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Effects of Synergistic Signaling by Phytochrome A and Cryptochrome1 on Circadian Clock–Regulated Catalase Expression

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Persistent oscillation in constant conditions is a defining characteristic of circadian rhythms. However, in plants transferred into extended dark conditions, circadian rhythms in mRNA abundance commonly damp in amplitude over two or three cycles to a steady state level of relatively constant, low mRNA abundance. In Arabidopsis, catalase *CAT3* mRNA oscillations damp rapidly in extended dark conditions, but unlike catalase *CAT2* and the chlorophyll *a/b* binding protein gene *CAB*, in which the circadian oscillations damp to low steady state mRNA abundance, *CAT3* mRNA oscillations damp to high steady state levels of mRNA abundance. Mutational disruption of either phytochrome- or cryptochrome-mediated light perception prevents damping of the oscillations in *CAT3* mRNA abundance and reveals strong circadian oscillations that persist for multiple cycles in extended dark conditions. Damping of *CAT3* mRNA oscillations specifically requires phytochrome A but not phytochrome B and also requires the cryptochrome1 blue light receptor. Therefore, we conclude that synergistic signaling mediated through both phytochrome A and cryptochrome1 is required for damping of circadian *CAT3* mRNA oscillations in extended dark conditions.

INTRODUCTION

Circadian rhythms are widespread among eukaryotes and prokaryotes (Edmunds, 1988; Johnson et al., 1996). The primary defining characteristic of these rhythms is their endogenous nature: under "free-running" conditions in which the organism is deprived of environmental time cues, circadian rhythms persist with an innate period that is approximately, although seldom exactly, 24 hr (Pittendrigh, 1981a, 1981b; Edmunds, 1988). The period length is compensated against changes in ambient temperature and remains constant throughout the physiological range of the organism. The length of the free-running period is characteristic of the organism, although period length is often modulated by light intensity. In response to environmental time cues such as light pulses, circadian rhythms exhibit predictable and reproducible shifts in the phase of expressed rhythms that result in the entrainment of the rhythm to precisely 24-hr-period lengths, corresponding to the period of rotation of the earth on its axis.

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The spectrum of circadian rhythms that have been documented in plants is broad and includes leaf and petal movement, odor production, stomatal aperture, and gas exchange (Sweeney, 1987). The expression of many plant genes has been shown to be regulated by the biological clock in plants, and examples of both transcriptional and post-transcriptional regulation are known (reviewed in Piechulla, 1993; McClung and Kay, 1994; Anderson and Kay, 1996). Most studies of circadian gene expression in plants have concentrated on genes, such as *CAB* and *RCA*, whose products (chlorophyll *a/b* binding proteins and ribulose-1,5-bisphosphate carboxylase/oxygenase activase, respectively) play important roles in photosynthesis (McClung and Kay, 1994). mRNA abundance for these genes oscillates in plants maintained in a light and dark cycle, and these oscillations persist for many cycles in plants transferred into continuous light, with the circadian phase of maximal mRNA abundance during the subjective day (for examples, see Millar et al., 1992b, 1995; Pilgrim and McClung, 1993; Zhong et al., 1994).

In plants transferred into extended dark conditions, these rhythms in mRNA abundance commonly damp in amplitude over two or three cycles to a steady state level of relatively constant, low mRNA abundance. Although persistence in constant conditions is a defining characteristic of circadian rhythms, this lack of persistence of the rhythmic oscillation in mRNA abundance in extended dark conditions can be

reconciled with continued clock function in extended dark conditions (Kay and Millar, 1993). The expression of these clock-controlled genes, including *CAB*, is inducible by light through Pfr phytochrome. The circadian clock acts as a permissive regulator, allowing Pfr-mediated positive regulation during the subjective day, and mRNA abundance of *CAB* is maximal during the day. In plants transferred from a light and dark cycle into extended dark conditions, the depletion of pools of Pfr phytochrome leads to the loss of the transcriptional activator required for high-level expression and the rapid damping of the circadian oscillations in mRNA abundance to a low level of expression (Kay et al., 1989; Kay and Millar, 1993; Millar et al., 1995).

