Environmental and Genetic Effects on Circadian Clock–Regulated Gene Expression in Arabidopsis

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Expression patterns of the cold–circadian rhythm–RNA binding (*CCR*) and chlorophyll *a/b* binding (*CAB*) protein genes have circadian rhythms with phases that are different from each other and are affected differently by cold (4°C) treatment. Cycling of *CCR* and *CAB* RNA levels was observed in Arabidopsis seedlings grown for 5 days at 4°C under a light/dark photoperiod, although the cycling had reduced amplitude compared with normal growth conditions (20°C). *CCR* RNA levels were elevated in the cold, whereas *CAB* RNA levels were reduced in the cold relative to levels in control seedlings. Cold pulses (4°C for 12 or 20 hr) under continuous light affected the rhythms of *CCR* and *CAB* RNA levels in similar ways. The 12-hr cold pulse caused a 4-hr phase delay in both rhythms, whereas the 20-hr cold pulse resulted in a 12-hr phase delay in both rhythms. The *timing of CAB expression1* (*toc1*) mutation shortened the period of the *CCR* rhythm, matching previous results for the regulation of the *CAB–luciferase* (*CAB–luc*) transgene in this mutant. The results suggest that *CCR* and *CAB* share clock machinery but are regulated by downstream components that are affected differently by the cold. Also, the circadian clock regulating these genes in Arabidopsis has a cold-sensitive phase under continuous light conditions.

INTRODUCTION

A circadian rhythm is defined as a daily rhythm that persists in the absence of external time cues (i.e., under free-running conditions) and has a period of ~24 hr (Pittendrigh, 1993). Circadian rhythms have been observed at the organismal level as well as at the molecular level and are generated by the activity of an endogenous circadian clock (Pittendrigh, 1993; McClung and Kay, 1994). Because organisms use circadian rhythms to anticipate daily events, it is necessary for the circadian clock to adjust to changes in the day/night cycles; this adjustment is known as entrainment (Pittendrigh, 1993). The environmental signals that are used by the clock for entrainment are called Zeitgeber, which translates from the German as "time-givers." A common Zeitgeber in plants is the onset of illumination (McClung and Kay, 1994).

Some organisms contain multiple circadian clocks. In bean, circadian rhythms have been observed with free-running periods of different lengths (Hennessey and Field, 1992), which is only possible if there are different clocks (Pittendrigh, 1993). The golden hamster has two clocks, which are physically separated: one is in the brain and the other in the retina (Tosini and Menaker, 1996). An organism can also have rhythms with different phases (i.e., the timing of peaks and valleys; Pittendrigh, 1993; McClung and Kay, 1994). Currently, the output pathways connecting the oscillations of the clock to overt rhythms have not been identified

in plants, although a role has been suggested for calcium (Anderson and Kay, 1996). Thus, it is not known whether rhythms with different phases in the same organism are due to coupling to different clocks or to different output pathways from a single clock. The molecular basis of the circadian clock is being elucidated in Drosophila (reviewed in Hastings, 1994) and *Neurospora crassa* (Aaronson et al., 1994). From these organisms, genes encoding clock components have been cloned. Clock mutants have been identified in hamster (Ralph and Menaker, 1988), cyanobacteria (Kondo et al., 1994), mouse (Vitaterna et al., 1994), and Arabidopsis (Millar et al., 1995a).

Several plant genes have been identified that have a circadian rhythm to their expression patterns, with daily peaks and valleys in their RNA levels (reviewed in McClung and Kay, 1994). The role of light as an entraining signal for the regulation of clock-controlled genes has been well investigated in plant systems (Pilgrim et al., 1993; Zhong et al., 1994; Boldt and Scandalios, 1995; Kolar et al., 1995; Millar et al., 1995b). In addition to light, temperature can also be an entraining signal in plants. In mustard, the cycling of RNA levels from two clock-regulated genes was entrained to rhythmic temperature pulses when the plants were grown under conditions of continuous light (LL) (Heintzen et al., 1994). Rhythmic temperature pulses in pea (Kloppstech et al., 1991) and barley (Beator et al., 1992) induced cycling of RNA from clock-controlled genes in plants maintained in continuous dark (DD). Martino-Catt and Ort (1992) showed that prolonged cold treatment (4°C) of tomato inhibited chlorophyll

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a/b binding (CAB) protein RNA and protein cycling, suggesting that extreme temperature can be stressful to circadian clocks in plants. Prolonged low-temperature treatment also stopped the clock driving the bioluminescence rhythm in Gonyaulax polyedra (von der Heyde et al., 1992) and caused phase delays in the conidiation rhythm of N. crassa (Gooch et al., 1994). As with tomato, the temperature of both of these organisms is set by the ambient temperature.

