Identification of an *Arabidopsis thaliana* Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase (RCA) Minimal Promoter Regulated by Light and the Circadian Clock

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Transcription of the *Arabidopsis thaliana* gene encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase (RCA) is organ-specific, light-responsive, and regulated by the circadian clock. RCA is transcribed throughout the green parts of the plant, but not in roots and petals. Responses elicited by short pulses of light indicate that the light response is mediated, at least in part, by phytochrome. Analysis of transgenic tobacco and *Arabidopsis* carrying RCA 5' untranscribed regions fused to reporter genes (uidA, encoding β-glucuronidase, or cat, encoding chloramphenicol acetyltransferase) indicate that elements sufficient to confer organ-specific, light-responsive, and clock-regulated transcription are localized within 317 base pairs upstream of the site of transcription initiation. A clock-responsive element sufficient to confer a low-amplitude (approximately 2-fold) circadian oscillation lies within 317 base pairs of the transcription start, but other elements necessary for high-amplitude (approximately 10-fold) circadian oscillation lie upstream of −317 and are removed by deletion from −970 to −317.

The first steps of the competing reactions of photosynthetic carbon assimilation and photorespiration are catalyzed by Rubisco, an enzyme located in the chloroplast stroma (Woodrow and Berry, 1988). The Rubisco holoenzyme is assembled in a catalytically inactive form and is activated by posttranslational modification, the binding of CO₂ and Mg²⁺ to a Lys residue near the catalytic site of the Rubisco large subunit (Miziorko and Lorimer, 1983). Under physiological conditions, this activation process is catalyzed by Rubisco activase in two sequential, light-requiring steps (Portis, 1992). It is thought that Rubisco activase promotes the dissociation of ribulose bisphosphate and other inhibitory sugar phosphates from the Rubisco active site in a process requiring the hydrolysis of ATP (Portis, 1992). In vivo, Rubisco activase is essential for the activation of Rubisco; mutants lacking Rubisco activase are conditionally lethal and require elevated CO₂ concentrations for growth (Somerville et al., 1982). In transgenic tobacco, antisense inhibition of Rubisco activase expression at the levels of both mRNA and protein accumulation reduces the rate of CO₂ assimilation and plant growth (Jiang et al., 1994).

The expression of Rubisco activase is highly regulated. Data gathered in several species indicate that the expression of RCA is organ-specific, developmentally regulated according to leaf age, and light-inducible (Zielinski et al., 1989; Rundle and Zielinski, 1991; Orozco and Ogren, 1993; Watillon et al., 1993). The expression of RCA of several species, including *Arabidopsis thaliana* (Pilgrim and McClung, 1993), tomato (Martino-Catt and Ort, 1992), and apple (Watillon et al., 1993), is regulated by the circadian clock, and in Arabidopsis and tomato this clock regulation has been shown by nuclear run-on analysis to include a transcriptional component (Martino-Catt and Ort, 1992; Pilgrim and McClung, 1993). It is likely that these complex expression patterns reflect the combined results of the interactions of multiple trans-acting protein factors with multiple cognate cis-acting DNA elements (Benfey et al., 1990; Schindler and Cashmore, 1990; Schindler et al., 1992). Although clock-regulated gene expression is widespread in plants (Kay and Millar, 1993; McClung, 1993; Piechulla, 1993; McClung and Kay, 1994), much less is known about the regulation of gene expression by the circadian clock than is known about, for example, the regulation of gene expression by light (Millar et al., 1994; Terzaghi and Cashmore, 1995). In this work we characterize the transcription of the Arabidopsis RCA gene in response to organ-specific signals, to light, and to the circadian clock. We demonstrate that a small, 330-bp promoter fragment of the Arabidopsis RCA gene is sufficient to confer normal transcriptional responses to organ-specific cues, to light, and to the circadian clock.