An independent approach to explore circadian clock function in extended dark conditions would be to investigate genes that are not positively regulated by phytochrome. The Arabidopsis *CAT3* catalase gene provides such a model. Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6), an iron porphyrin protein, is present in all aerobic organisms and converts H_2O_2 to O_2 and H_2O, thus protecting cells from the damaging effects of H_2O_2. Plant catalases play diverse roles in germination, photorespiration, and resistance to oxidative stress (McClung, 1997) and possibly in mediating signal transduction involving H_2O_2 as a second messenger (Baker and Orlandi, 1995; Neuenschwander et al., 1995; Ryals et al., 1995; Low and Merida, 1996). In Arabidopsis, catalase is encoded by a small family of three *CAT* genes (Frugoli et al., 1996). *CAT1* mRNA abundance is not controlled by the circadian clock (Frugoli et al., 1996). However, we have demonstrated a circadian oscillation in *CAT2* mRNA abundance with a morning-specific phase of maximal abundance and have established a role for phytochrome in the light induction of *CAT2* expression (Zhong et al., 1994). The third Arabidopsis catalase gene, *CAT3*, is also regulated by the circadian clock, but the oscillation in *CAT3* mRNA abundance differs from that described for *CAT2* in that the evening-specific phase of maximal abundance of *CAT3* mRNA is out of phase with the peak in *CAT2* mRNA abundance (Zhong and McClung, 1996). Furthermore, *CAT3* mRNA abundance does not increase in response to light (E.L. Connolly, M. Learned, H.H. Zhong, and C.R. McClung, unpublished observations). In continuous light, circadian oscillations in *CAT3* mRNA abundance persist with an evening-specific phase for at least five cycles (Zhong and McClung, 1996).

In the present study, we show that *CAT3* mRNA oscillations damp rapidly in extended dark conditions. Unlike *CAT2* and *CAB*, in which the circadian oscillations damp to low steady state levels of mRNA abundance (Zhong et al., 1994; Millar et al., 1995), *CAT3* mRNA oscillations damp to high steady state levels of mRNA abundance. We have examined the effects of mutants with defects in photoperception or in the transduction of photic signals and show that mutational disruption of either phytochrome- or cryptochrome-mediated light perception prevents damping of the oscillations in *CAT3* mRNA abundance and reveals strong oscillations that persist for multiple cycles in extended dark conditions. Damping of *CAT3* mRNA oscillations specifically requires phytochrome A but not phytochrome B. Therefore, we conclude that synergistic signaling mediated through both phytochrome A and cryptochrome1 is required for damping of circadian *CAT3* mRNA oscillations in extended dark conditions.

RESULTS

Amplitude of Circadian Oscillations in *CAT2* and *CAT3* mRNA Abundance Damp in Extended Dark Conditions

Because *CAT3* expression is not induced by light (E.L. Connolly, M. Learned, H.H. Zhong, and C.R. McClung, unpublished observations), we hypothesized that Pfr phytochrome abundance would not be limiting to *CAT3* mRNA abundance and thus that circadian oscillations in *CAT3* mRNA abundance would fail to damp in extended dark conditions. Accordingly, Arabidopsis ecotype Columbia seedlings were grown in light and dark cycles for 7 days and then transferred into extended dark conditions. Samples were harvested at 4-hr intervals for the last 48 hr (days 6 and 7) of the light and dark cycles and then for 72 hr in extended dark conditions. As we have described previously, *CAT3* mRNA abundance also oscillates in the light and dark cycle, with a peak at a Zeitgeber time (ZT) of 12 hr (Zhong and McClung, 1996). In extended dark, *CAT3* mRNA abundance rapidly increases and oscillations in abundance are not detected (Figure 1B and Table 1). During extended dark conditions, *CAT3* mRNA abundance stays high and relatively constant. Similar results were obtained with a second Arabidopsis ecotype, Landsberg *erecta* (Table 1). Consistent with our previous observations (Zhong et al., 1994), *CAT2* mRNA oscillates in light and dark cycles with an ~24-hr period length, and *CAT2* mRNA peaks in abundance at ZT of 0 to 4 hr (Figure 1A). As expected over two or three circadian cycles in extended dark conditions, the circadian oscillations in *CAT2* mRNA abundance decrease in amplitude and the steady state level of mRNA abundance of *CAT2* is low.

We asked whether the oscillation in *CAT3* mRNA abundance could be restored in plants in extended darkness by light pulses administered at subjective dawn (ZT of 24 hr) on the first day in extended dark conditions. A 15-min pulse of either white light or red light was sufficient to rescue one circadian oscillation in *CAT3* mRNA accumulation in extended dark conditions (Figures 1D and 1F). This is consistent with a role of phytochrome in the generation of the oscillation in *CAT3* mRNA abundance, although other photoreceptors may also be involved. A 15-min pulse of white light produced an acute induction of *CAT2* mRNA abundance in extended dark conditions, but the damping of the rhythm was otherwise unaffected (Figure 1C). However, a 15-min pulse of red light had little effect on *CAT2* mRNA abundance in ex-