Cold–circadian rhythm–RNA binding1 (CCR1) and CCR2 are members of a family of genes encoding glycine-rich RNA binding proteins (Carpenter et al., 1994). CCR1 and CCR2 mRNA levels cycled according to a circadian rhythm, with minimal accumulation at 1000 hr and maximal accumulation at 1800 hr (Zeitgeber times 3 and 11 [ZT3 and ZT11], where ZT0 is lights on; 0700 hr was ZT0 in these experiments). Cycling continued in LL or DD conditions (Carpenter et al., 1994). Under short-term (within a 24-hr period) conditions of low temperature (4°C) in a light/dark (LD) photoperiod and LL, CCR1 and CCR2 RNA levels increased at the appropriate peak in the cycle and were higher than in control plants during the valley of the cycle.

The phase of the rhythm of CCR expression is different from that of CAB, with a peak at ZT11 versus ZT7, respectively. Also, the responses of the two gene families to cold are different (Martino-Catt and Ort, 1992; Carpenter et al., 1994), although both genes have not been studied in the same plant. These differences suggest that the circadian regulation of CCR and CAB RNA cycling could be mediated by either two different clocks or by a single clock with different output pathways. A comparative study between the regulation of CCR and CAB expression in the same plant was initiated to determine whether they share clock components and thereby to gain insight into the organization of circadian-regulated gene expression.

In this report, we show that a prolonged low-temperature treatment of 7-day-old seedlings (5 days at 4°C) in LD had different effects on CCR and CAB RNA levels, whereas short cold pulses (12 or 20 hr at 4°C) of seedlings growing under LL affected the clock regulation of CCR and CAB expression in similar ways. Clock regulation of CCR expression was affected in the putative clock mutant timing of CAB expression1 (toc1) (Millar et al., 1995a), exhibiting a phenotype similar to that for CAB expression in the same mutant. These results suggest that the clock regulation of CCR and CAB expression is mediated by separate downstream pathways that are differentially sensitive to cold but that the genes share clock components.

RESULTS

Effects of Low Temperature on Cycling of RNA Levels from Clock-Regulated Genes

We demonstrated previously that CCR RNA levels increased in plants exposed to 4°C for 24 hr relative to levels in control

plants (20°C; Carpenter et al., 1994). To determine whether the cold-induced increase might be a result of a loss of cycling of RNA levels, CCR RNA levels were compared between cold-treated (4°C) and control (20°C) plants grown in LD conditions. LD conditions were chosen for this experiment to reveal possible cold effects on regulatory components that may be downstream of the clock itself. RNA gel blots from control and cold-treated seedlings were probed with CCR2, and the results are shown in Figure 1A. CCR2 expression levels were quantitated and normalized for any loading differences; the relative values are shown in Figure 1B. In cold-treated plants, CCR2 RNA levels were elevated during the first 36 hr, even at points in the circadian cycle when low levels of CCR2 RNA were present in control plants. Cycling of CCR2 RNA levels in the cold-treated plants was evident after 48 hr at 4°C (Figure 1B). Cycling of CCR2 RNA from cold-treated plants had a reduced amplitude and higher absolute levels than in control plants. Similar results were obtained using a probe to CCR1 (data not shown). These results indicate that CCR2 and CCR1 RNA levels can cycle when plants are grown in the cold; however, the cycling was disrupted in the cold-treated plants during the first 48 hr of the cold treatment.