Abbreviations: CAB, gene encoding chlorophyll a/b binding proteins; CaMV, cauliflower mosaic virus; cat, chloramphenicol acetyltransferase; CRE, clock-responsive element; LRE, light-responsive element; MUG, 4-methylumbelliferyl-β-D-glucuronide; RBGS, gene encoding Rubisco small subunit; RCA, gene encoding Rubisco activase; uidA, gene encoding GUS.
MATERIALS AND METHODS

DNA Constructs

All nucleic acid manipulations employed standard methods (Ausubel et al., 1995). For construction of RCA-uidA fusions, a series of RCA promoter fragments were fused with the uidA reporter gene in pBI101.1 (Jefferson et al., 1987). First, the out-of-frame AUG initiator codon in the SpAI recognition site in the pBI101.1 polylinker was eliminated by digestion with HindIII and SalI, treatment with the Klenow fragment of DNA polymerase I to fill in the overhangs, and re-ligation to form pR410. This recreated the HindIII site but eliminated the SpAI, PstI, and SalI sites. A set of nested deletions prepared during the DNA sequence determination of the RCA genomic locus (Orozco et al., 1993) was then ligated into the HindIII site of pR410 to yield a series of RCA-uidA constructs in which transcription of uidA was dependent upon a series of RCA upstream sequences that had been progressively truncated from the 5' end. In each case, the 3' base of the RCA fragment was nucleotide +13 and +9, relative to the two endogenous Arabidopsis thaliana RCA transcription initiation sites (Werneke and Ogren, 1989). A parallel series of RCA-cat transcriptional fusions was prepared in the binary vector pBIN19 (Bevan, 1984). For these constructs, the cat gene was subcloned as a BamHI fragment from GRE-CAT (Schena et al., 1991) into pR106 (Liu and Sanford, 1993) such that cat was transcribed from the CaMV 35S promoter sequences, again at the HindIII site at +13/+9 relative to the endogenous RCA transcriptional start sites. All junction sites were verified by DNA sequence analysis.

Generation of Transgenic Plants

Binary vector constructs were transformed into Agrobacterium tumefaciens strain GV3101pMP90 (Koncz and Schell, 1986) by freeze-thaw. Agrobacterium-mediated transformation of Arabidopsis ecotype Columbia was performed by vacuum infiltration of adult plants (Bchtold et al., 1993; Bent et al., 1994). The seeds harvested from infiltrated plants were plated on selection plates containing Murashige and Skoog (1962) medium containing Murashige and Skoog (1962) mineral salts supplemented with Suc (3%), myo-inositol (2 μg/mL), thiamine (0.2 μg/mL), pyridoxine HCl (0.2 μg/mL), nicotinic acid (0.2 μg/mL), and kanamycin (50 μg/mL), and solidified with 0.9% agar.

Light Treatments

RCA mRNA abundance was determined in Arabidopsis seedlings grown in continuous light or in continuous dark as described previously (Zhong et al., 1994). For light-pulse experiments, 10-d-old, etiolated, transgenic Arabidopsis seedlings carrying the RCA-uidA 5' deletion series constructs or untransformed Arabidopsis ecotype Columbia seedlings were exposed to 5- or 15-min pulses of white light, red light, far-red light, or red light followed by far-red light. Seedlings were then returned to the dark. Seedlings were harvested at 3, 12, and 24 h after the light pulse. Red light (18 μmol m⁻² s⁻¹, between 600 and 700 nm) was provided by four white fluorescent lights (model TL-741, Philips, Somerset, NJ) filtered through a 6-cm depth of 1.5% CuSO₄ (to absorb the heat) and red plexiglass (model no. 2423, Rohm and Haas, Philadelphia, PA). Far-red light (10 μmol m⁻² s⁻¹, between 700 and 800 nm; <0.01 μmol m⁻² s⁻¹, between 300 and 700 nm) was provided by two 90-W halogen lamps (model no. GE 90 PASR/FL/HAL, General Electric) filtered through a water bath and plexiglass (model no. FRF700, Westlake Plastics, Lenni, PA). Fluence rates (for red and white light) were monitored with a quantum photometer (model no. LI-189, Li-Cor, Lincoln, NE). In addition, each light source, including that for far-red light, was tested by scanning from 300 to 800 nm with a radiometer-photometer (model no. LI-1800, Li-Cor), and the fluences delivered to the plants were derived from these scans.

