (BG10), 15 min (BG15) and 20 min (BG20). (b) Monoexponential fit of data from (a) showing the fraction of blue light-induced GUS activity that can be suppressed by a pulse of green light given directly after the blue light pulse or after various lengths of dark intervals between the two pulses. Given are mean values ± SE of three biological replicates.
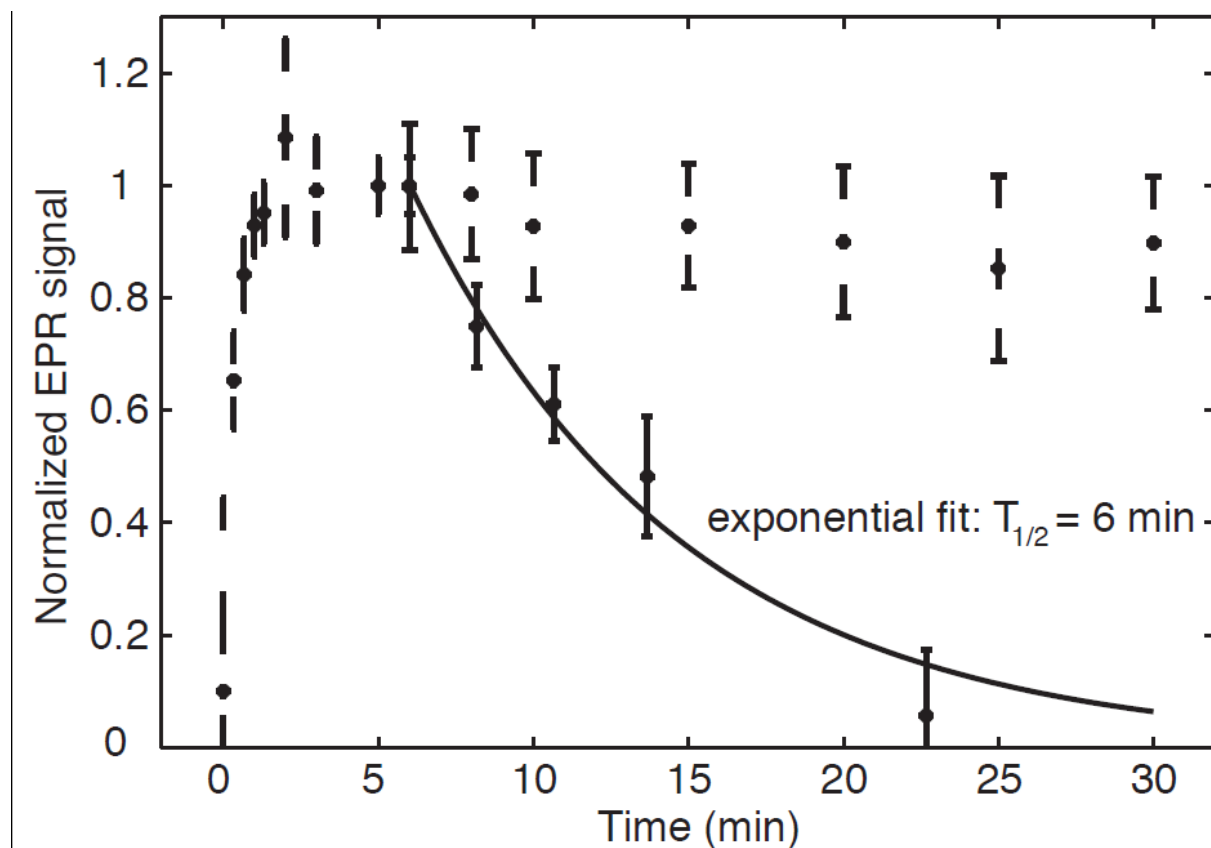
Figure 4. Formation and dark conversion of the flavin radical of cry1 in insect cells. Insect cells overexpressing Arabidopsis cry1 were kept at 8°C and treated with a 5-min pulse of blue light (λ_{max} 450 nm; 440 µmoles m^{-2} s^{-1}) followed by dark incubation. EPR signals of the FAD radical state were determined and normalized. Exponential fits for radical formation and dark adaptation resulted in half-lives for the FAD_{ox} state of 15 s in blue light and 6 min (solid line) for the radical state in darkness, respectively.