**CIRCADIAN CLOCK ASSOCIATED1 Transcript Stability and the Entrainment of the Circadian Clock in Arabidopsis**

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The circadian clock is an endogenous mechanism that generates rhythms with an approximately 24-h period and enables plants to predict and adapt to daily and seasonal changes in their environment. These rhythms are generated by molecular oscillators that in Arabidopsis (*Arabidopsis thaliana*) have been shown to consist of interlocking feedback loops involving a number of elements. An important characteristic of circadian oscillators is that they can be entrained by daily environmental changes in light and temperature. Previous work has shown that one possible entrainment point for the Arabidopsis oscillator is the light-mediated regulation of expression of one of the oscillator genes, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*). In this article, we have used transgenic plants with constitutive *CCA1* expression to show that light also regulates *CCA1* transcript stability. Our experiments show that *CCA1* messenger RNA is relatively stable in the dark and in far-red light but has a short half-life in red and blue light. Furthermore, using transgenic plants expressing chimeric *CCA1* constructs, we demonstrate that the instability determinants in *CCA1* transcripts are probably located in the coding region. We suggest that the combination of light regulation of *CCA1* transcription and *CCA1* messenger RNA degradation is important for ensuring that the Arabidopsis circadian oscillator is accurately entrained by environmental changes.

Many organisms have circadian clocks, endogenous 24-h systems that allow them to anticipate the daily changes in their environmental conditions (Bell-Pedersen et al., 2005). In plants, the circadian system is important for the regulation of a wide variety of processes from leaf and petal movements to stomatal opening, protein phosphorylation (Yakir et al., 2007), and transcript accumulation from up to 16% of the genome (Harmer et al., 2000; Schaffer et al., 2001; Michael and McClung, 2003; Edwards et al., 2006).

In the model plant, Arabidopsis (*Arabidopsis thaliana*), the oscillator that generates circadian rhythms consists of interlocking positive/negative feedback loops of several genes including *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB EXPRESSION1* (*TOC1*; Millar et al., 1995; Wang et al., 1997; Schaffer et al., 1998; Wang and Tobin, 1998). *CCA1* and *LHY* expression rises before dawn and suppresses the expression of *TOC1* by binding to its promoter. In the evening, when *CCA1* and LHY levels decrease, *TOC1* expression rises. TOC1 then activates *CCA1* and *LHY* expression by an as-yet-unknown mechanism (Alabadi et al., 2001). Other genes such as *GIGANTEA* (*GI*), *EARLY FLOWERING4*, *LUX ARRHYTHMO*, *TIME FOR COFFEE*, and *ARABIDOPSIS PSEUDORESPONSE REGULATOR3* (APRR3)*5/7/9* (APRR3)*5/7/9* might operate in the oscillator or close to it (Doyle et al., 2002; Hazen et al., 2005; Locke et al., 2005; Nakamichi et al., 2005; Edwards et al., 2006; Gould et al., 2006; Ding et al., 2007; McWatters et al., 2007). An important adaptive feature of the circadian clock is its ability to be entrained by environmental signals such as changes in light or temperature. This can be achieved by affecting one or more of the stages of the oscillator feedback loop (e.g. changing transcription rates, posttranscriptional events, translation rates, or posttranslational events). Several putative entrainment points have been described for the Arabidopsis oscillator. One such entrainment point is the activation of *CCA1* and *LHY* transcription by light (Wang and Tobin, 1998; Kim et al., 2003). Another well-characterized effect light has on the Arabidopsis oscillator is the regulation of proteasomal degradation of TOC1 and APR5 proteins that is mediated by *ZEITLUPE* (*ZTL*), an F-box motif and Kelch domain protein (Mas et al., 2003; Kiba et al., 2007). ZTL itself is stabilized by a blue light-mediated interaction with GI (Kim et al., 2007). Finally, the translation rate of *LHY* may be light regulated; light causes an increase in the translation of *LHY* in transgenic plants expressing *LHY* from a constitutive promoter (Kim et al., 2003).

In other organisms, there is evidence for entrainment by posttranscriptional events and RNA modification.
For example, in Neurospora crassa, there is a transcribed antisense sequence for the oscillator component frequency (frq), which is expressed with an opposite phase to the sense sequence of frq and appears to play a role in entrainment by light signals (Kramer et al., 2003). In addition, frq shows temperature-dependent alternative splicing, creating FRQ proteins with different lengths (Colot et al., 2005). The Drosophila melanogaster oscillator component PERIOD (PER) has an alternative splicing region in its 3′ untranslated region (UTR). The ratio of spliced and unspliced PER mRNA is controlled by temperature and light and can affect the rate of PER accumulation and activity rhythms (Majercak et al., 1999, 2004).

