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Genes Encoding Glycine-Rich *Arabidopsis thaliana* Proteins with RNA-Binding Motifs Are Influenced by Cold Treatment and an Endogenous Circadian Rhythm[1]

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We have characterized the expression of two members of a class of *Arabidopsis thaliana* glycine-rich, putative RNA-binding proteins that we denote *Ccr1* and *Ccr2*. Southern blot analysis indicates that *Ccr1* and *Ccr2* are members of a small gene family. Both *Ccr1* and *Ccr2* mRNA levels were influenced by a circadian rhythm that has an unusual phase for plants, with maximal accumulation at 6:00 PM and minimal accumulation at 10:00 AM. The level of CCR1 protein, however, remained relatively constant throughout the cycle. The transcript accumulation patterns of the *Ccr1* and *Ccr2* genes differed considerably from conditions that affect the expression of similar genes from maize, sorghum, and carrot. Levels of *Ccr1* and *Ccr2* mRNAs were unchanged in wounded plants, increased at least 4-fold in cold-stressed plants, and decreased 2- to 3-fold in abscisic acid-treated plants. *Ccr1* transcript levels decreased in response to drought, whereas *Ccr2* transcript levels increased under the same conditions. Based on the presence of additional *Ccr* transcripts in dark-grown plants, we propose that *Ccr* transcripts may be subjected to a light- or dark-mediated regulation.

GRPs make up one of the major classes of cell wall proteins in plants (Condit and Meagher, 1986; Keller et al., 1988). The expression of plant GRPs can be tissue specific (Quigley et al., 1991; de Oliveira et al., 1993), developmentally regulated (Lei and Wu, 1991; de Oliveira et al., 1993), and responsive to a wide variety of external stimuli, such as virus infection (van Kan et al., 1988; Fang et al., 1991), wounding (Condit and Meagher, 1986; Keller et al., 1988), salicylic acid, water stress (de Oliveira et al., 1990), and light (Kaldenhoff and Richter, 1989).

Several GRPs that lack an amino-terminal signal peptide in their deduced primary structure and, therefore, presumably play a nonstructural role in the cell have been identified in plants. These proteins, which have been described in *Arabidopsis thaliana* (de Oliveira et al., 1990; van Nocker and Vierstra, 1993), maize embryos (Gómez et al., 1988) and leaves (Didierjean et al., 1992), sorghum seedlings (Cretin and Puigdomenech, 1990), and mature carrot roots (Sturm, 1992), contain two distinct domains: an amino-terminal RNA-binding domain and a Gly-rich carboxy-terminal domain. Genes encoding nonstructural GRPs are also responsive to environmental stress. mRNA specifying one maize protein accumulates in response to wounding and water stress in leaves and in response to ABA or water stress in embryos (Gómez et al., 1988). Transcripts for the carrot RRM protein accumulate in response to wounding and, to a lesser extent, treatment with ABA (Sturm, 1992). Although the cellular function(s) of these proteins remains unclear, these results suggest a stress-related role for nonstructural GRPs that may involve interaction with specific or nonspecific RNA molecules.

Clues leading to a function for these nonstructural GRPs may come from studies of proteins from other organisms that contain a similar RNA-binding sequence. This RRM (for recent reviews, see Dreyfuss et al., 1988; Bandziulis et al., 1989; Mattaj, 1989; Keene and Query, 1991; Kenan et al., 1991) is composed of 80 amino acids, including two highly conserved sequences, an octomer designated RNP1 and a hexamer designated RNP2. Aromatic amino acids within these highly conserved elements have been implicated in direct RNA interactions (Merrill et al., 1988). A number of proteins with known or postulated RNA-binding functions and that contain between one and four amino-terminal RRM sequences have been found in organisms ranging from *Escherichia coli* to man (Kim and Baker, 1993).