It is possible that the effects of cold treatment on CCR2 RNA cycling represent general low-temperature effects on clock-regulated gene expression in Arabidopsis, such as a loss of normal mRNA turnover. Also, if the circadian regulation of CCR and CAB RNA levels have pathways in common, then the effects of cold treatment observed in Figure 1 may also be observed for CAB expression. Therefore, gel blots from Figure 1A were reprobed with the Arabidopsis CAB1 gene, and the results are shown in Figure 2. The CAB1 probe may detect other CAB family members (see Methods); therefore, the general term CAB has been used to describe the results obtained using this probe. CAB RNA levels cycled in control plants with a 24-hr period and normal amplitude. In the cold-treated plants, CAB peak expression was reduced, and the cycling of CAB RNA levels was not as tightly coupled to the photoperiod as was the cycling in control plants.

These results (Figures 1 and 2) indicate that the components regulating CCR and CAB expression are affected differently by growth at 4°C. In that these effects were observed in plants grown under LD conditions (which were receiving external time cues), it is likely that these cold effects represent changes in downstream components rather than in the circadian clock itself.

Effects of Cold Treatments on the Circadian Clock in Arabidopsis

To study the effects of cold on the circadian clock in Arabidopsis, short cold pulses (4°C) of different duration were given to Arabidopsis seedlings immediately after a transfer from LD to LL. Samples were collected during and after the cold pulse. If the circadian clock stops at 4°C, as suggested
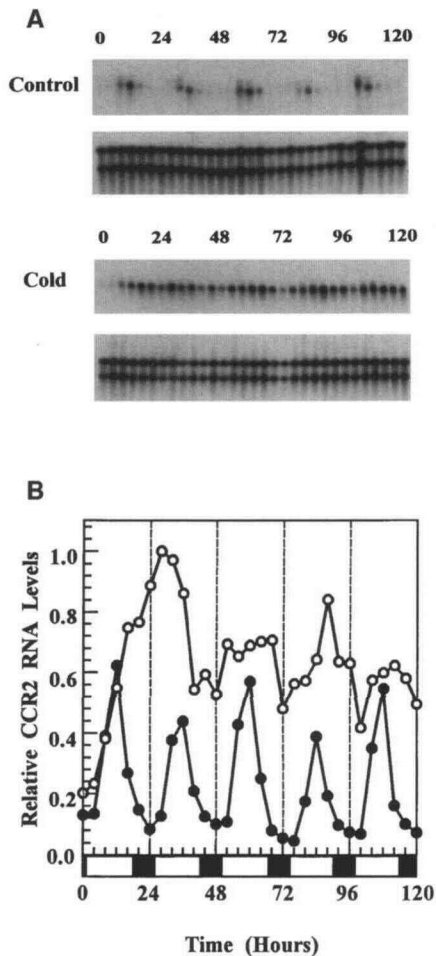
A

	0	24	48	72	96	120

Control

	0	24	48	72	96	120

Cold

B

Figure 1. *CCR2* Expression in Seedlings Exposed to Continuous Cold Treatment.

Seven-day-old seedlings were grown under a 16-hr/8-hr LD cycle. The seedlings were kept at 20°C (Control) or transferred to 4°C (Cold; 1 hr before lights on) for the duration of the experiment. Samples were collected at 4-hr intervals (the first sample was harvested immediately before transfer to 4°C). The experiment was performed twice with nearly identical results; one representative data set is shown.

(A) Gel blots showing *CCR2* RNA from the control and seedlings grown at 4°C. The times of harvesting, in hours, are shown above the lanes. The results from the loading control hybridization with the rDNA probe (Jorgensen et al., 1987) are shown below each hybridization with *CCR2*.

(B) Normalized values are shown for *CCR2* RNA levels (●, 20°C control; ○, 4°C treated). The bars below the x-axis indicate the light treatment during the experiment. The x-axis indicates the time at 4°C. RNA levels were quantitated as described in Methods.