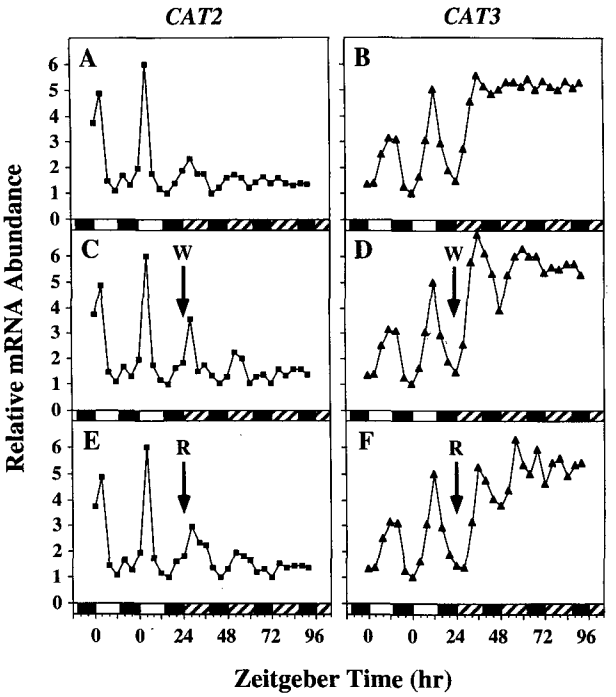

Figure 1. Circadian Oscillations in *CAT2* and *CAT3* mRNA Abundance Are Damped in Extended Dark Conditions; Oscillations in *CAT3* but Not in *CAT2* mRNA Are Revealed by Brief (15 Min) Pulses of White or Red Light Applied at the Beginning of the Subjective Day.

(A) Circadian oscillations in *CAT2* mRNA abundance persist in seedlings transferred from a light and dark cycle into extended dark conditions. The light regime is indicated by the bars at the base of the graph. The white bars indicate light conditions, the black bars indicate dark conditions, and the hatched bars indicate subjective day conditions (during extended dark conditions).

(B) Circadian oscillations are not detected in *CAT3* mRNA abundance in extended dark conditions; *CAT3* mRNA oscillations damp in amplitude to continuous high levels.

(C) to **(F)** Effects of pulses of white (**[C]** and **[D]**) or red (**[E]** and **[F]**) light administered at subjective dawn (indicated by arrows) on *CAT* mRNA abundance in plants transferred from light and dark into extended dark conditions. **(C)** shows that a single 15-min pulse of white light (W; ~125 μmol m^{-2} sec^{-1}) increases the amplitude of the first cycle in *CAT2* mRNA abundance in continuous dark but does not affect the amplitude of the second cycle. A single 15-min pulse of white light, as shown in **(D)**, restores one circadian oscillation in *CAT3* mRNA abundance in extended dark conditions. **(E)** shows that a single 15-min pulse of red light (R; ~18 μmol m^{-2} sec^{-1}) has little effect on the damping of oscillations in *CAT2* mRNA in extended dark conditions. A single 15-min pulse of red light, as shown in **(F)**, restores one circadian oscillation in *CAT3* mRNA abundance in extended dark conditions. Sampling times are expressed in hours as ZT, which is the number of hours after the onset of illumination. Zeitgeber (literally "time giver") is a term for any environmental signal, here the onset of light, that resets the circadian clock. Each point represents the mean value from three independent experiments; that is, RNA was analyzed from three sets of tissue, each of which was harvested in an independent experiment. The standard errors were always <20% and usually <10%.

tended dark conditions (Figure 1E), suggesting either that activation of phytochrome alone is insufficient to mediate the acute response of *CAT2* mRNA abundance or that the fluence employed was below the threshold required to elicit the acute response.

Phytochrome A Is Required for Damping the Circadian Oscillations in *CAT3* mRNA Abundance in Extended Dark Conditions

To assess the role of phytochrome in damping the oscillations in *CAT2* and *CAT3* mRNA abundance during extended dark conditions, we examined a series of mutants with specific defects affecting phytochrome function. The long hypocotyl *hy1* mutant is defective in chromophore synthesis and is thus deficient in functional phytochromes (Koornneef et al., 1980; Parks and Quail, 1991), although the mutation is leaky and the mutant retains some phytochrome function (Whitelam and Smith, 1991). In *hy1* seedlings transferred to extended dark conditions, the oscillations in *CAT3* mRNA abundance failed to damp and persisted for three cycles with an ~24-hr period (Figure 2B and Table 1). Therefore, functional phytochrome is required for damping of *CAT3* mRNA levels in extended dark conditions. A 15-min white light pulse administered at subjective dawn on the first cycle in extended dark conditions had no effect on these persistent oscillations (data not shown). In the *hy1* mutant growing in a light and dark cycle, *CAT2* mRNA abundance oscillated

Table 1. Periodicity of *CAT3* mRNA Oscillations in Extended Dark Conditions

Lines	Period (hr)
Wild type	
Columbia	NR[a]
Landsberg *erecta*	NR
Mutants[b]	
hy1-1	24.2 ± 3.2
phyA-201	20.2 ± 2.6
phyB-1	NR
phyA-201 phyB-5	23.4 ± 3.4
hy8-1 phyB-1	24.8 ± 3.4
hy4-2.23N	23.5 ± 2.5
hy1-1 hy4-101	20.1 ± 4.3
hy5-1	25.8 ± 2.9

[a] NR, not rhythmic (no rhythmic component detected at 90% probability). Periods (±SD) were estimated by a coupled fast Fourier transform–nonlinear least squares (FFT-NLLS) multicomponent cosine estimation algorithm (see Methods).