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 (Nagy et al., 1988a) with the modifications previously described (Zhong et al., 1994). Aliquots of total RNA (5 μg) were loaded on reinforced nitrocellulose (Schleicher & Schuell) using a MiniGel II apparatus (Schleicher & Schuell). The membranes were then baked at 80°C under vacuum for 1 h. Hybridization conditions
were as previously described (Zhong et al., 1994). Following hybridization, mRNA abundance was quantified using a PhosphorImager with ImageQuant (Molecular Dynamics, Sunnyvale, CA). An Arabidopsis RCA probe was generated by labeling a 1.67-kb EcoRI fragment of pRCA4.2 (Werneke et al., 1988). For analysis of the expression of the RCA-cat gene fusions in transgenic plants, a 1.8-kb HindIII fragment containing the cat coding sequence (Schena et al., 1991) was labeled as a hybridization probe.

Histochemical GUS Assay

Fresh samples were immersed in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 (Sigma) and infiltrated under vacuum for 2 min. Samples were rinsed twice with 50 mM sodium phosphate buffer and then stained with 1 mM 5-bromo-4-chloro-3-indoyl glucuronide in 50 mM phosphate buffer at 37°C (Jefferson, 1987). The staining solution was then removed and the samples were dehydrated by sequential changes of 25, 50, and 70% ethanol.

Image Processing

Photographic slides were scanned and digitized using an Eikonix model 1435 (Eastman Kodak). Brightness, contrast, and color balance were adjusted using Adobe Photoshop version 3.0 (Adobe Systems, Mountain View, CA). Composite pictures were printed using a silver halide printer (Pictography 3000, Fuji Photofilm USA, Elmsford, NY).

Quantitative GUS Assay

Seedlings were homogenized in extraction buffer (50 mM sodium phosphate buffer [pH 7.0], 1 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol) and clarified by centrifugation at 14,000g for 10 min at 4°C. After centrifugation, the samples were frozen at −80°C. Samples were assayed for GUS activity in extraction buffer containing 1 mM 4-methylumbelliferyl-β-D-glucuronide (Jefferson, 1987). Fluorescence was measured in a minifluorometer (model no. TKO100, Hoefer, San Francisco, CA). Protein concentrations were determined using the Bio-Rad protein assay.

RESULTS

Transcription of the RCA Promoter Is Organ-Specific

RCA mRNA was detected in leaves and inflorescences, including the stems, siliques, and flowers, but it was not detected in roots (Fig. 1). This pattern of RCA transcription was verified by histochemical staining for GUS activity of transgenic Arabidopsis seedlings expressing RCA-uidA fusions (Fig. 2). In addition, histochemical staining revealed strong RCA-GUS expression in pollen (Fig. 2F). That a 1330-bp fragment of the RCA upstream untranscribed region is sufficient to confer organ-specific expression on the GUS reporter gene indicates that the RCA promoter is transcribed in an organ-specific manner. Deletion from the 5' end of this promoter fragment established that a minimal promoter fragment extending from −317 to +13 was sufficient to drive GUS expression and that this 330-bp promoter fragment retained organ-specific expression (Fig. 2, C and E) in at least 12 independent transformants, but expression levels were reduced about 2-fold compared with expression levels supported by the longer promoter fragments (Fig. 3). Smaller promoter fragments extending from −140 to +13 or from −83 to +13 failed to drive detectable GUS expression in transgenic tobacco (data not shown). Both of these constructs retain a putative TATA sequence (−29 TATAAAT −23) and a potential CAAT sequence (−54 CGAAT −50), indicating that these sequences are not sufficient to drive transcription at rates greater than those seen in the promoterless control. That some element(s) in this region of the RCA promoter is essential for promoter activity was confirmed by deletion from the 3' end of the longest promoter fragment tested; a fragment extending from −1317 to −147 (i.e. from which the region from −147 to +13 was deleted) failed to yield GUS activity above that of the promoterless control (data not shown).

