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Functional dissection of circadian clock- and phytochrome-regulated transcription of the *Arabidopsis CAB2* gene

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ABSTRACT **Both the circadian clock and phytochrome regulate expression of the *Arabidopsis* genes encoding the light-harvesting chlorophyll *a*/*b*-binding proteins (*CAB* genes). Phytochrome activates *CAB* transcription, and it has been proposed that the circadian clock negatively regulates *CAB* transcription. The tobacco nuclear proteins CUF-1 (*CAB* upstream factor 1) and CGF-1 (*CAB* GATA factor 1) bind the *Arabidopsis CAB2* promoter, and the CGF-1 binding site is contained within a minimal clock- and phytochrome-regulated region of the promoter. We have used *in vivo cab2*::luciferase gene bioluminescence markers containing site-directed mutations in the CUF-1 and CGF-1 binding sites to define the role of these proteins in *CAB2* regulation and to further delineate the terminal genomic targets of the phytochrome and circadian clock signal transduction pathways. Results from these studies confirm that CUF-1 is not required to generate the circadian clock- or phytochrome-responsive *CAB2* expression pattern but rather functions as a positive factor to increase *CAB2* expression levels. CGF-1 interaction with the *CAB2* promoter mediates the acute increase in *CAB2* expression in response to phytochrome activation and contributes to the light-induced high-amplitude circadian oscillation in *CAB2* expression.**

Expression of the *CAB* gene family, encoding the light-harvesting chlorophyll *a*/*b*-binding proteins of photosystem II, is regulated at the level of transcription both by the family of red/far-red light photoreceptors, the phytochromes (1), and by the circadian clock (2, 3). A model describing the interactions between phytochrome and the circadian clock to regulate *CAB* transcription (2) has been proposed based on the experimental observations of the effect of different light conditions on the oscillation in *CAB* expression in both green and etiolated tissue (4–6). In this model, light signals mediated by phytochrome are proposed to both positively regulate *CAB* transcription and regulate the phase and period of the circadian oscillation; and the circadian clock is proposed to negatively regulate *CAB* transcription (Fig. 1). An aspect of this model which has not been determined is whether the signal transduction pathways originating from the clock and phytochrome interact directly with the same or distinct cis-acting *CAB* promoter elements or whether the pathways converge prior to binding of a transcription factor(s) to the *CAB* promoter to regulate transcription.

We have previously characterized two tobacco nuclear factors, CUF-1 and CGF-1 (7), which interact in a sequence-specific manner with the promoter of the clock-regulated (4, 5) and phytochrome-regulated (1) *Arabidopsis CAB2* gene. CUF-1, which binds *CAB2* promoter sequences between −139 and −115, is a member of the intermediate class of ACGT-binding proteins with affinity for both G-box and TGACGT/C motifs (7). The participation of ACGT cis-acting sequences and their cognate binding proteins have been implanted in the light-regulated expression of specific plant genes (8, 9). CGF-1 binds a repeated GATA motif (nt −74 to −42 of the *CAB2* promoter) conserved in *CAB* promoters from several species (10). The binding-site specificity of CGF-1 is distinct from that of ASF-2 and GAF-1 (7) which bind GATA motifs in the cauliflower mosaic virus 35S (11) and *RBCS-3A* (12) promoters, respectively. However, CGF-1 does bind DNA target sequences similar to those of the box II-binding protein GT-1 (7), which has been demonstrated to confer light-regulated transcription upon a heterologous promoter (13).

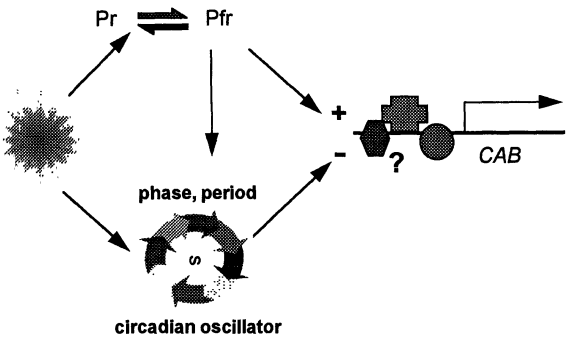

FIG. 1. Model of the phytochrome- and circadian clock-regulated pathways which control *CAB* gene expression. Light signals, via phytochrome, positively regulate the level of *CAB* transcription. In addition, phototransduction pathways must exist for regulation of the phase and period of the circadian oscillator. The circadian clock in turn negatively regulates *CAB* transcription.