[b] Mutant lines were homozygous for the indicated alleles. Alleles used are *hy1-1*, *hy3-1* (*phyB-1*), *hy4-2.23N*, *hy5-1* (Koornneef et al., 1980), *phyA-201* (Nagatani et al., 1993), *phyB-5* (Reed et al., 1993), *hy8-1* (Parks and Quail, 1993), and *hy4-101* (Liscum and Hangarter, 1991).

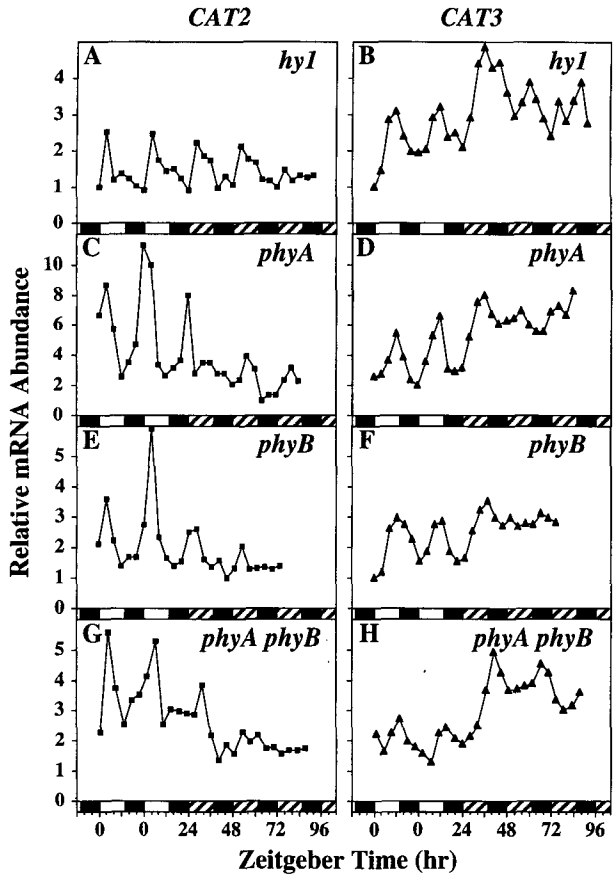

Figure 2. Effects of Mutations Affecting Phytochrome on Damping of Circadian Oscillations in *CAT2* and *CAT3* mRNA Abundance in Extended Dark Conditions.

(A) and **(B)** In the long hypocotyl *hy1* mutant, circadian oscillations in *CAT2* mRNA abundance damp in extended dark conditions **(A)**, but circadian oscillations in *CAT3* mRNA abundance persist in extended dark conditions **(B)**.

(C) and **(D)** In a mutant (*phyA*) lacking phytochrome A, circadian oscillations in *CAT2* mRNA abundance damp in extended dark conditions **(C)**, but circadian oscillations in *CAT3* mRNA abundance persist in extended dark conditions **(D)**.

(E) and **(F)** In a mutant (*phyB*) lacking phytochrome B, circadian oscillations in *CAT2* mRNA abundance **(E)** and in *CAT3* mRNA abundance **(F)** damp in extended dark conditions.

(G) and **(H)** In a double mutant (*phyA phyB*) lacking both phytochrome A and phytochrome B, circadian oscillations in *CAT2* mRNA abundance damp in extended dark conditions **(G)**, but circadian oscillations in *CAT3* mRNA abundance persist in extended dark conditions **(H)**.

Experimental conditions and symbols are as described for Figures 1A and 1B. Each point represents the mean value from three independent experiments (see legend to Figure 1).

at reduced amplitude (approximately two- to threefold versus four- to fivefold in the wild type; Figures 1A and 2A). Thus, phytochrome is required for the generation of *CAT2* mRNA oscillations of wild-type amplitude. This is consistent with our earlier observation that *CAT2* was positively regulated by phytochrome (Zhong et al., 1994). Damping of the oscillations in *CAT2* mRNA abundance in extended dark conditions was unaltered in *hy1* relative to wild-type Columbia seedlings. A white light pulse failed to elicit an acute induction of *CAT2* mRNA (data not shown), suggesting that phytochrome is required for this acute response, as has been shown for the acute response to light by *CAB2* (Anderson and Kay, 1995).