As with the plant RRM-GRPs, RRM proteins from other organisms are also characterized by distinctive carboxy-terminal domains that can be either acidic, rich in a single amino acid such as Gly or Pro, or contain putative zinc fingers or nucleotide-binding motifs (Swanson and Dreyfuss, 1988; Keene and Query, 1991). Many of these auxiliary domains are proposed to function in protein-protein interactions (Bandziulis et al., 1989), whereas others appear to form separate domains with RNA-binding activity (Kenan et al., 1991). The presence of introns in identical locations within eukaryotic genes that encode RRM proteins (Li et al., 1991; this report) is indicative of an ancient origin for the RRM. Members of this family of RNA-binding proteins have been implicated in a variety of roles in RNA metabolism, including transcription termination (Dombroski and Platt, 1988; Gott-

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Abbreviations: Cab, chlorophyll *a*/*b*-binding protein; CCR, cold, circadian rhythm, and RNA binding; Col-0, ecotype Columbia wild-type; GRP, glycine-rich protein; RRM, RNA recognition motif; TCV, turnip crinkle virus.

lieb and Steitz, 1989), mRNA stability (Minvielle-Sebastia et al., 1991), splicing (Query et al., 1989; Scherly et al., 1990; Ge et al., 1991), ribosomal biogenesis (Lapeyre et al., 1987), translational initiation (Milburn et al., 1990), and sex determination (Bell et al., 1988; Amrein et al., 1990).

In an attempt to determine a function for the plant RRM-GRPs, we have initiated a detailed study of two *A. thaliana* genes that encode RRM-GRPs and appear to be members of a small gene family. Transcripts of the *A. thaliana* genes accumulated in all tissues examined and were influenced by a plant circadian rhythm, with maximal and minimal mRNA accumulation at unusual times in the diurnal cycle. Expression of the *A. thaliana* genes differed from expression patterns of the maize, sorghum, and carrot homologs when exposed to external stimuli such as wounding, drought, ABA, and cold. Differential expression patterns in response to the various stress conditions were also evident between the two *A. thaliana* genes. Preliminary data suggest that *Ccr* mRNAs accumulate differentially in light-grown versus dark-grown plants.

MATERIALS AND METHODS

Plant Growth and Treatment Conditions

Arabidopsis thaliana ecotype Columbia wild type (Col-0) was a gift from F. Ausubel (Massachusetts General Hospital, Boston, MA). Plants were grown in controlled environmental chambers with a 16-h light/8-h dark photoperiod (180 μE s^{-1} m^{-2}; the light cycle began at 7:00 AM) at a constant 20°C unless specified otherwise. Plants with four fully expanded leaves (approximately 2.5 weeks postgermination) were individually inoculated by dipping a glass rod into inoculation buffer containing TCV RNA and rubbing each leaf with a single stroke as previously described (Simon et al., 1992). Mock-treated plants were rubbed in an identical fashion with buffer minus the viral RNAs. For ABA treatments, 3-week old plants were sprayed until run-off with 50 mM ABA (mixed isomers) in 0.02% Tween-20 at 6:00 AM. Control plants were sprayed with 0.02% Tween-20 without ABA. Plants were covered with plastic wrap and placed in a growth chamber at 22°C. At 10:00 AM, roots were excised, and plants were blotted to remove moisture and then stored frozen at −80°C. For wound treatments, plants were sprinkled with diatomaceous earth, and leaves were rubbed vigorously with a glass rod until visible wounding was achieved. Control plants were untreated. Plants were harvested at 10:00 AM, 22 h later. For drought treatment, plants were grown under standard conditions for 6 d and either watered normally (control) or not watered. Plants subjected to drought conditions had visibly wilted and lost 60% of their fresh weight when compared with rehydrated plants. Plants were harvested at 10:00 AM. For cold treatment, plants were incubated at 4°C under constant illumination of 20 μE s^{-1} m^{-2} at 10:00 AM and harvested 24 h later. Control plants were grown under the same light conditions at 22°C. For the cold time course, the same conditions were used except that the plants were subjected to cold stress beginning at 6:00 AM and harvested every 4 h.