Light-inducible Transcription Is Conferred by a 330-bp Fragment of the RCA Promoter

Etiolated Arabidopsis seedlings accumulated little RCA transcript, but RCA transcript became abundant in light-grown seedlings, accumulating above etiolated levels 2 d after imbibition (Fig. 4). The full-length (−1317 to +13) and the truncated (−317 to +13) Arabidopsis RCA promoter fragments conferred this regulation on the GUS reporter in transgenic Arabidopsis. GUS activity was readily detected in light-grown seedlings (Fig. 2, A and C). In contrast, little GUS activity was detected in etiolated seedlings (Fig. 2B; data not shown for the −317 to +13 construct).

To examine the responsiveness of RCA expression to illumination of etiolated seedlings, wild-type Arabidopsis
Figure 2. Histochemical localization of GUS expression in transgenic Arabidopsis seedlings carrying RCA-GUS fusion constructs. A, Arabidopsis seedling (magnified 5.1X) expressing an RCA (-1317 to +13)-uidA construct grown in continuous light for 7 d. B, As in A, but etiolated by growth in continuous dark (7 d). C, Arabidopsis seedling (magnified 11.9X) expressing an RCA (-317 to +13)-uidA construct grown in continuous light for 7 d. D, Arabidopsis flower (magnified 8.5X) expressing a RCA (-1317 to +13)-uidA construct showing staining in sepals and anthers. E, As in D, but expressing an RCA (-317 to +13)-uidA construct. F, Anther (magnified 51X) of transgenic Arabidopsis expressing the RCA (-1317 to +13)-uidA construct.

(Columbia ecotype) seedlings were grown in complete darkness for 10 d and then subjected to 15-min light pulses. Using a RCA-specific probe, we detected the accumulation of endogenous RCA transcripts 3 h (Fig. 5) and 12 h (data not shown) after irradiation. The induction of RCA expression was greater at 3 h than at 12 h after the light pulse (data not shown). All light treatments, including white, red, far red, red followed by far red, and blue light, stimulated the accumulation of RCA mRNA relative to plants maintained in the dark. Therefore, the accumulation of RCA mRNA upon illumination is mediated at least in part by phytochrome, although these results do not exclude a role for a blue-light receptor.

To determine if the light-mediated induction of RCA expression includes a transcriptional component, we grew in darkness transgenic Arabidopsis seedlings carrying various RCA-uidA fusions, and then tested for phytochrome responsiveness by providing a 5-min pulse of white, red, far red, or red followed by far-red light. GUS activity was determined at 3-, 12-, and 24-h intervals following the light pulses and induction was greatest 3 h after illumination; these data are presented in Figure 6.