by Martino-Catt and Ort (1992) in tomato, the seedlings that experience the cold pulse would have rhythms that were out of phase (in proportion to the duration of the cold pulse) with control seedlings that did not receive any cold pulse. The

relative *CCR2* RNA levels in seedlings treated with cold for 12 or 20 hr and nontreated controls are shown in Figure 3. *CCR2* RNA levels in control seedlings continued to cycle during LL with a period of 24 hr but with a reduced amplitude relative to the first cycle after transfer from LD conditions (0 to 24 hr; Figure 3). A 12-hr cold pulse during free-running conditions (LL) resulted in a 4-hr phase delay in the *CCR2* rhythm during the 24 hr immediately after the treatment (Figure 3A). The cycling in *CCR2* RNA levels after the 20-hr cold treatment (Figure 3B) also showed a phase delay after transfer to control temperature (20°C). This delay resulted in a *CCR2* rhythm in the 20-hr cold-pulsed seedlings that was ~180° out of phase (i.e., 12 hr) with the rhythms in seedlings subjected to a 12-hr cold pulse and control seedlings. This altered phase in the 20-hr cold-pulsed seedlings was maintained throughout the experiment. Twenty hours of cold did not affect the eventual steady state period or amplitude of the cycling of *CCR2* RNA levels (Figure 3B). In addition, *CCR2* RNA levels elevated in the cold dropped dramatically to near control levels within 4 hr after the seedlings were returned to the control temperature. The results for gel blots probed with *CCR1* were very similar to the results for *CCR2* (data not shown).

The effects of cold on the clock regulation of *CAB* RNA cycling were also examined. The normalized values for *CAB* RNA levels in control and cold-treated seedlings are shown in Figure 4. As expected, *CAB* RNA levels in control plants cycled with a 24-hr period and had slightly reduced amplitude under LL relative to LD conditions (the first cycle in
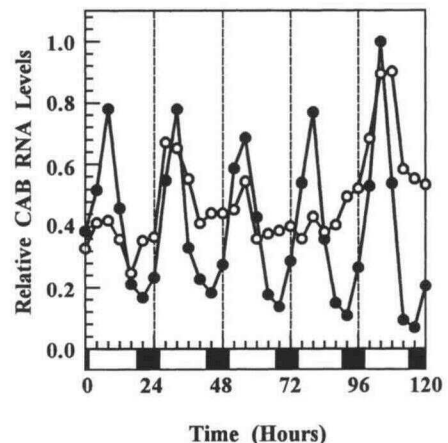
Figure 2. *CAB* Expression in Seedlings Exposed to Continuous Cold Treatment.

The normalized values are shown for *CAB* RNA levels (●, 20°C control; ○, 4°C treated). The gel blots from Figure 1 were stripped and reprobed with the *CAB1* gene, and the results were quantified (see Methods). The x-axis indicates the time at 4°C. The bars below the x-axis indicate the light treatment during the experiment. The experiment was performed twice with nearly identical results; one representative data set is shown.
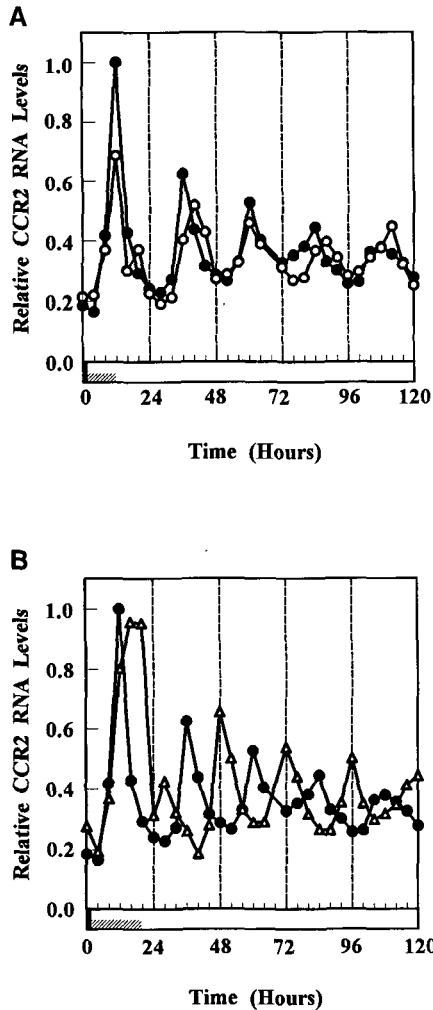
Figure 3. CCR2 RNA Levels in Seedlings Exposed to Cold for 12 and 20 Hr.