A minimal clock- and phytochrome-responsive *Arabidopsis CAB2* fragment extending from −111 to −33 and containing the CGF-1 binding site has been previously identified (7). The −111 to −33 *CAB2* fragment confers a robust circadian oscillation of expression upon a heterologous cauliflower mosaic virus 35S promoter fused to the firefly luciferase (*luc*) reporter gene with an ≈2-fold amplitude and red-light-induced (*luc*) expression in etiolated seedlings (7). These results suggest that CUF-1 binding is not required to generate the complex pattern of gene expression mediated by the circadian clock and phytochrome. The CGF-1 binding site or other sequences within the −111 to −33 fragment are implicated as possible genomic targets for the circadian clock and/or phytochrome signal transduction pathways. In order to assay more precisely the roles of CUF-1 and CGF-1 in the regulation of *CAB2* expression, and in particular to identify the cis-acting target sites for clock and phytochrome regulation, site-directed mutations of the CUF-1 and CGF-1 binding sites which interfere with binding of the respective tobacco nuclear factors *in vitro* were identified. These same mutations were generated in *CAB2* promoter fragments fused to the *luc* reporter gene and assayed for their effect on clock- and phytochrome-regulated expression in transgenic tobacco *in vivo*.

MATERIALS AND METHODS

Gel Retardation Assays. Gel retardation assays of CUF-1 and CGF-1 binding activities and competition analyses were

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performed as described (7). Synthetic oligonucleotides corresponding to the top and bottom strand of the CUF-1 binding site (nt −138 to −112) and a mutant CUF-1 binding site (CUF-M, in which the ACGT tetranucleotide core has been changed to AATT), the CGF-1 binding site (nt −78 to −44), and a mutant CGF-1 binding site (CGF-G3M, containing a G → C mutation in each of the three repeated GATA motifs) were synthesized, annealed, and used as competitors. The wild-type and the CUF-M mutant versions of the −199 to +1 *CAB2* fragment were isolated as *Bam*HI–*Hin*dIII fragments, purified by gel electrophoresis, and used as competitors. The wild-type and the CGF-G3M mutant versions of the −111 to −33 *CAB2* fragment were amplified by polymerase chain reaction (PCR), gel purified, and used as competitors. Dried gels were exposed to x-ray film and the autoradiographs were scanned and imported into ADOBE PHOTOSHOP (Adobe Systems, Mountain View, CA). Total image contrast was adjusted by means of the autocontrast function and the image was printed as a dye sublimation print.

Generation of Transgenic Tobacco Lines Containing Site-Directed Mutant *cab2* Promoter–*luc* Fusions. The CUF-M and CGF-G3M top-strand oligonucleotides described above were used to introduce the respective mutations into each of two contexts of the native *CAB2* promoter: −199 to +1 and −142 to +1. The respective *CAB2* promoter fragments in plasmid pBluescript II KS: (−) (Stratagene) were mutagenized with the Muta-Gene phagemid *in vitro* mutagenesis kit (Bio-Rad) and mutant clones were verified by nucleotide sequence analysis. The mutant *cab2* fragments were isolated as *Bam*HI–*Hin*dIII fragments and cloned into the binary vector pMON721 (Monsanto) fused to the *luc* reporter gene as described in Fig. 2. The mobilization of binary vector constructs into the *Agrobacterium* strain ABI, construct verification, and the generation and selection of transgenic tobacco plants containing the *cab2*::Ω::*luc* fusions were performed as described (7). The generation and characterization of transgenic tobacco lines containing the 5′ *cab2* deletions (−322 to +1, −199 to +1, −142 to +1, and −111 to +1 *cab2*::Ω::*luc*) were described previously (7).