To distinguish which phytochrome is required for damping of *CAT3* mRNA levels in extended dark conditions, we examined mutants specifically defective in either phytochrome A (*phyA*) or in phytochrome B (*phyB* [*hy3*]) as well as double mutants lacking both phytochromes A and B (*phyA phyB*). Both the *phyA* allele, *hy8-1* (Parks and Quail, 1993), and the *phyB* allele, *phyB-1* (originally called *hy3-Bo64* [Koornneef et al., 1980]), are strong (null) alleles that eliminate phytochrome A and phytochrome B function, respectively (Dehesh et al., 1993; Reed et al., 1993). Loss of phytochrome B had no effect on circadian oscillations in *CAT2* mRNA abundance and only resulted in a modest reduction in the damped abundance of *CAT3* mRNA (Figures 2E and 2F and Table 1). Similarly, loss of functional phytochrome A had little effect on either amplitude or circadian oscillation of *CAT2* mRNA abundance (Figure 2C and Table 1). However, loss of functional phytochrome A completely eliminated the damping of the circadian oscillations in *CAT3* mRNA abundance in extended dark conditions and allowed the detection of statistically significant oscillations with circadian period (Figure 2D and Table 1). Two different lines carrying strong (null) mutations in both *phyA* and *phyB* (*hy8-1 phyB-1* and *phyA201 phyB-5* [Nagatani et al., 1993; Reed et al., 1993, 1994]) each showed persistent oscillations with the wild-type period in *CAT3* mRNA abundance in extended dark conditions (Figure 2H and Table 1), similar to those of the single *phyA* mutant. However, damping of oscillations in *CAT2* mRNA abundance was unaffected in the *phyA phyB* double mutants (Figure 2G). The period length of the oscillations in *CAT3* mRNA abundance in the single *phyA* mutant was shorter than was the period in the double *phyA phyB* mutants or in the wild type, although the difference was not statistically significant (Table 1). Thus, we conclude that damping of *CAT3* oscillations in extended dark conditions specifically requires phytochrome A but does not require phytochrome B.

Cryptochrome1 Is Required for Damping the Circadian Oscillations in *CAT3* mRNA Abundance in Extended Dark Conditions

The *HY4* locus encodes the blue light receptor cryptochrome1 (CRY1; Ahmad and Cashmore, 1993), and *hy4* is defective in a

number of blue light responses (Koornneef et al., 1980; Ahmad and Cashmore, 1996). The circadian expression of *CAT2* in light and dark cycles and in extended dark conditions was unaltered (Figure 3A) in seedlings homozygous for the *hy4-2.23N* allele, a strong mutation that lacks functional CRY1 protein (Ahmad and Cashmore, 1993). However, damping of *CAT3* mRNA oscillations in extended dark conditions was not observed in the *hy4* mutant (Figure 3B). The circadian behavior of *CAT2* and *CAT3* mRNA abundance in an *hy1 hy4* double mutant, which is depleted in active phytochromes and which lacks the CRY1 blue light receptor, was similar to that seen in the *hy4* mutant. *CAT2* showed low-amplitude (approximately two- to threefold) oscillations in light and dark cycles, and the amplitude of these oscillations damped to low-level expression in extended dark conditions (Figure 3C). In contrast to the damping seen in the wild type, in this *hy1 hy4* double mutant, the oscillations in *CAT3* mRNA abundance persisted for at least three circadian cycles in extended dark conditions (Figure 3D). The period length of the *CAT3* mRNA oscillations was not significantly different from that of the wild type in either the *hy4* or the *hy1 hy4* mutants (Table 1). Therefore, we conclude that damping of *CAT3* oscillation in extended dark conditions specifically requires CRY1 in addition to phytochrome A.

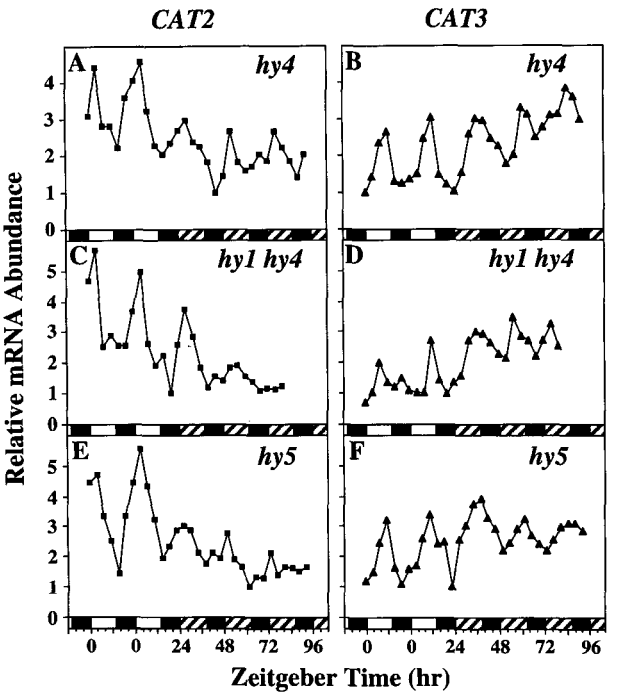

Figure 3. Effects of Mutations Affecting Cryptochrome or Light Signal Transduction on Damping of Circadian Oscillations in *CAT2* and *CAT3* mRNA Abundance in Extended Dark Conditions.