In general, RNA stability has an important role in the regulation of gene expression in eukaryotic cells (Ross, 1995; Meyer et al., 2004). One of the best characterized elements affecting RNA stability is the AUUUA sequence that appears, at differing frequencies and often in overlapping patterns, in the 3′ UTR of eukaryotic transcripts like c-fos and interleukin-3. This group of sequences (also known as AU-rich elements) can accelerate the decay of an mRNA containing them (Chen and Shyu, 1995). Another sequence affecting RNA stability is the so-called downstream element (DST), discovered first in the 3′ UTR of auxin-inducible SMALL AUXIN-UP RNA family of soybean (Glycine max; McClure et al., 1989). The DST sequence was shown to destabilize mRNA in tobacco (Nicotiana tabacum) cells and probably plays a similar role in RNA stability in Arabidopsis (Newman et al., 1993; Gil and Green, 1996; Johnson et al., 2000). Furthermore, a DST element is present in some circadian-regulated genes, and, in some cases, destabilization of mRNA by DST is under circadian clock control (Lidder et al., 2005). mRNA stability is also frequently regulated by small complementary RNAs. Several such small RNA pathways have been characterized in plants. The best characterized is the miRNA pathway involving small RNA molecules derived from endogenous noncoding transcripts with an extensive stem and loop structure (Brodersen and Voinnet, 2006).

Some mRNAs in plants show altered half-lives in response to external signals. For example, the FERREDOXIN-1 transcript in pea (Pisum sativum) is destabilized by darkness. This process is controlled by multiple CAUU sequences in the 5′ UTR (Petracek et al., 1998; Bhat et al., 2004). In Arabidopsis and pea, some LIGHT-HARVESTING CHLOROPHYLL-BINDING transcripts are destabilized by blue light perceived by the phototropin-1 receptor (Anderson et al., 1999; Folta and Kaufman, 2003). Furthermore, in beans (Phaseolus vulgaris), PROLINE-RICH PROTEIN1 mRNA is destabilized by treatment with a fungal elicitor (Zhang et al., 1993).

In this article, we show that CCA1 transcript stability is regulated by light. We suggest that the combination of light regulation of CCA1 transcription and RNA degradation is important for ensuring that the circadian oscillator is accurately entrained by environmental changes.

RESULTS

Light Regulates CCA1 Posttranscriptionally

To determine whether light has posttranscriptional effects on CCA1 levels in addition to its known role in activating CCA1 expression (Wang et al., 1997; Wang and Tobin, 1998; Kim et al., 2003), we examined CCA1 transcript levels in transgenic plants expressing CCA1 driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (CCA1-ox 034; described in Wang and Tobin, 1998). Although in these plants CCA1 should be constitutively expressed, we observed that CCA1 mRNA levels cycled according to the light/dark period. Figure 1A shows that after the lights came on, there was a sharp decrease in total CCA1 transcript levels and that the levels stayed low until the lights went off again. In CCA1-ox 034 plants maintained in constant dark, CCA1 mRNA levels were consistently high (Fig. 1, B and C). By contrast, the levels of CCA1 mRNA were significantly lower in plants grown in constant light conditions (Fig. 1C). Thus, it appears that the levels of CCA1 transcript are light dependent, with CCA1 mRNA being degraded significantly more quickly in the light than in the dark. Although the TUB2 control used for the quantitative real-time PCR has been reported to be circadian regulated (Edwards et al., 2006), the changes are very small and do not significantly affect our results (Supplemental Fig. S1, A and B).

There might be, however, alternative explanations for the differences in CCA1 transcript levels that we observed. One possible explanation is that there is a light-dependent difference in transcription rates that is a result of the specific integration site of the transgene. To address this question, we examined CCA1 transcript levels in an independent transgenic line, CCA1-ox 038 (Wang and Tobin, 1998), by both quantitative real-time PCR and northern analysis. Our results (Fig. 1D; Supplemental Fig. S2) show that the CCA1 mRNA levels were low in the light and high in the dark, ruling out the possibility of light-dependent insertion site differences in transcription.