As expected, Arabidopsis carrying a promoterless control construct did not respond to any of the light treatments (Fig. 6, PL). Each of the RCA promoter constructs tested (-1317, -970, and -317) showed light inducibility. The -1317 and -970 RCA promoter fragments responded similarly in terms of the level of induction in response to light; white light achieved 7- to 9-fold induction, which is greater than that observed with endogenous RCA mRNA (Fig. 5). Presumably, this reflects the greater stability of GUS reporter protein activity relative to endogenous RCA mRNA stability. Similarly, Sun et al. (1993) observed greater light induction with reporter gene constructs than with endogenous Arabidopsis CAB mRNA. Red light was as effective for induction of RCA transcription as white light. Far-red light also induced RCA transcription but was less effective than red light. Induction in response to red light followed by far-red light was quantitatively similar to that in response to far-red light alone. With deletion of the RCA promoter sequences to -317, the magnitude of the response to light pulses was lower (approximately 5-fold) than that seen with the -1317 and -970 constructs, but the -317 construct responded to each of the light treatments (Fig. 6). These results, taken together with the inductive effects of the various light treatments on endogenous RCA mRNA abundance in Arabidopsis, suggest a role for phytochrome in the response of RCA transcription to illumination. We found that 330 bp (from -317 to +13) of the RCA 5' region is sufficient to confer
light inducibility in etiolated seedlings, and is mediated at least in part by phytochrome.

330 bp of the RCA Promoter Is Sufficient to Confer Circadian Regulation

We used the cat reporter to test two RCA-cat fusions for circadian regulation because previous work had indicated that cat mRNA was sufficiently unstable in transgenic tobacco to allow the detection of circadian transcription from CAB promoters (Nagy et al., 1988b; Fejes et al., 1990). Three independently transformed lines were tested for each construct. We found that when the RCA fragment from -970 to +13 was used to transcribe the cat reporter a robust oscillation (approximately 10-fold in amplitude) was observed in a light-dark cycle, and that this oscillation persisted with similar amplitude for at least two circadian cycles in plants transferred into continuous light (Fig. 7). In contrast, when the cat gene was driven by the CaMV 35S promoter, we detected a high level of cat mRNA but observed no circadian oscillations in cat mRNA abundance (data not shown), as has been reported elsewhere (Millar et al., 1992b; Anderson et al., 1994). Circadian regulation of cat transcription was retained after deletion to -317 of the RCA promoter fragment, although amplitude of the oscillation was reduced from approximately 10-fold to approximately 2-fold (Fig. 7). As was observed with RCA-uidA fusions (Fig. 2) the level of expression of the cat reporter gene when transcription was driven by the -317 RCA promoter fragment was reduced relative to the expression seen with the -970 RCA promoter fragment.

DISCUSSION

The expression of Arabidopsis RCA is highly regulated and responds to organ-specific signals, to light, and to the circadian clock. To determine the role of DNA sequence elements upstream of the transcriptional initiation site of the Arabidopsis RCA gene, a series of progressively truncated fragments from within the 5' region of the RCA gene were fused to uidA or cat reporter genes. Analysis of these gene fusions indicates that the expression pattern of the Arabidopsis RCA gene reflects regulation at the level of transcription and does not require elements residing within the transcribed portions (beyond +13) of the RCA locus.
within the spinach RCA promoter abolished expression (Orozco and Ogren, 1993). A tobacco nuclear protein, CUF-1, binds to the G-box-like ACGT core in the clock-regulated Arabidopsis CAB2 promoter (Anderson et al., 1994). Mutational analysis indicated that CUF-1 binding contributes to high-level CAB2 transcription but does not contribute to phytochrome or clock regulation (Anderson et al., 1994). In the Arabidopsis CAB1 promoter deletion of a site overlapping an ACGT core that is bound by a distinct binding activity, CA-1, results in the loss of detectable expression (Sun et al., 1993). In addition to a possible role of the G-box (or the related ACGT core) in RCA expression

The longest construct tested (−1317 to +13) and a shorter fragment (−970 to +13) each recapitulate endogenous expression in terms of organ specificity and light induction (Figs. 1, 2, 4, 5, and 6). Deletion to −317 resulted in reduced GUS expression (Fig. 3), suggesting the deletion of a critical element with a function that limits RCA expression. The sequences between −317 and +13 contain elements sufficient to confer organ-specificity, light induction in etiolated seedlings, and circadian regulation. Although each of these regulatory properties is lost with deletion of the sequences between −317 and −140, we cannot be certain that elements responsible for these three levels of regulation all lie within that 177-bp fragment. Sequences between −317 and −140 are required for transcription above the background defined by a promoterless control. It is possible that some or all of the regulatory properties are retained in the −140 to +13 fragment but remain undetected because of the loss of a separate positively acting, enhancer-like element required for expression above background. For example, the G-box element (Gilmartin et al., 1990) is essential for transcription of a number of genes and there is a consensus G-box at position −265 to −258 in the Arabidopsis RCA promoter. Deletion of a similar G-box consensus element