(A) and **(B)** In a mutant (*hy4*) lacking the cryptochrome1 blue light receptor, circadian oscillations in *CAT2* mRNA abundance damp in extended dark conditions **(A)**, but circadian oscillations in *CAT3* mRNA abundance persist in extended dark conditions **(B)**.

(C) and **(D)** In a double mutant (*hy1 hy4*) lacking spectrally active phytochrome and the cryptochrome1 blue light receptor, circadian oscillations in *CAT2* mRNA abundance damp in extended dark conditions **(C)**, but circadian oscillations in *CAT3* mRNA abundance persist in extended dark conditions **(D)**.

(E) and **(F)** In a *hy5* mutant affected in signal transduction, circadian oscillations in *CAT2* mRNA abundance damp in extended dark conditions **(E)**, but circadian oscillations in *CAT3* mRNA abundance persist in extended dark conditions **(F)**.

Experimental conditions and symbols are as described for Figures 1A and 1B. Each point represents the mean value from three independent experiments (see legend to Figure 1).

HY5 Is Required for Damping the Circadian Oscillations in *CAT3* mRNA Abundance in Extended Dark Conditions

The HY5 gene product is required for both phytochrome- and cryptochrome-mediated signaling (Koornneef et al., 1980; Chamovitz and Deng, 1996). The *hy5* mutation had little effect in the circadian oscillations in *CAT2* mRNA abundance (Figure 3E). However, the *hy5* mutation prevented the damping of the circadian oscillations in *CAT3* mRNA, which persisted for at least three circadian cycles in extended dark conditions with a period that was not statistically different from that of the wild type (Figure 3F and Table 1). Thus, HY5 is required for damping the *CAT3* mRNA oscillation in the dark, consistent with its role in mediating both phytochrome and cryptochrome signaling.

DISCUSSION

In this study, we provide evidence that signaling from both CRY1 and PHYA is required for damping of the circadian oscillations in *CAT3* mRNA abundance in plants transferred from a light and dark cycle into extended dark conditions. A defining characteristic of circadian rhythms is persistence in constant conditions in which the organism is deprived of external time cues. Nonetheless, when plants are transferred into extended dark conditions, circadian oscillations in *CAT3* mRNA abundance are rapidly replaced by a continuous high level of mRNA abundance. Circadian oscillations in mRNA abundance of several other genes, including *CAT2*, *CAB*, and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RBCS*), have previously been shown to damp in extended dark conditions, although the damping for these genes is distinct from the damping of *CAT3* mRNA oscillations in that the steady state mRNA abundance after several cycles in extended dark conditions is low (Millar et al., 1992b, 1995; Pilgrim and McClung, 1993; Zhong et al., 1994). Another difference in the characteristics of the circadian oscillations of *CAT3* versus *CAB* is that the period length for *CAT3* oscillations in every genotype examined remains close to 24 hr,

whereas the period length of oscillations in *CAB* expression in extended dark conditions lengthens to ~30 hr (Millar et al., 1992a, 1995; Millar and Kay, 1996).

The circadian oscillations in *CAT3* mRNA abundance also differ significantly from those in *CAT2*, *CAB*, and *RBCS* mRNA abundance in that the phase of maximal *CAT3* mRNA abundance is in the evening (Zhong and McClung, 1996), whereas *CAT2*, *CAB*, and *RBCS* mRNA abundance peaks much earlier in the light period (Millar et al., 1992b; Pilgrim and McClung, 1993; Zhong et al., 1994; Millar and Kay, 1996). However, damping of circadian oscillations to a steady state level of high mRNA abundance is not a general property of evening-specific genes, because oscillations in mRNA abundance of maize *Cat3* (Redinbaugh et al., 1990) as well as of two other evening-specific clock-controlled Arabidopsis genes, *CCR1* and *CCR2* (Carpenter et al., 1994), do not damp to high-level expression when transferred from light and dark into extended dark conditions (Boldt and Scandalios, 1995; J.A. Kreps, personal communication). Thus, the damping of circadian oscillations of *CAT3* mRNA abundance in extended dark conditions to high levels is novel among clock-regulated genes in plants. Interestingly, oscillations in the mRNA abundance of the newly identified clock-controlled Drosophila gene *Dreg-2* also damp to high abundance upon entering total darkness (Van Gelder et al., 1995). *Dreg-2* mRNA abundance oscillates in light and dark cycles, but in extended dark conditions, it increases in the dark and remains at a high level without further fluctuation. Unlike *CAT3*, *Dreg-2* mRNA is maximally abundant in the morning. The regulation of *Dreg-2*, other than by the circadian clock, is not known.