Seven-day-old seedlings were grown under a 16-hr/8-hr LD cycle, and the light cycle was switched to LL at the end of a light period at the beginning of the experiment. One hour before lights on, the seedlings were either kept at 20°C (control) or transferred to 4°C for 12 or 20 hr. Samples were collected at 4-hr intervals (the first sample was harvested immediately before transfer to 4°C). The bars below the x-axis indicate the light treatment during the experiment. The experiment was performed twice with nearly identical results; one representative data set is shown.

(A) Normalized values are shown for CCR2 RNA levels (●, 20°C control; ○, 12 hr at 4°C), and the time of the cold pulse is shown as a hatched box.

(B) Normalized values are shown for CCR2 RNA levels (●, 20°C control; △, 20 hr at 4°C), and the time of the cold pulse is shown as a hatched box.

Figure 4). The normal CAB daily increase was severely reduced in the cold (at a time of 4 to 20 hr). Twelve hours of cold caused a 4-hr phase delay in the CAB rhythm during the first cycle after the cold pulse (Figure 4A). Twenty hours of cold also caused a phase delay in the CAB rhythm relative to
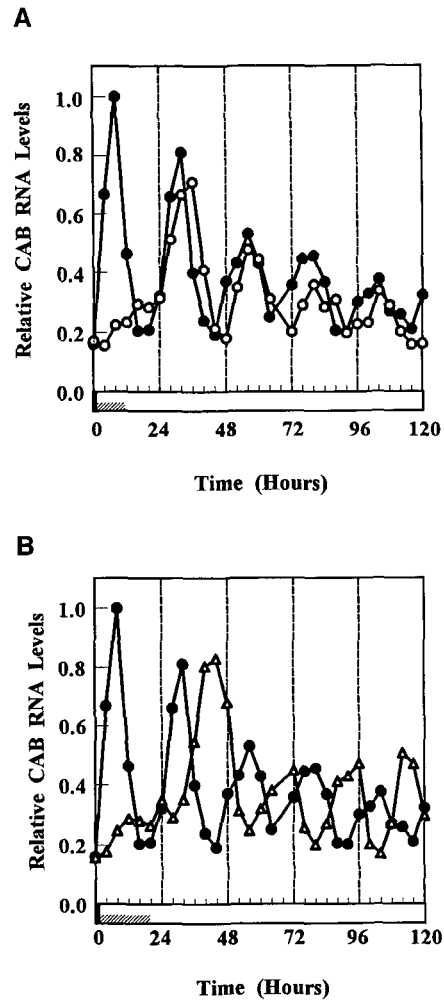

Figure 4. CAB RNA Levels in Seedlings Exposed to Cold for 12 and 20 Hr.

The normalized values are shown for CAB RNA. The blots used for Figure 3 were stripped and reprobed with the CAB gene, and the results were quantified (see Methods). The bars below the x-axis indicate the light treatment during the experiment. The experiment was performed twice with nearly identical results; one representative data set is shown.

(A) Normalized values are shown for CAB RNA levels (●, 20°C control; ○, 12 hr at 4°C), and the time of the cold pulse is shown as a hatched box.

(B) Normalized values are shown for CAB RNA levels (●, 20°C control; △, 20 hr at 4°C), and the time of the cold pulse is shown as a hatched box.

control, but otherwise the rhythm in the cold-treated seedlings had the same period and nearly the same amplitude as the control (Figure 4B). The CAB rhythm in the 20-hr cold-treated seedlings never recovered the same phase as the control rhythm within the time frame of the experiment (Figure 4B).

Together, these results indicate that cold pulses have very similar effects on the clock regulation of CCR and CAB, even though overall CCR and CAB expression levels were inversely affected during the cold pulses.

CCR2 RNA Cycling in toc1 and Wild-Type Plants Grown in LL

The results shown in Figures 1 and 2 indicate that pathways downstream from the clock, that regulate CCR and CAB expression, were differentially sensitive to cold treatments. The results shown in Figures 3 and 4 indicate that the clock(s) regulating the two genes was similarly affected by cold during LL. To determine more directly whether CCR and CAB share clock regulatory components, CCR RNA levels were analyzed in the Arabidopsis toc1 mutant and its transgenic parental line. Both the mutant and its parental line express the luciferase (luc) gene under control of the CAB2 promoter from Arabidopsis (CAB–luc), which results in LUC enzyme levels cycling with the same clock regulation as CAB expression (Millar et al., 1995a). Transgenic plants homozygous for toc1 have a LUC activity rhythm with a 20.9-hr period when plants are grown under LL versus a 24.7-hr period in the wild-type parental line (Millar et al., 1995a).