Screening *Arabidopsis* Genomic Libraries

An *A. thaliana* genomic library (ecotype Landsberg) constructed in the vector EMBL4 was a gift from E. Meyerowitz (California Institute of Technology, Pasadena, CA), and an *A. thaliana* genomic library (ecotype Col-0 in EMBL4) was a gift from C. Town (Case Western Reserve University, Cleveland, OH). The Landsberg library was differentially screened (Ausubel et al., 1991) using radiolabeled cDNA prepared from poly(A)$^+$ RNA isolated from leaves of *A. thaliana* plants 4 d postinoculation with TCV or mock treated with inoculation buffer. The Col-0 library was screened using radiolabeled *Ccr2* cDNA. Despite repeated attempts, we were unable to identify phage containing *Ccr2* genomic sequences from the Landsberg genomic library.

Construction and Screening of an *Arabidopsis* cDNA Library

Double-stranded cDNA was prepared using standard methods (Ausubel et al., 1991) from 10 μg of poly(A)$^+$ RNA isolated from *A. thaliana* ecotype Col-0 plants 4 d postinoculation with TCV. The cDNA was treated with *Eco*RI methylase, ligated to *Eco*RI linkers, and digested with *Eco*RI before fractionation in a 1.2% agarose gel. cDNA larger than 900 bp was electroeluted, ligated to predigested λ ZapII *Eco*RI arms, and packaged for plating according to the supplier's suggested protocols (Stratagene). An aliquot consisting of 2 × 10^5 individual recombinant clones was amplified for subsequent screening procedures. A 2.8-kb *Eco*RI-*Kpn*I fragment, from the genomic *Ccr1* clone, was labeled using random primers (Sen and Murai, 1991) and used as a probe to screen the cDNA library. Two positive clones containing unique inserts were obtained from the 150,000 clones screened.

DNA Sequencing

Genomic and cDNA clones were sequenced by the chain termination method (Sequenase, United States Biochemical) following construction of nested deletions by *Exo*III treatment (Ausubel et al., 1991).

DNA Isolation and Genomic Southern Blots

DNA was isolated from *A. thaliana* ecotype Col-0 as described by Bernatzky and Tanksley (1986). DNA (2 μg) was digested with various restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel. The DNA was then transferred to a nylon membrane (Zetaprobe, Bio-Rad) and hybridized to an 883-bp *Sst*I-*Rsa*I fragment derived from the genomic *Ccr1* clone, or the complete *Ccr2* cDNA insert, which was labeled using random primers (Sen and Murai, 1991). Hybridization conditions were 5× SSC, 5× Denhardt's solution, 0.2% SDS, 10 mM $NaPO_4$, 0.5 mg mL^{-1} of single-stranded DNA at 68°C overnight. The blot was then washed for 20 min at 68°C in succession with 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS; and 0.5× SSC, 0.1% SDS.

RNA Isolation and Northern Blots

Total RNA was isolated from leaves using a LiCl procedure as previously described (Simon et al., 1992). Poly(A)$^+$ RNA

was isolated following two passages over an oligo(dT)-cellulose column (Ausubel et al., 1991). Poly(A)$^+$ RNA (8 μg) or total RNA (4 μg) was loaded in each lane. RNA was subjected to electrophoresis on standard formaldehyde gels (Sambrook et al., 1989) and then transferred to a NitroPlus 2000 membrane according to the manufacturer's suggested procedure (Micron Separations, Inc., Westboro, MA). Blots were probed and washed as previously described (Simon et al., 1992). Final wash conditions were 0.1× SSPE, 0.1% SDS for 20 min at 50°C. Autoradiograms were scanned with a two-dimensional gel analysis system (Microscan 1000; Technology Resources, Nashville, TN), and the data were integrated using software provided by the manufacturer.

Cloning and Expression of CCR1 Protein in *E. coli*

To obtain a full-length reading frame clone of *Ccr1* (without intron sequence), the 3′ end of the cDNA clone extending from the unique *Bgl*II site through the poly(A) tail was ligated to the 5′ end sequence from the genomic 2.8-kb *Eco*RI-*Kpn*I fragment, also digested with *Bgl*II. The resultant clone served as a substrate in a PCR reaction primed with oligonucleotides that generated a full-length reading frame plus 11 bp of 3′ untranslated sequence flanked by *Bam*HI and *Eco*RI sites, 5′ and 3′, respectively. The PCR fragment was ligated into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia) for expression as a fusion protein with GSH *S*-transferase. The fusion protein was purified by affinity chromatography using immobilized GSH (Smith and Johnson, 1988).