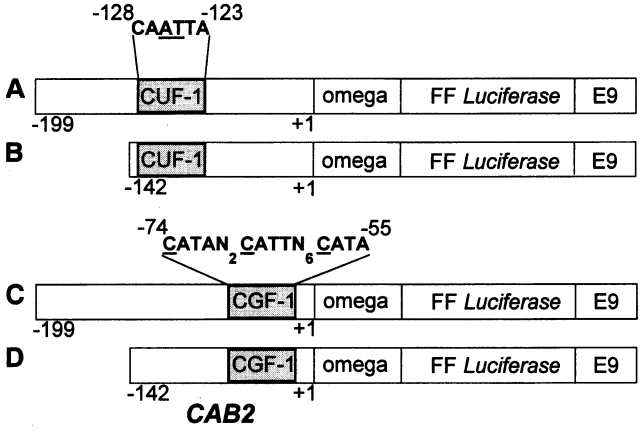

FIG. 2. *cab2*::Ω::*luc* constructs used for *in vivo* assay of *CAB2* expression in transgenic tobacco. *CAB2* promoter fragments (nt −199 to +1 and −142 to +1) containing site-directed mutations in the CUF-1 and CGF-1 binding site were fused to the tobacco mosaic virus translational enhancer [omega (Ω)] sequence, fused to the firefly (FF) luciferase coding region followed by the poly(A) addition sequence from pea *RBCS-E9* in the polylinker of the binary vector pMON721 (not shown) to create the constructs: −199 to +1 CUF-M *cab2*::Ω::*luc* (*A*), −142 to +1 CUF-M *cab2*::Ω::*luc* (*B*), −199 to +1 CGF-G3M *cab2*::Ω::*luc* (*C*), and −142 to +1 CGF-G3M *cab2*::Ω::*luc* (*D*). Locations of the CUF-1 and CGF-1 binding sites are indicated by stippled boxes, and the mutated core binding-site sequences are indicated above the respective sets of constructs. Nucleotides which differ from wild type are underlined.

Analyses of Promoter Activities in Transgenic Plants. T_2 seeds from T_1 lines carrying the *cab2*::Ω::*luc* fusions were grown on solid Murashige–Skoog medium containing 3% sucrose. All growth conditions and manipulations of light- and dark-grown plants were as described (7). *luc* expression was measured with 2-hr time resolution in transgenic seedlings sprayed with 1 mM luciferin in 0.01% Triton X-100 prior to each time point by *in vivo* imaging and quantitation of luciferase bioluminescence as mean photons per seedling per min (5, 7), using an intensified camera and photon-counting image processor (Hamamatsu Photonic Systems, Bridgewater, NJ). The mean and standard error of the mean were calculated for the amplitude of the circadian oscillation and for the peak 2/peak 1 ratio of *luc* expression in 5′ deletion, CUF-M, and CGF-G3M *cab2*::Ω::*luc* lines as described in *Results*. The means were compared by Welch's *t* test and significance was taken to be 5%.

RESULTS

CUF-M and CGF-G3M Mutations Reduce Factor–DNA Binding *in Vitro*. Gel retardation and competition assays were performed to characterize the effect of site-directed mutations of the CUF-1 and CGF-1 binding sites in the *CAB2* promoter on protein–DNA interactions *in vitro*. The CUF-1 oligonucleotide effectively competed for formation of the CUF-1 complex at 25× molar excess (Fig. 3, lane 3), whereas the CUF-M oligonucleotide, containing the ACGT → AATT mutation in the core of the binding site, did not compete for binding to CUF-1 at the same molar excess (lane 4). Similarly, formation of the CUF-1 complex was blocked with the −199 to +1 *CAB2* fragment at 25× molar excess (lane 5) to an extent comparable to competition with the CUF-1 oligonucleotide. The −199 to +1 CUF-M *cab2* fragment did not compete for binding to CUF-1 (lanes 9–12) at even 200× molar excess (lane 12), demonstrating that the CUF-M mutation effectively abolishes CUF-1 interaction with the *CAB2* promoter *in vitro*.