RNA gel blots from toc1 and the transgenic parental seedlings that were grown in LD conditions and then transferred to LL were probed with CCR2, and the expression levels are shown in Figure 5. CCR2 RNA cycling in the toc1 mutant had a period similar to that in the wild type during the first 24 hr in LL. However, the toc1 shorter period phenotype was evident for CCR2 RNA cycling during subsequent growth in LL. Similar results were obtained when the blots were probed with CCR1 (data not shown). The shorter period and normal amplitude of cycling phenotype of CCR gene expression in toc1 was identical to the phenotype observed using a LUC assay (Millar et al., 1995a). This result indicates that the toc1 mutation defines a gene product that is required for normal clock-regulated expression of both CCR and CAB.

DISCUSSION

The results presented here address how a plant circadian system may be organized so that it can regulate genes in such a way that one set, as exemplified by the CCR genes, is expressed at one time of the day and another gene, CAB, is expressed at a different time of the day.

Cycling of CCR RNA levels was affected in Arabidopsis grown at 4°C for 5 days under an LD photoperiod (Figure 1).

This prolonged cold treatment resulted in elevated CCR2 RNA levels and disrupted cycling during the first 36 hr. The increase in absolute CCR2 RNA levels during cold treatment could be due to the loss of a cold-sensitive negative regulatory component such as an RNase or a transcriptional repressor or due to a positive component such as a cold-mediated induction signal. In any case, cycling was not observed during the first 24 hr but was detectable by 48 hr in the cold. It is possible that the increase in CCR2 RNA levels masked the clock-regulated expression from this gene. The cycling of CCR2 RNA levels in cold-grown seedlings indicates that the machinery downstream from the clock, that regulates CCR RNA cycling, can function at 4°C. It is possible that the ability of the clock in Arabidopsis to overcome cold effects may be part of this plant's tolerance of chilling.

CAB expression was also affected in cold-grown plants grown under an LD photoperiod (Figure 2). Contrary to the results for the CCR genes, CAB RNA levels were reduced initially in plants grown at 4°C. Cycling of CAB RNA was detected in RNA from cold-grown plants, but the rhythm had a low amplitude. The results for CCR and CAB indicate that expression from clock-regulated genes can cycle in Arabidopsis plants grown at 4°C. When the effects of 4°C on CAB
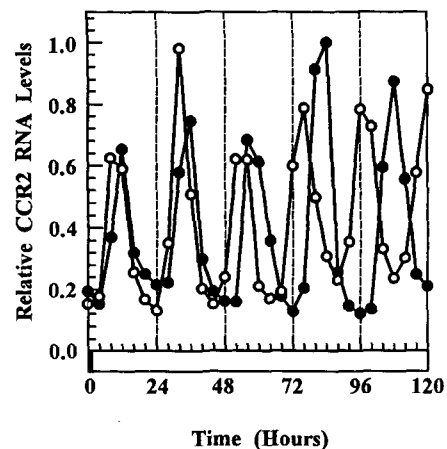

Figure 5. CCR2 RNA Levels in the toc1 Mutant and Its Wild-Type Parental Line.

Seven-day-old seedlings were grown under a 16-hr/8-hr LD cycle, and the light cycle was switched to LL at the end of a light period. Tissue samples were collected every 4 hr, and RNA was isolated and CCR2 levels quantitated as described in Methods. "Wild type" refers to the transgenic parental line expressing the CAB–luc construct (Millar et al., 1995a). The bar below the x-axis indicates the light treatment during the experiment. Normalized values are shown for CCR2 RNA levels (○, toc1; ●, wild type). RNA levels were quantitated as described in Methods. The experiment was performed twice with nearly identical results; one representative data set is shown.

expression were studied in tomato plants grown in DD, no cycling of *CAB* RNA was detected and no repression was observed (Martino-Catt and Ort, 1992). The differences between our results and the previously published work may be explained by different experimental designs (LD versus DD) and different organisms: Arabidopsis, a chilling-tolerant plant, versus tomato, a chilling-intolerant plant.