Another explanation for the changes in CCA1 transcript levels under different light conditions could be that light alters the transcription rate from the CaMV 35S promoter itself. Although, as far as we know, there is no evidence showing a light- or dark-dependent regulation of transcription from the CaMV 35S promoter, we wanted to rule out this possibility. We therefore examined mRNA levels of three different transcripts, CONSTANS (CO), AtRabG3e (Rab7), and BREVIPIDECILLUS (BP; Lincoln et al., 1994; Onouchi et al., 2000; Mazel et al., 2004), expressed from the CaMV 35S promoter in transgenic plants (see “Materials and Methods”). As shown in Figure 2A, CO transcript levels were not significantly altered in light and dark. This result is consistent with previous reports showing CO mRNA levels are not light dependent (Valverde et al., 2004). Rab7 and BP transcripts
also remain high and did not show significant changes in light and dark (Fig. 2, B and C). Thus, expression from the CaMV 35S promoter does not appear to be regulated by light.

To further confirm that the differences we observe in CCA1 mRNA accumulation in dark and light are due to differences in the rate of mRNA degradation, we inhibited transcription using cordycepin (3'-deoxy-adenosine). The cordycepin was applied to whole CCA1-ox 034 plants (as described in the “Materials and Methods”) after 10 h of growth in the dark. The plants were subsequently transferred to light or left in the dark and the levels of CCA1 transcripts examined. Figure 3 shows that in plants transferred to light, the levels of CCA1 mRNA decreased faster than in plants left in the dark.

We also calculated the half-life of CCA1 mRNA in light and dark. In general, most sense mRNA degradation obeys first-order kinetics (Lam et al., 2001) and CCA1 transcript degradation can be fitted with first-order kinetics ($R^2$ greater than 0.9 for both light and dark). Using the equation $t_{1/2} = 0.693/k_d$ (Petracek et al., 1998) to calculate the half-life of CCA1 mRNA, we found that in plants transferred to light, CCA1 mRNA half-life is 1.5 h. In the dark, however, CCA1 transcript degradation rate is doubled, and the half-life is about 3 h. Using a binomial test, we calculated that the levels of CCA1 mRNA in the light are significantly lower than CCA1 mRNA levels in the dark ($P < 0.015$).

CCA1 is highly homologous to another oscillator gene, LHY (55% ClustalW score for the two cDNAs and more than 70% ClustalW score for the MYB domain). Previous work has shown that in plants expressing LHY from the constitutive CaMV 35S promoter (lhy-1TNI04), LHY transcript levels did not cycle under conditions of alternating light and dark (Kim et al., 2003). However, under the conditions that we used in our experiments, we found that LHY transcript levels are lower in the light than in the dark although, unlike CCA1, do not show a clear square wave form of accumulation (Fig. 4 compared to Fig. 1, A and D). Thus, it seems that LHY transcripts may be light regulated but possibly in a different way from CCA1 transcripts.

**Red and Blue, But Not Far-Red, Light Promotes CCA1 Transcript Degradation**

Plants have specific photoreceptors that are sensitive to different wavelengths of light. To determine which wavelengths and, thus, which photoreceptors are important for CCA1 mRNA degradation, the levels of CCA1 transcript were measured in CCA1-ox 034 plants transferred from dark to red, blue, or far-red light. Figure 1 shows that in plants expressing CCA1 transcript under the control of constitutive CaMV 35S promoter, CCA1 mRNA levels are higher in the dark than in the light. Three-week-old CCA1-ox 034 and CCA1-ox 038 plants were kept in light:dark cycles (14 h light:10 h dark). The levels of CCA1 mRNA were determined by northern analysis (A, B, and D; shown above the graph) or quantitative real-time PCR (C) and plotted on a graph relative to the maximum levels of expression. Aliquots of 1.5 μg of total RNA from each sample were run on an agarose gel to check for quality and to verify quantification (A, B, and D; shown above the graph). The white and black bars represent light and dark periods, respectively. The experiments were repeated at least twice.

**Figure 1.** In plants expressing CCA1 transcript under the control of constitutive CaMV 35S promoter, CCA1 mRNA levels are higher in the dark than in the light. Three-week-old CCA1-ox 034 and CCA1-ox 038 plants were kept in light:dark cycles (14 h light:10 h dark). The levels of CCA1 mRNA were determined by northern analysis (A, B, and D; shown above the graph) or quantitative real-time PCR (C) and plotted on a graph relative to the maximum levels of expression. Aliquots of 1.5 μg of total RNA from each sample were run on an agarose gel to check for quality and to verify quantification (A, B, and D; shown above the graph). The white and black bars represent light and dark periods, respectively. The experiments were repeated at least twice.
light or kept in the dark. Figure 5A shows that in the dark the levels of CCA1 transcripts were constantly high. However, by 1 h after exposure to red or blue light there was a sharp decrease in CCA1 transcript to less than 40% of the levels observed in the dark. By contrast, CCA1 mRNA levels remained high in far-red light. When dark-adapted plants were first exposed to red light before being transferred to far red, CCA1 transcript levels decreased in red light and increased again in far red to the levels observed in the dark (Fig. 5B). Taken together, our results show that red and blue light induce CCA1 mRNA degradation and that this effect is reversed by far-red light.