CGF-1 binding to the −111 to −33 *CAB2* probe (Fig. 4*A*, lane 2) was effectively inhibited by the unlabeled −111 to −33 *CAB2* fragment (lanes 3 and 4) and the CGF-1 oligonucleotide (lanes 5–7). In comparison, the CGF-G3M oligonucleotide, in which the first G of each of the three repeated GATA motifs required for CGF-1 binding was mutated to C, did not compete appreciably for binding to CGF-1 even at 200× molar excess (lane 10). The CGF-G3M mutation in the −111 to −33 *cab2*

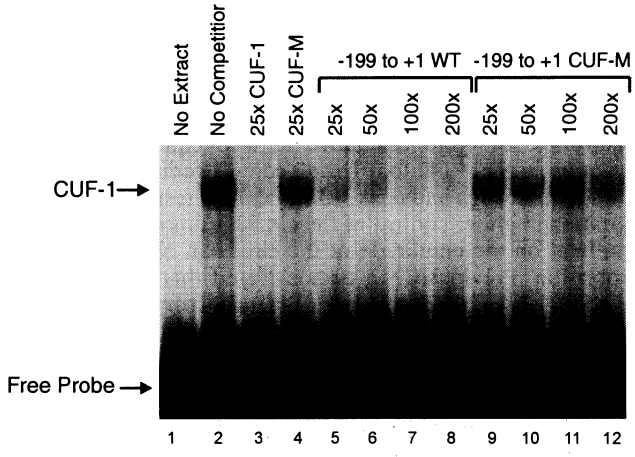

FIG. 3. The CUF-M mutation disrupts CUF-1 binding *in vitro*. Gel retardation assays were performed with 10 fmol of the radiolabeled CUF-S oligonucleotide as a probe. For lanes 2–12, assay mixtures contained 2 μg of tobacco nuclear proteins; lane 2 shows the CUF-1 complex in the absence of competitor DNA. The fold molar excess of oligonucleotide and *CAB2* fragment competitors is indicated above each lane. WT, wild type.

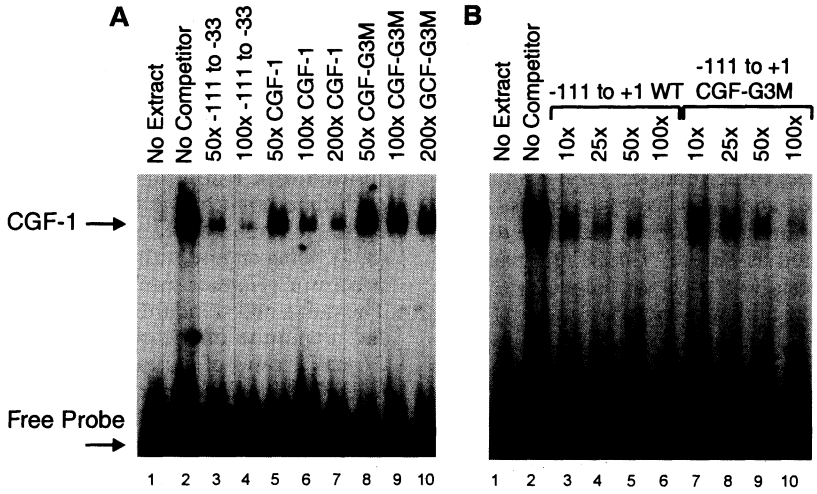

FIG. 4. The CGF-G3M mutation attenuates CGF-1 binding *in vitro*. Gel retardation assays were performed with 10 fmol of the radiolabeled −111 to −33 *CAB2* fragment as a probe. For lanes 2–10, assay mixtures contained 0.4 and 0.8 μg of tobacco nuclear proteins in *A* and *B*, respectively. Lanes 2 show the CGF-1 complex in the absence of competitor DNA. The fold molar excess of oligonucleotide and *CAB2* fragment competitors is indicated above each lane. WT, wild type.

context resulted in a moderate but reproducible decrease in CGF-1 binding *in vitro* (Fig. 4*B*, lanes 7–10), relative to the wild-type −111 to −33 *CAB2* fragment (lanes 3–6).