In two clock mutants (*Clock*, Vitaterna et al., 1994; *toc1*, Millar et al., 1995a), the altered-period phenotypes are only observed under free-running conditions (DD and LL, respectively), indicating that exogenous time cues can produce rhythms with periods similar to that of the wild type in organisms with defective clocks. Given that the cold-grown plants in Figures 1 and 2 were receiving an external time cue (the LD cycle), the cold-mediated changes in expression for these genes may represent effects on downstream components of the clock(s) rather than on the clock(s) itself. If this is the case, then the *CCR* and *CAB* genes are connected to a central clock through different downstream components that are distinguishable by their sensitivities to cold. Confirmation of this model (see below) will require the isolation and characterization of clock factors that directly interact with these genes and/or mRNAs.

We also looked at the effects of cold treatments on the clock (Figures 3 and 4). In the case of clock regulation of *CCR2*, a 12-hr cold pulse caused a 4-hr delay in the cycling during three of the four cycles analyzed (Figure 3A). The 20-hr cold pulse resulted in a 12-hr delay in the *CCR* rhythm, and the altered phase was maintained throughout the experiment (Figure 3B) but with the same period as the control and 12-hr treatments. These results show that the clock regulating *CCR2* cycling was affected more by a 20-hr cold pulse than by a 12-hr cold pulse under LL.

Overall, the effects of cold on the clock regulation of *CAB* RNA cycling were very similar to those for the *CCR* genes. A 12-hr cold pulse inhibited the normal peak in *CAB* RNA and produced a 4-hr phase delay that was observed in two of the four cycles analyzed (Figure 4A). The 20-hr treatment, however, had a pronounced effect on the cycling of *CAB* RNA (Figure 4B). In this case, the rhythm of *CAB* cycling did not recover a normal phase relative to the control treatment within the time window of the experiment. By the last 24-hr cycle in the experiment, the *CAB* rhythm in the 20-hr–treated seedlings appeared to be assuming a new phase that was different from the rhythms in the 12-hr and control treatments.

It is noteworthy that *CCR2* and *CAB*, whose rhythmic expression is out of phase with each other, had the same responses to cold: a 4-hr phase delay after a 12-hr cold pulse and a 12-hr phase delay after the 20-hr cold pulse. This observation supports the model (see below) that *CAB* and *CCR* share clock components. It is possible that a phase delay of <4 hr was maintained after the 12-hr cold pulse, but this was not reproducibly observed throughout the experiment because of the limits of resolution in this experiment (i.e., harvesting every 4 hr and quantitating RNA gel

blots). However, the 12-hr phase delay due to the 20-hr cold pulse was clearly present throughout the time window of the experiment.

Because the seedlings used in the experiments shown in Figures 3 and 4 were assayed in constant light, the only timing information came from the endogenous clock. The altered phase of *CAB* and *CCR* cycling after a 20-hr cold treatment therefore suggests that there may be a cold-sensitive phase of the clock that was hit by the 20-hr but not the 12-hr cold pulse. It is also possible that the 20-hr but not the 12-hr temperature shift acted as a Zeitgeber, as was observed for cyclic temperature shifts in other plants (Kloppstech et al., 1991; Beator et al., 1992; Heintzen et al., 1994). However, those experiments were performed using seedlings that had not experienced an entraining photoperiod (i.e., either etiolated or germinated and grown in LL) and were not expressing discernible rhythms before the temperature cycles. In our study, the seedlings had been growing under an entraining LD cycle before the cold pulse and had an established circadian rhythm before the single cold pulse. Additional experiments using cold pulses of the same length applied at different times in the cycle are required to address these issues. Although cold affected the clock in Arabidopsis, the effect did not seem to be a stoppage of the clock for the duration of the cold treatment, as was observed in tomato (Martino-Catt and Ort, 1992).