cis-Elements Determining CCA1 Transcript Stability

To start to determine the mechanism(s) for regulating CCA1 transcript stability, we looked for the location of the cis-acting stability elements. Elements affecting posttranscriptional regulation of transcripts are often located in the untranslated regions of genes, the 5′ and 3′ UTRs, and introns (Mignone et al., 2002). Therefore, it might be expected that the elements determining CCA1 mRNA stability are found in the untranslated regions of CCA1.

We made a novel construct (CCA1::eIF4A-ox) to test the contribution of the untranslated regions of CCA1 mRNA to stability. CCA1::eIF4A-ox contains the coding region of CCA1 and 19 nt (out of 237 nt) of 5′ UTR under the control of the CaMV 35S promoter. Furthermore, in the CCA1::eIF4A-ox construct, the 3′ UTR of CCA1 has been replaced with the 3′ UTR from AteIF4A, a highly stable transcript (Gutierrez et al., 2002). Plants were transformed with the CCA1::eIF4A-ox construct and homozygous lines recovered. Figure 6, A and B, shows that in two independent transgenic lines of CCA1::eIF4A-ox, CCA1 transcripts were still unstable in the light. Our results indicate that the elements that control different stability are most likely to be at least partly localized in the coding region rather than in the 5′ and 3′ UTRs and introns.

DISCUSSION

It has been known for several years that the plant circadian clock is entrained by light signals that affect the transcription of its components, such as CCA1 and LHY (Wang and Tobin, 1998; Kim et al., 2003). More
recently, evidence suggests that there are also additional levels of light entrainment that affect posttranscriptional events (e.g. TOC1 and GI protein stability and LHY translation rate; Kim et al., 2003; Mas et al., 2003; David et al., 2006). Here, we present evidence that light has an additional role in posttranscriptional regulation by controlling the stability of CCA1 RNA.

The light-induced changes in CCA1 mRNA stability were followed using plants expressing CCA1 under the control of the constitutive CaMV 35S promoter. As we have demonstrated, expression of CO, Rab7, and BP transgenes from this promoter is not regulated by light. Other groups have shown similar results (Kim et al., 2003; Valverde et al., 2004; David et al., 2006). However, CCA1 transcript levels under the control of the CaMV 35S promoter cycle according to the light/dark conditions. In the dark, CCA1 mRNA levels are constantly high, while in the light, they rapidly decline to about 40% of the dark levels. We showed that the changes in CCA1 mRNA levels are not dependent on the site of transgene integration and hypothesize that it is the result of light-dependent changes in transcript stability. Our measurements do not distinguish between endogenous CCA1 mRNA and CCA1 mRNA from the transgene. However, because endogenous CCA1 expression is induced by light, albeit to lower levels in CCA1-ox 034 plants than wild type, endogenous CCA1 mRNA would, if anything, mask the reduction in CCA1 transcripts we observed in the light (Wang et al., 1997). Our results that CCA1 mRNA is less stable in light are consistent with an earlier report that showed CCA1 transcript levels increasing in CCA1-ox 034 plants transferred from light to constant dark (Wang and Tobin, 1998).