Effect of the CUF-M and CGF-G3M Mutations on Circadian Clock-Regulated *cab2*::Ω::*luc* Expression *in Vivo*. The identification of site-directed mutations which interrupt specific nuclear factor–*CAB2* promoter interactions *in vitro* provides an effective tool for the assay of the role of CUF-1 and CGF-1 binding to the *CAB2* promoter in regulating expression *in vivo*. Low-light video imaging of *luc* bioluminescence in seedlings of independent transgenic tobacco lines with −199 to +1 wild type, CUF-M, and CGF-G3M *cab2*::Ω::*luc* transferred to continuous light at "ZT" 0 revealed a circadian oscillation in *luc* reporter gene expression for each fusion (Fig. 5). [ZT (*Zeitgeber* time) is the time in hours from the onset of illumination; thus ZT 0 is at dawn.] Similar results were observed for −142 to +1 wild-type, CUF-M, and CGF-G3M *cab2*::Ω::*luc* seedlings (data not shown). However, quantitative differences in the expression patterns in the mutant lines relative to that in representative wild-type −199 to +1 or −142 to +1 *cab2*::Ω::*luc* seedlings were observed. Overall, the CUF-M mutation in either *CAB2* promoter context (−199 to +1 or −142 to +1) had no effect on the phase or period of the oscillation in bioluminescence (Fig. 5*A* and data not shown). In the −199 to +1 CUF-M *cab2*::Ω::*luc* lines the expression level at both the trough and the peak of the oscillation calculated at ZT ≈18 and 30, respectively, was reduced an average of ≈2-fold relative to wild type. The CUF-M mutation in the −142 to +1 *cab2* context reduced expression at both the trough and peak ≈32-fold. As a result, the CUF-M mutation in either context of the *CAB2* promoter had little or no effect on the amplitude of the circadian oscillation in *luc* expression. Previously, we demonstrated that progressive 5′ deletion of the *CAB2* promoter sequences from −322 to −142 had no significant effect on the amplitude of the oscillation in *luc* expression, which averaged ≈15-fold (7). The amplitude of the circadian oscillation in *luc* expression in the −111 to +1 *cab2*::Ω::*luc* lines, which lack the CUF-1 binding site, was 2-fold, suggesting that although CUF-1 is not required, it may contribute to the high-amplitude oscillation (7).

In comparison to the 5′ deletion results, the amplitudes of the *luc* oscillation, calculated for the −199 to +1 CUF-M *cab2*::Ω::*luc* lines at 12.5 ± 1.8 ($P = 0.1014$) and the −142 to +1 CUF-M *cab2*::Ω::*luc* lines at 16.2 ± 3.0 ($P = 0.7724$), were not significantly different from the average amplitude observed for the 5′ deletion endpoints from −322 to −142 of the wild-type *CAB2* promoter. The absence of any alteration in the period, phase, or amplitude of the circadian oscillation in the CUF-M lines, although the expression level was reduced relative to wild-type, suggests CUF-1 is a general transcriptional activator which positively influences the overall *CAB2* expression level.

Like the CUF-M mutation, the CGF-G3M mutation had no effect on the phase or period of the circadian oscillation in *luc* expression relative to wild type in either context of the *CAB2* promoter (Fig. 5*B* and data not shown). In contrast to the CUF-M mutation, the CGF-G3M mutation differentially reduced the peak and trough levels. Peak and trough levels were