The possible inhibiting effect of cold treatment on a negative regulator of *CCR* expression (see above) was reinforced by the finding that *CCR* RNA levels dropped to near control levels within 4 hr after the transfer from 4°C back to 20°C. This suggests that an RNase or a transcriptional repressor was reactivated. However, when tissue samples from cold-grown and control plants were analyzed for changes in major Arabidopsis RNase activities by using a substrate-based gel assay (Yen and Green, 1991), no differences were detected between cold-grown and control extracts (M.L. Abler and P.J. Green, personal communication). This result suggests that any loss in putative RNase activity in the cold was transient or did not affect the major activities that were monitored.

A short-period phenotype was detected with the *CCR* genes in the *toc1* mutant (Figure 5), similar to that observed with *CAB* and *CAB–luc* by Millar et al. (1995a). Although *CCR* and *CAB* gene expression have different circadian phases, both are affected in the same manner by the *toc1* mutation. This result also suggests that the expression of *CCR* and *CAB* is regulated by the same clock.

Based on the results presented here, it is possible to propose a model in which a central clock regulates the expression of both the *CCR* and *CAB* genes through separate output pathways. The clock receives input from the environment, such as light and temperature, and one output from the clock is control over *CCR* and *CAB* expression. This clock requires wild-type function of the TOC1 protein for normal circadian regulation, and it has a cold-sensitive phase under LL conditions. Also, the separate output pathways are

distinguishable based on their responses to low temperature (4°C), with cold inhibiting *CAB* expression and increasing *CCR* RNA levels.

METHODS

Plant Growth and Treatment Conditions

The plants and seedlings used were either the wild type (*Arabidopsis thaliana* ecotype Columbia, originally from F. Ausubel, Massachusetts General Hospital, Boston, MA) or the *toc1* mutant and its transgenic parental line in the Arabidopsis C24 genomic background (kindly provided by S.A. Kay, The Scripps Research Institute, La Jolla, CA). Arabidopsis seedlings were grown by using surface-sterilized seeds, according to the protocol of Valvekens et al. (1988), and then sowing them onto 0.8% agar plates fortified with Murashige and Skoog salts (MS; Murashige and Skoog, 1962) and 3% sucrose. The plates were wrapped with Parafilm (American National Can, Greenwich, CT), covered with aluminum foil, stratified at 4°C for 72 hr, and then placed in the growth chamber during the end of the dark cycle and incubated under conditions described for each figure (Figures 1 to 5). The lighting was a mixture of cool-white fluorescent and regular incandescent light bulbs with a fluence rate of 120 to 150 μmol m^{-2} sec^{-1}. For each time point, all of the seedlings from one Petri dish were harvested and put at −80°C.

For the cold treatment described in Figures 1 through 4, Arabidopsis ecotype Columbia seedlings were grown on MS agar plates under a 16-hr/8-hr photoperiod, with 120 μmol m^{-2} sec^{-1} of light at 20°C. Seven-day-old seedlings were transferred to 4°C 1 hr before lights on and kept at that temperature throughout the experiment (Figures 1 and 2) or for the duration of the cold pulse (Figures 3 and 4); the control seedlings were kept at 20°C. The first samples were harvested immediately before transfer to the cold. For Figures 3 and 4, the samples for the last time points at 4°C were harvested just before the return to 20°C.

RNA Isolation and Gel Blotting

Total RNA was isolated from frozen tissue by using a LiCl procedure as previously described (Simon et al., 1992). RNA gel blots were probed with the cold–circadian rhythm–RNA binding (*CCR*) genes as previously described (Carpenter et al., 1994). The probe used to detect chlorophyll *a/b* binding (*CAB*) protein RNA was a gel-purified DNA fragment consisting of the 537-bp *CAB1* open reading frame from the plasmid pATHAB14C, kindly provided by A.J. Millar (University of Warwick, Coventry, UK). The *CAB1* probe used in this study does not result in gene-specific hybridization (Millar and Kay, 1991); therefore, the term *CAB* was used to indicate the lack of family member specificity. Quantification of hybridization levels was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The results from rDNA probings (Jorgensen et al., 1987) were used to normalize for loading differences within a given experiment. The values for each lane were normalized further to the highest value within an experiment, which was defined as 1.0, and then plotted as relative RNA levels. Gel images in Figure 1 were made using Adobe Photoshop, version 4.0 (Adobe Systems, Inc., San Jose, CA).

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