The use of the transcription inhibitor, cordycepin, enabled us to demonstrate that in vivo CCA1 transcripts are less stable in light than in dark. We also used the cordycepin results to estimate the CCA1 mRNA half-life. The half-life values are, however, only approximate due to the limited sample time frequency. Based on our cordycepin results, CCA1 transcript half-life is around 1.5 h in the light; thus, CCA1 represents one of the unstable to moderately unstable Arabidopsis genes, a group that comprises only about 4% of Arabidopsis genome (Gutierrez et al., 2002). In the dark, however, CCA1 mRNA is significantly more stable. Our cordycepin experiments also suggest the differences in CCA1 transcript levels in light and dark are not the result of differential transcription regulation controlled by cis-acting elements within in the CCA1 coding region. Finally, the cordycepin experiments show that light-dependent transcription is not necessary for CCA1 mRNA degradation. Thus, if small RNAs are involved in this process, they are not transcribed in vivo.
need for additional control at the level of transcript stability? It is possible that the differences in \textit{CCA1} mRNA decay rates we observed in light and dark are important for maintaining an accurately entrained circadian clock by allowing plants to synchronize \textit{CCA1} expression precisely to sunrise. Thus, in wild-type plants grown in light:dark cycles, \textit{CCA1} expression starts early in the morning, before sunrise (Wang and Tobin, 1998). Then at daybreak, a change in \textit{CCA1} transcript stability causes a rapid disappearance of \textit{CCA1} mRNA. The combination of these two events, induction of transcription and transcript degradation, will result in a sharper peak of \textit{CCA1} mRNA accumulation, allowing a more precise detection of early morning, irrespective of seasonal changes. Consistent with the idea that mRNA stability is important for the pattern of oscillator transcript accumulation, experiments carried out in mice have shown that increasing the stability of a chimeric construct based on \textit{Period3}, an oscillator gene, significantly broadens the peak of expression (Kwak et al., 2006). Light-mediated changes in transcription rate and transcript stability can also be important for modifying the effects of environmental noise in clock entrainment. Kramer et al. (2003) have shown that in \textit{Neurospora}, \textit{frq} antisense transcripts are activated by light and are probably important for limiting the response of the clock to extremes in the environment.

In summary, we have found that light controls \textit{CCA1} mRNA degradation. Our findings, together with recent results from other groups, suggest that there are multiple entrainment points in the Arabidopsis circadian system (e.g. light activation of \textit{CCA1} and \textit{LHY} transcription and \textit{LHY} translation and light repression of \textit{TOC1} and \textit{GI} degradation; Wang and Tobin, 1998; Kim et al., 2003; Mas et al., 2003; David et al., 2006). There is thus a complex network of light regulation throughout the day to ensure tight control of oscillator function and entrainment.

**MATERIALS AND METHODS**

**Plant Materials and Growth**

\textit{Arabidopsis} (\textit{Arabidopsis thaliana}) ecotype Columbia-0 (Col-0) was used for all experiments unless stated otherwise. \textit{CCA1}-ox 034 and \textit{CCA1}-ox 038 contain an insertion of \textit{CaMV35S::CCA1} cDNA in Col-0 (Wang and Tobin, 1998). This insertion includes 23 nt of the 5’ UTR, the coding region, and all the 3’ UTR of \textit{CCA1}. \textit{35S::CO} contains an insertion of a genomic coding sequence under control of the 35S promoter in Col-0 (Onouchi et al., 2000). \textit{AtRab7-7} contains an insertion of 35S::\textit{Rab7} cDNA in Wassilewskija (Mazel et al., 2004). 35S::\textit{BP} contains an insertion of \textit{BP} cDNA under control of the 35S promoter in Nossen (Lincoln et al., 1994). \textit{Lhy1TN104} contains an insertion of the \textit{LHY} genomic sequence under control of the 35S promoter in Landsberg erecta (Schaffer et al., 1998).

All seeds were imbibed and cold treated at 4°C for 4 d before germination. All plants, except those used for transformations, were grown in petri dishes on Murashige and Skoog (Weigel and Glazebrook, 2002) medium from Duchefa Biochemie supplemented with 1% Suc (w/v). Plants used for transformations were grown on soil. Plants used for examining mRNA levels in different light conditions were grown under 14:10 light:dark (125 \(\mu\)E m\(^{-2}\) s\(^{-1}\)) cycles for 3 weeks before being transferred to constant dark, red light (50 \(\mu\)E m\(^{-2}\) s\(^{-1}\)), blue light (50 \(\mu\)E m\(^{-2}\) s\(^{-1}\)), far red (50 \(\mu\)E m\(^{-2}\) s\(^{-1}\)), or left in
light:dark. All experiments were performed at a constant 23°C. Philips fluorescent lights, TLD 38W/29 and TLD18W/33CW, and LED lights provided lighting for plant growth.