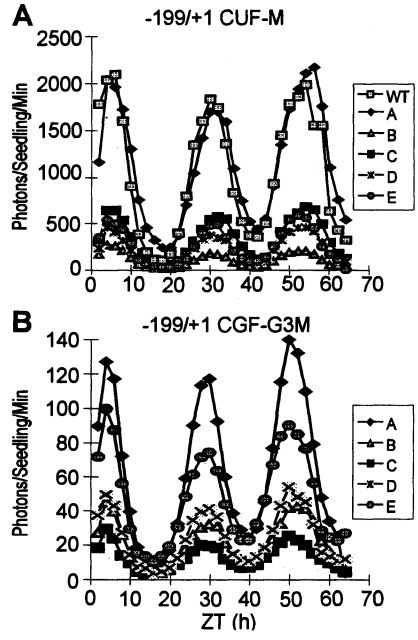

FIG. 5. CUF-M and CGF-G3M *cab2*::Ω::*luc* fusions are circadian-regulated *in vivo*, but at reduced expression levels relative to wild type (WT). The mean number of photons per seedling per min was monitored for 8-day-old light/dark-grown independent transgenic tobacco lines transferred to continuous light at ZT 0 (see text for definition of ZT). *cab2*::Ω::*luc* fusions were as follows: −199 to +1 CUF-M, five lines (*A*), −199 to +1 CGF-G3M, five lines (*B*). Data from a representative wild-type −199 to +1 *cab2*::Ω::*luc* line are shown for comparison (*A*).

reduced ≈32- and ≈15-fold, respectively, in the −199 to +1 context and ≈100- and ≈10-fold, respectively, in the −142 to +1 context, resulting in a statistically significant reduction in the mean amplitude of the circadian oscillation. The mean amplitudes for the −199 to +1 and −142 to +1 CGF-G3M *cab2*::Ω::*luc* lines were 8.3 ± 1.3 (P = 0.0003) and 1.5 ± 0.4 (P = 0.0001), respectively. The −142 to +1 CGF-G3M *cab2* mutation reduced *luc* expression to the extent that the trough levels were above background in only two of six lines tested. The specific amplitude-reducing effect of the CGF-G3M mutation to preferentially reduce peak compared with trough levels of the circadian oscillation in *luc* expression strongly suggests CGF-1 is a positive regulator of the amplitude of the circadian oscillation in *CAB2* expression.

Effect of the CUF-M and CGF-G3M Mutations on Phytochrome-Regulated *cab2*::Ω::*luc* Expression *in Vivo*. In etiolated tobacco seedlings, *CAB* transcription oscillates with a low amplitude and a period of ≈30 hr (5). Brief red-light treatment induces a characteristic cyclic *CAB* expression pattern with a brief peak at ≈4 hr, a second broader peak at ≈20 hr (5, 7), and subsequent peaks at up to 52 hr (5) after the onset of the light treatment. This red-light-induced *CAB* expression in etiolated seedlings is attenuated by subsequent irradiation with far-red light, and the response is therefore thought to be mediated by phytochrome (2). The brief peak in *CAB* expression at 4 hr is proposed to be an acute response to phytochrome activation independent of the circadian clock, with the subsequent peaks corresponding to the high-amplitude cyclic oscillation in *CAB* expression induced by phytochrome activation (2).

Etiolated seedlings containing the CUF-M and CGF-G3M mutations in both the −199 to +1 and −142 to +1 contexts of the *CAB2* promoter showed an acute response in *luc* expression to phytochrome activation relative to the dark controls. Typical results are shown for representative −199 to +1 wild-type, CUF-M, and CGF-G3M *cab2*::Ω::*luc* lines (Fig. 6); similar results were observed for the −142 to +1 *cab2*::Ω::*luc* lines (data not shown). As with circadian clock regulation of expression, the CGF-G3M mutation results in a quantitative difference in the pattern of *luc* expression, relative to wild type, which was not observed in the CUF-M lines. The −199 to +1 wild-type and CUF-M *cab2*::Ω::*luc* lines showed the characteristic response to red-light irradiation (Fig. 6 *A* and *B*, respectively). However, in the −199 to +1 CGF-G3M *cab2*::Ω::*luc* seedlings the initial peak in *luc* expression at 4 hr in response to red light was dramatically attenuated relative to the subsequent peak at 20 hr (Fig. 6*C*). The ratio of *luc* expression calculated as the mean photon counts per seedling per min of the second peak relative to the first peak (peak 2/peak 1 ratio) was used to compare the effect of the respective mutations on the acute response to phytochrome activation on *CAB2* expression (Fig. 7). The peak 2/peak 1 ratio was ≈2 for all representative 5′ *cab2* deletion lines, including the −111 to +1 *cab2*::Ω::*luc* line, in which the CUF-1 binding site has been deleted (Fig. 7). The peak 2/peak 1 ratios for two independent −199 to +1 CUF-M *cab2*::Ω::*luc* and −142 to +1 CUF-M *cab2*::Ω::*luc* lines were not significantly different from their respective wild-type lines, suggesting that CUF-1 does not contribute to the pattern of phytochrome-responsive *CAB2* expression. In comparison, two independent −199 to +1 CGF-G3M *cab2*::Ω::*luc* lines gave a significant increase in the peak 2/peak 1 ratio, to 13.5 ± 2.9 (P = 0.0056) and 10.6 ± 1.4 (P = 0.0230), respectively (Fig. 7). These increases in the peak 2/peak 1 ratio were due to the attenuation of the first peak of *CAB2* transcription (Fig. 5*C*). For a single −142 to +1 CGF-G3M *cab2*::Ω::*luc* line the mean peak 2/peak 1 ratio, 8.0 ± 2.9, was ≈4-fold higher than for the wild-type −142 to +1 *cab2*::Ω::*luc* line. However, this difference is not quite significant (P = 0.088) and is likely to be due to the increased variability in the low peak 1 levels, which were at or near the