Mutant analysis clearly establishes that regulation of *CAT3* mRNA abundance in extended dark conditions responds to CRY1- and phytochrome A–mediated signaling. Disruption of either signaling pathway is sufficient to allow the detection of circadian oscillations in *CAT3* mRNA abundance in extended dark conditions. Because both signaling pathways are required for the damping of *CAT3* mRNA oscillations, we suggest that either one signaling pathway potentiates the other or that the two signals act synergistically. Another possible explanation is that elements of the CRY1 and phytochrome A signaling pathways form a common complex that fails to form or to function in mutants lacking either CRY1 or phytochrome A. Synergism between distinct signaling pathways has been previously described in plants. For example, methyl jasmonate and soluble sugars synergistically stimulate vegetative storage protein gene expression in soybean (Mason et al., 1992). Methyl jasmonate and ethylene synergistically stimulate osmotin transcription in tobacco (Xu et al., 1994). Abscisic acid and osmotic stress synergistically regulate *Em* gene expression in rice (Bostock and Quatrano, 1992). Synergism between the far-red light–absorbing forms of phytochromes A and B is responsible for the light-mediated reduction in gravitropism in Arabidopsis seedlings (Poppe et al., 1996). Recently, synergistic interaction between blue, UV-A, and UV-B light signaling was shown to induce chalcone synthase gene expression in Arabidopsis (Fuglevand et al., 1996). Synergism, termed coaction, between red and blue light signaling pathways is well documented (Mohr, 1994). One recent example describes synergism mediated through phytochrome B (but not phytochrome A) and CRY1 in the regulation of Arabidopsis hypocotyl elongation (Casal and Boccalandro, 1995). Another study demonstrates that phytochrome A acts synergistically with CRY1 in Arabidopsis hypocotyl elongation and anthocyanin accumulation (Ahmad and Cashmore, 1997).

The damping in amplitude of circadian oscillations of light-inducible genes, such as *CAB*, to low-level expression after several cycles in extended dark conditions has been attributed to the loss of Pfr phytochrome, which serves as a relatively long-lived, positive regulator of *CAB* expression whose activity is gated by the circadian clock (Kay and Millar, 1993). One possible explanation for the damping of *CAT3* mRNA oscillations in extended dark conditions is that light activates a negative regulator of *CAT3* mRNA abundance that is necessary for a decrease in *CAT3* mRNA abundance during the next circadian cycle; depletion of a light-activated repressor would result in continuously high mRNA abundance. A light pulse administered at subjective dawn on the first day in extended dark conditions rescues a single trough in *CAT3* mRNA abundance during the next circadian cycle, ~24 hr later. Therefore, either the negative regulator or the light-derived signal must be sufficiently stable to function during the next circadian cycle but sufficiently unstable such that it is depleted in subsequent circadian cycles.

A second explanation of the damping of *CAT3* mRNA oscillations in extended dark conditions is that a positive regulator of *CAT3* mRNA abundance accumulates during extended dark conditions, leading to high levels of *CAT3* mRNA and obscuring the circadian rhythm in *CAT3* mRNA abundance. However, this positive regulator must not accumulate or become active until the first subjective day in extended dark conditions, because during the night phase of a light and dark cycle and during the first subjective night in extended dark conditions, *CAT3* mRNA decreases in abundance. The loss of damping in the various photoperception mutants indicates that the accumulation or the activity of this positive regulator requires intact phytochrome A and CRY1 signal transduction pathways, including HY5.

Regardless of whether the accumulation of *CAT3* mRNA to high levels during extended dark conditions results from the depletion of a negative regulator or the accumulation of a positive regulator, the expression of robust circadian oscillations in *CAT3* mRNA abundance during extended dark conditions in the mutants suggests that it is the loss of a signal during extended dark conditions that prevents the accumulation of *CAT3* mRNA. This implies that there is signaling mediated through phytochrome A– and CRY1-dependent pathways during extended dark conditions that induces the mechanism responsible for the accumulation of *CAT3* mRNA. Although it is tempting to speculate that this might

indicate a physiological role for the Pr form of phytochrome A and for unstimulated CRY1, it is also plausible that a signal involving Pfr and light-stimulated CRY1 is initiated at lights off or during the first hours of darkness. The effects of this signal would then persist until antagonized by a subsequent light signal.