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Light-regulated transcription of progressively truncated RCA promoter fragments. Effects of irradiation with white, red, far-red, and red followed by far-red light on transcription driven by different RCA promoter fragments. Arabidopsis seedlings carrying various RCA-uidA fusions were grown for 10 d in continuous dark and then either exposed to a 5-min pulse of light treatment as indicated and returned to the dark for 3 h, or not irradiated. GUS activity was measured fluorometrically in extracts prepared from irradiated and from nonirradiated seedlings. Effects of light pulses are shown for four constructs: −1317, the full-length (−1317 to +13) RCA promoter fragment; −970, the −970 to +13 RCA promoter fragment; −317, the −317 to +13 RCA promoter fragment; and PL, a vector control containing a promoterless uidA gene. Twelve lines derived from independent transformation events were analyzed for each construct. Fold induction by light is defined as the ratio of GUS activity in the irradiated versus the nonirradiated seedlings. Data, presented as mean ± se for the pools of 12 lines, are shown for the plants returned to the dark for 3 h following the light pulses. Similar results were observed when plants were returned to the dark for 12 and 24 h following the light pulses (data not shown).

![Figure 7](https://example.com/figure7.png)

**Figure 7.** A short (330 bp) region of the Arabidopsis RCA promoter is sufficient to confer circadian transcription on a reporter gene. Tobacco seedlings (14 d old) transformed with RCA-cat fusions were transferred from a light-dark cycle into continuous light, and cat mRNA was measured at timed intervals. A, The RCA promoter fragment extending from −970 to +13 conferred robust high-amplitude (approximately 10-fold) cycling on the reporter cat gene that persisted in continuous light (●). B, The shorter RCA promoter fragment extending from −317 to +13 (○) also conferred robust circadian oscillation, albeit at reduced amplitude (approximately 2- to 3-fold). cat mRNA abundance was quantified with a PhosphorImager (Molecular Dynamics) and is presented relative to rRNA abundance, which was relatively constant throughout the experiment (data not shown). Data for each construct are presented as mean ± se for at least three independently transformed lines. For each line, the minimum cat transcript abundance (relative to rRNA) was defined as 1.0 (arbitrary units) and all other points are expressed relative to that value. Thus, comparison of absolute cat transcript abundances between the two constructs based on these data is not valid. However, as indicated in Figure 3 promoter activity of the −970 fragment is stronger than that of the −317 fragment.
sequences between −140 and +13, such as TATA and/or CAAT boxes but possibly other sequences, are required for transcription above the background defined by a promoterless control.

It is not yet clear whether organ-specific expression of Arabidopsis RCA results from a positively acting element(s) in green organs or from a negatively acting element leading to transcriptional repression in nongreen organs (roots and petals). For example, the as-2 sequence (a GATA motif), bound by the factor ASF-2, confers leaf-specific expression in transgenic tobacco (Lam and Chua, 1989; Gotor et al., 1993). However, a short deletion spanning a GT element in the spinach RCA promoter resulted in roots with 100-fold increased expression, suggesting the presence of a silencer in roots (Orozco and Ogren, 1993). However, specific mutation (by base substitution) of the GT element had no effect, indicating that the GT element per se was not acting as a silencer (Orozco and Ogren, 1993). This result possibly implicates a change in spacing between two or more elements as being responsible for the increased expression in roots (Orozco and Ogren, 1993).