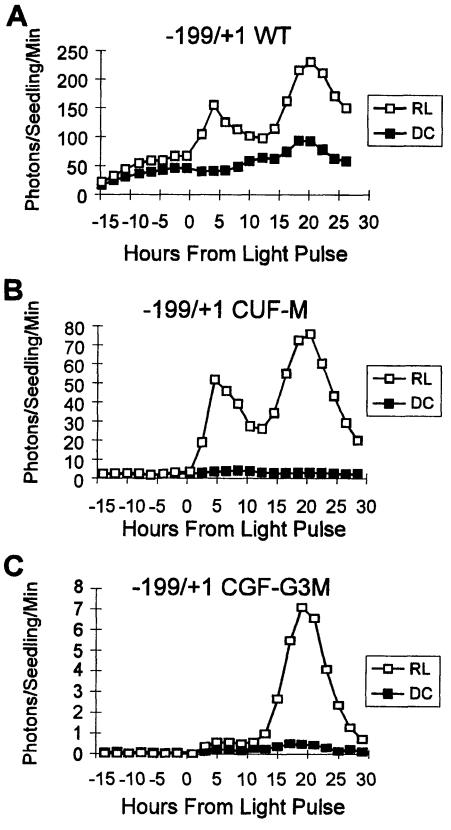

FIG. 6. The CGF-G3M mutation attenuates the initial peak in *CAB2* transcription in response to phytochrome activation. The mean number of photons per seedling per min was monitored for 7-day-old etiolated transgenic tobacco seedlings which received a 2-min red-light pulse at time 0 (RL) and from dark controls (DC). The *cab2*::Ω::*luc* lines were as follows: −199 to +1 (*A*), −199 to +1 CUF-M (*B*), and −199 to +1 CGF-G3M (*C*). Data are from one representative experiment. WT, wild type.