RNA Analysis

RNA extraction and northern analysis were carried out as previously described (Green and Tobin, 1999). As a control to ensure that we could directly compare results from northerns and quantitative real-time PCR, we analyzed a set of CCA1 mRNA by both techniques. Supplemental Figure S1 shows that in our experimental system, northerns and quantitative real-time PCR (qPCR) were very similar results. The primers used to make RNA probes for the northern analyses were: CCA1 forward: GTGACGCTGCTAGTGTGCT, CCA1 reverse: TGTAAATACGACTACATATGGAAGATCGACCGCTTGTGATGC, TUB2 forward: CTTAAGAATTCGGATTGCTTGTACACT, and TUB2 reverse: TGTAAATACGACTACATATGGAAGATCGACCGCTTGTGATGC. For quantitative real-time PCR, RNA samples were treated with DNase (DNA-free from Ambion) according to the manufacturer’s instructions. DNA-free RNA samples were then used as a template to produce cDNA using Reverse-IT Max 1st Strand Synth kit from Ambion with random-hexamer primers according to the manufacturer’s instructions. cDNA samples were diluted 5-fold and used as templates for the real-time PCR reaction by using Absolute SYBR Green ROX mix from Ambion according to the manufacturer’s instructions. Reactions were performed in a Rotagene real-time PCR machine. The primers for quantitative real-time PCR were: CCA1 forward, TTCAGATAAGCTCGGGAGATG, CCA1 reverse, AGTACCGCAGGAAACAAC; CO forward, ATATGCCTTCCTAGCCAGCTA, CO reverse, ACTCCTGCTCAACCACCCGAT; Rab7 forward, AGAGCAGCCGTGCTGCAATACT, Rab7 reverse, TGTGAGTTGCTGGTGGTTACAT, TUB2 reverse, GGTTAGCCCTTTCATAAG; TUB2 forward, GGTTAGCCCTTTCATAAG; TUB2 reverse, GGTTAGCCCTTTCATAAG.

RNA Stability Measurements

At the end of a dark period, 3-week-old plants were transferred to incubation buffer containing 1 mM PIPES, pH 6.25, 1 mM sodium citrate, 1 mM KCl, and 15 mM Suc. After 30 min, 3’-desoxyadenosine (cordycepin; Sigma) was added to a final concentration of 0.6 mM. A vacuum treatment was applied for 45 s. The manipulations were carried out in a darkroom under a green safe light. Plants were then kept in the dark for another 20 min before being transferred to light (125 mM m⁻² s⁻¹) or kept in the dark. Samples were taken at intervals as described in the text. As a control for cordycepin activity, the levels of an unstable transcript encoded by the expansin-like gene AEG34970 were measured (Gutierrez et al., 2003). As expected, AEG34970-encoded mRNA levels dropped rapidly in both light and dark following cordycepin treatment (data not shown).

Preparation of the CCA1::eIF4A-ox Construct

The pBS-CCA1 plasmid, containing the CCA1 coding region cloned into the pBlueScript II KS+ (p: 58061) plasmid between the PstI and BamHI sites, was obtained from Elaine Tobin, UCLA. The eIF4A 3’ UTR was amplified by reverse transcription-PCR and cloned into the pBlueScript between the EcoRI and BamHI sites to create pBS-eIF4A. pBS-CCA1 was digested with XhoI to isolate the CCA1 coding region, which was then cloned into the XhoI site on pBS-eIF4A. The direction of the insertion was confirmed by sequencing. The plasmid was then digested with BamHI and religated to minimize the space between the CCA1 coding region and the eIF4A 3’ UTR. The resulting plasmid was called pBS-CCA1::eIF4A.

To generate additional cloning sites, pHy-Bar binary vector for plant transformation obtained from Elaine Tobin, UCLA, was digested with BamHI and XhoI and religated with the linker: forward, GATCTTCTGAGATCCTCTAGAG, reverse, TTTTGGTTGCTGACCGCTTGTGATGC, pBS-CCA1::eIF4A was digested with XhoI and XhoI and the CCA1::eIF4A fragment was inserted between XhoI and XhoI to create pHy-Bar-CCA1::eIF4A, which contains the CCA1::eIF4A-ox construct.

Plant Transformation

GV3101::pPM900RK Agrobacteria containing the binary vector pHY-Bar-CCA1::eIF4A was cultured in Luria-Bertianni medium at 28°C with agitation until OD₆₀₀nm = 1. Three-week-old flowering Arabidopsis plants were dipped in floral dip medium for 5 min (Weigel and Glazebrook, 2002). Plants were left horizontally in the dark for 24 h, then grown for three more weeks until the seeds were ready for harvesting. Transformed plants were identified by their resistant to 1% Basta (glufosinate ammonium).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Quantitative PCR analysis of CCA1 mRNA levels is not affected by minor oscillations in TUB2.

Supplemental Figure S2. Estimations of CCA1 mRNA levels by northern and by quantitative real-time PCR are comparable.

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