Illumination induces RCA transcription in etiolated seedlings; a minimal promoter fragment (−317 to +13) retains light responsiveness. That this induction is mediated by brief pulses of red or far-red light suggests a phytochrome-mediated response (Mancinelli, 1994). However, we have not ruled out a possible role of blue-light response systems in the regulation of RCA transcription. The −317 promoter is less light-responsive than the −1317 and −970 promoters (Fig. 5), which suggests that the −317 promoter is missing one or more LREs present in the longer promoters. It should be noted, however, that these data do not establish that the effects of illumination of etiolated seedlings on RCA transcription represent a direct induction by light. Illumination of etiolated seedlings results in dramatic developmental changes (Kendrick and Kronenberg, 1994), and it is possible that the effects on RCA transcription result from these light-induced changes in growth and development.

The −970 to +13 RCA promoter fragment also displays circadian regulation. The amplitude of the circadian oscillations in cat mRNA abundance detected with the −970 to +13 promoter fragment is approximately 10-fold. A number of other studies have established that short regions of the wheat cab-1 (Nagy et al., 1988b; Fejes et al., 1990) and the Arabidopsis CAB1, CAB2, and CAB3 promoters (Millar and Kay, 1991; Millar et al., 1992a) contain elements sufficient for phytochrome and circadian regulation. The GATA motif (I-box) and its binding factors from tomato nuclear extracts have been implicated in both light and circadian regulation of rbcS, cab, and nitrate reductase promoters (Borello et al., 1993). Interaction of the tobacco factor CGF-1 with a GATA motif in the Arabidopsis CAB2 promoter has been implicated in phytochrome and circadian regulation (Anderson et al., 1994; Anderson and Kay, 1995). Carré and Kay (1995) have localized the clock-responsive region of the CAB2 promoter to a 36-bp region. One element within that region, AAATCCAATG, is conserved at 9 of 10 positions (−214 AAATCCAATG −205) in the RCA promoter.

Mutation of the CAB2 AATCC region reduces the ability of oligonucleotides to compete for the binding of the Tac protein factor. In the Lhcb2*1 promoter of Lemna gibba, a similar element (AACCAAG) termed REa is required for phytochrome regulation, although an adjacent element of similar sequence to REa apparently is not functional (Ke-hoe et al., 1994; Degenhardt and Tobin, 1996). However, REa has not been tested for circadian regulatory function. An A-rich region immediately upstream from the AATCCAAAG element in the Arabidopsis CAB2 promoter binds other protein factors such as CUF2 and CUF3 (Carré and Kay, 1995). In the RCA promoter a similar A-rich region is found immediately upstream of the AAATCCATG, and there are two other A-rich regions that lie farther upstream within the −317 promoter fragment. Functional analysis in which site-directed mutations in each of these potential CREs are tested within the context of minimal and full-length RCA promoters will be required to evaluate their roles in the circadian regulation of the Arabidopsis RCA gene.

An Arabidopsis RCA promoter fragment deleted to −317 retains circadian regulation with the wild-type period (approximately 24 h in continuous light), but the amplitude of the oscillations in mRNA abundance is reduced to approximately 2-fold. Similarly, minimal Arabidopsis CAB2 promoter fragments that are sufficient to drive circadian transcription in tobacco and in Arabidopsis also show reduced amplitude oscillations (Anderson et al., 1994; Anderson and Kay, 1995; Carré and Kay, 1995). One hypothesis to explain these observations is that there are multiple CREs that act additively or multiplicatively to confer high-amplitude oscillations. Alternatively, there may be multiple positively acting LREs that are negatively controlled (gated) by one or more CREs. These multiple LREs act additively or multiplicatively to confer high-amplitude oscillations, but all are subject to CRE regulation. Our data indicate that at least one LRE and one CRE reside in the region from −317 to +13 of the RCA promoter. The identification and characterization of these elements and of their cognate binding factors is the subject of ongoing investigation.

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