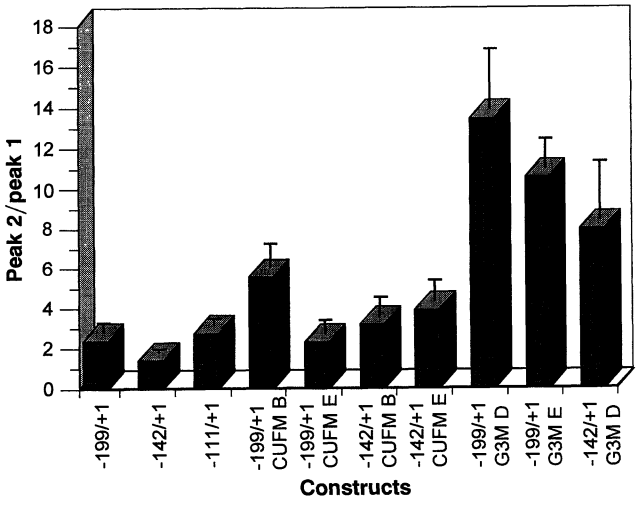

FIG. 7. Quantitation of the effect of the CUF-M and CGF-G3M mutations on phytochrome-responsive *CAB2* expression. The peak 2/peak 1 ratio was calculated for representative 5′ deletion, CUF-M, and CGF-G3M *cab2*::Ω::*luc* lines. The number of repetitions of the experiment for each line was as follows: n = 4 for −199 to +1, −142 to +1, −111 to +1, and −199 to +1 CUF-M lines B and E and −142 to +1 CUF-M lines B and E; n = 5 for −199 to +1 CGF-G3M lines D and E and −142 to +1 CGF-G3M line D.

background noise level. Attenuation of the first peak in *luc* expression in red-light-pulsed etiolated CGF-G3M mutants is consistent with a specific role of CGF-1 in mediating the initial acute peak in *CAB2* expression in response to phytochrome activation.

DISCUSSION

We have demonstrated in this study distinct functional roles for CUF-1 and CGF-1 in the regulation of *CAB2* transcription. The use of site-directed mutagenesis to specifically interrupt the CUF-1–*CAB2* interaction in the context of the native *CAB2* promoter provides a more accurate assay of cis- and trans-acting factor function in *CAB2* gene expression than 5′ deletion analyses, which may be complicated by the cumulative effects of the removal of multiple putative DNA–protein interactions. The abolition of CUF-1 binding *in vitro* by the CUF-M mutation correlates with reduced expression levels in the absence of any change in the pattern (i.e., timing or amplitude) of the circadian oscillation or phytochrome-responsive expression in CUF-M *cab2*::Ω::*luc* lines *in vivo*. These results demonstrate that CUF-1 is a general positive transcription factor which increases *CAB2* expression and is not required for clock- or phytochrome-regulated *CAB2* transcription.

Although the CGF-G3M mutation only moderately attenuates CGF-1 binding *in vitro*, this mutation has a dramatic effect *in vivo*, including quantitative effects on the amplitude of the circadian oscillation and attenuation of the acute response in *CAB2* transcription to phytochrome activation. The effect of the CGF-G3M mutation on *CAB2* expression *in vivo* can be understood in terms of the model for the interactions between phytochrome and the circadian clock (Fig. 1). One would predict an increase in trough levels—and possibly in peak expression levels, as well—if a promoter mutation disrupted the interaction of a negative circadian clock-responsive element with its respective protein factor. The effect of the CGF-G3M mutation to reduce *luc* reporter expression in green tissue, differentially reducing peak levels to a greater extent than the reduction in trough levels, is inconsistent with the function of the CGF-1 binding site as a negative circadian clock-responsive element. Instead, CGF-1 activates *CAB2* transcription in green tissue. The reduced amplitude in the CGF-G3M lines mimics damping of the oscillation in CAB expression observed in green seedlings transferred to continuous darkness (6, 14) and thus suggests that CGF-1 mediates light regulation of amplitude. Moreover, CGF-1 mediates the initial acute peak in *CAB2* transcription in response to phytochrome activation in etiolated seedlings, leading us to propose that CGF-1 is a phytochrome-responsive transcription activator. Tobin and coworkers (15) have recently demonstrated the functional requirement for two regions of a *Lemna Lhcb* gene promoter (*cabAB19*) necessary for phytochrome regulation, one of which contains a GATA motif similar to the CGF-1 binding site within the *Arabidopsis CAB2* promoter.

Furthermore, if phytochrome regulates the phase and period of *CAB* expression by modulating the circadian clock, as proposed in the model for *CAB* regulation (2), mutations which decrease or eliminate interaction of a phytochrome-responsive element with its respective trans-acting protein should have no effect on the phase or period of *CAB* transcription. The CGF-G3M mutation did not alter the phase or period of cyclic *luc* expression in etiolated seedlings or the circadian oscillation in *luc* expression in green seedlings relative to wild-type. This then provides support for phytochrome regulation of phase and period of the *CAB2* oscillation via input pathways to the circadian clock (Fig. 1).

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