In the mutants lacking functional phytochrome A or CRY1, the signal required to induce the accumulation of *CAT3* mRNA during extended dark conditions would be missing and the circadian oscillation in *CAT3* mRNA abundance would persist, clearly indicating that neither phytochrome A– nor CRY1-mediated signaling is essential for the manifestation of this robust circadian rhythm. In particular, the robust rhythmicity evident in the *hy1 hy4* double mutant, which lacks spectrally detectable functional phytochrome (Parks and Quail, 1991) as well as CRY1 (Ahmad and Cashmore, 1993), indicates that either residual phytochrome (*hy1* is leaky; Whitelam and Smith, 1991) or another distinct photoperception system is sufficient for the generation of robust circadian rhythms. Furthermore, the photoperception mutants tested entrain to a light and dark cycle apparently normally, indicating that other photoperception pathways that remain intact in these mutants are sufficient for entrainment.

Plants contain multiple photoreceptors and multiple pathways of light signal transduction (Bowler and Chua, 1994; Short and Briggs, 1994; Jenkins et al., 1995; Chamovitz and Deng, 1996; Furuya and Schäfer, 1996). It is becoming increasingly clear that these multiple pathways can function independently of one another but also are capable of interacting additively and synergistically (Mohr, 1994; Casal and Boccalandro, 1995; Ahmad and Cashmore, 1996; Fuglevand et al., 1996; Poppe et al., 1996). The results of this study emphasize that the circadian clock interacts with and modulates the responses to multiple light signaling pathways.

METHODS

Plant Materials

Seeds of *Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta* were used in these studies. Mutant seeds were obtained from R. Hangarter (Indiana University, Bloomington) or from the Arabidopsis Stock Center (Ohio State University, Columbus). Seeds were imbibed in water for 30 min and then surface sterilized by soaking in 95% ethanol for 10 min and then in 30% bleach and 0.1% Triton X-100 for 10 min. After five washes with sterile distilled water, the seeds were spread onto media containing Murashige and Skoog mineral salts (Murashige and Skoog, 1962), 10 g/L agar, and 2% sucrose. After stratification at 4°C in the dark for 3 days, seeds were incubated at 22 to 24°C.

Plant Growth and Light Conditions

Arabidopsis seedlings were grown under light–dark cycles (14 hr of light and 10 hr of dark) at 22°C for 7 days and then transferred into total darkness. White light (~125 μmol m^{-2} sec^{-1}) was provided by a combination of white fluorescent and incandescent lights. Red light (18 μmol m^{-2} sec^{-1}, between 600 to 700 nm) was provided by four white fluorescent lights (Philips TL-741; Eagle Electric Supply, Norwood, MA) filtered through a 6-cm depth of 1.5% $CuSO_4$ (to absorb the heat) and red Plexiglas (Rohm and Haas No. 2423; Cadillac Plastics, Manchester, NH). For light pulses, 6-day-old etiolated seedlings were exposed to 15-min pulses of white light or red light. After the different light treatments, seedlings were incubated in the dark for the indicated intervals. Fluence rates were monitored with a quantum photometer (model LI-189; LI-COR Inc., Lincoln, NE).

RNA Isolation and Slot Blot Analysis

Tissue was homogenized with a hand-held homogenizer (OMNI International, Waterbury, CT), and total RNA was isolated by phenol-SDS extraction and LiCl precipitation, as described by Zhong et al. (1994). RNA gel and slot blot analyses using *CAT2* and *CAT3* hybridization probes were as previously described (Zhong et al., 1994; Zhong and McClung, 1996). The catalase *CAT2* and *CAT3* hybridization probes do not cross-hybridize under the high-stringency conditions employed (Frugoli et al., 1996). rRNA (Richards and Ausubel, 1988) was used as the loading control. RNA abundance was quantified using a PhosphorImager with ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Time-series data were quantitatively evaluated by an iterative, coupled fast Fourier transform–nonlinear least squares (FFT-NLLS) multicomponent cosine estimation algorithm (Johnson and Frasier, 1985; Straume et al., 1991; Plautz et al., 1997). FFT-NLLS provides estimates of periods, phases, and amplitudes of rhythmic components, along with associated estimates of joint parameter confidence limits, as well as an index of determination regarding statistical significance of rhythmic amplitudes. FFT-NLLS self-initializes, using FFT spectral peaks as initial guesses for NLLS minimization, to reduce user-introduced bias and to facilitate automated implementation. FFT-NLLS accommodates noisy, short/sparse time series with occasional missing points and temporal nonstationarities (drifting measurements and nonconstant oscillatory amplitudes) while reliably detecting underlying rhythms. Linear regression–detrended time series are considered (zero mean and zero slope). FFT spectral peaks initialize a multicomponent-cosine NLLS minimization to the detrended data. Approximate nonlinear asymmetric joint confidence limits for all parameters (period, phase, amplitude, and the mean of the detrended time series) are evaluated at a user-specified level of confidence probability. The maximum number of statistically significant (i.e., with statistically significant [nonzero] amplitudes) cosine components supportable by the data is identified.

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