Antibody Production and Western Blot

Six-month-old rabbits were intradermally injected with approximately 1 mg of purified CCR1-GSH *S*-transferase fusion protein along with Freund's complete adjuvant. After two boosters of 0.5 mg of protein were injected into the rabbits, serum was collected and processed following standard techniques (Ausubel et al., 1991). For western blots, plants were grown and harvested under conditions identical with those used for the northern blot analyses. Proteins were extracted from whole plants as follows: 100 to 300 mg of frozen plant material were ground in liquid nitrogen, followed by further grinding in 30 mM Tris-HCl (pH 7.2). Debris was removed from the grindate using centrifugation, and the cleared extract was used for analysis. Protein concentration was determined using the Bradford (1976) assay. For the western blot, proteins were subjected to electrophoresis through 12% SDS-polyacrylamide gels and then transferred onto nylon membranes (Nytran) using standard electroblotting techniques (Ausubel et al., 1991). CCR1 protein was detected using the polyclonal antisera described above; a sample of the original antigen was included on each blot as a positive control. The blot was developed using a secondary antibody fused to horseradish peroxidase and stained with the appropriate substrate (4-chloro-1-naphthol, Kierkegaard and Perry).

RESULTS

Cloning and Sequencing of the *Ccr1* and *Ccr2* Genes

This study began as an effort to identify mRNAs that accumulate differentially in response to infection of *A. thaliana* (ecotype Col-0) with TCV. A genomic library prepared from *A. thaliana* ecotype Landsberg was differentially screened using either cDNA prepared from *A. thaliana* leaves inoculated with TCV 4 d previously (at the four-expanded leaf stage) or cDNA from plants mock treated with inoculation buffer. One hybridization-positive phage that rescreened successfully was subjected to restriction endonuclease analysis; a 1.1-kb *Rsa*I subfragment within a 2.8-kb *Eco*RI-*Kpn*I fragment hybridized strongly only to the cDNA from infected plants (data not shown).

The 2.8-kb *Eco*RI-*Kpn*I fragment was used to screen a cDNA library derived from mRNA isolated from Col-0 leaves 4 d after being inoculated with TCV. Of the 150,000 phage screened, two positive cDNA clones containing unique inserts were obtained. One cDNA clone that produced a strong hybridization signal contained a 715-bp inserted sequence and terminated in a poly(A) tract; this sequence was identical with a portion of the genomic sequence contained within the 1.1-kb *Rsa*I fragment, with the exception of a single gap in the cDNA sequence corresponding to a 283-bp A/T-rich segment, flanked by consensus intron-splicing signals (Fig. 1; White et al., 1992). The cDNA sequence was less than full length, containing one open reading frame that extended from one end to 153 bp from the poly(A) tract. The open reading frame terminated with a TAA codon, the most common termination codon in dicots (Cavener and Ray, 1991). Although the 3′ untranslated region was rich in thymidine residues, a plant consensus poly(A) addition signal was not present (Joshi, 1987).

Examination of the genomic sequence revealed the presence of an in-frame Met codon 40 nucleotides upstream of the 5′ end of the cDNA sequence and 24 nucleotides downstream from a termination codon. There are several lines of evidence that suggest that this is the initiation codon: the sequence immediately preceding the ATG, 5′-CTCAAAAAAAATG-3′, is very similar to the consensus sequence for translation initiation sites in dicots, 5′-AAAAAAAAAAAUG-3′ (Cavener and Ray, 1991); the sequence 5′-CTTATCA-3′, located 63 nucleotides upstream from the presumptive ATG initiation codon, is in agreement with the location and composition of the consensus plant transcription start site (5′-CTCATCA-3′; Joshi, 1987); the sequence 5′-TATAAA-3′ is located 18 nucleotides further upstream, in good agreement with the sequence and position of plant TATA boxes (Joshi, 1987). We have designated this gene "*Ccr1*." A second cDNA clone hybridized weakly to the genomic *Eco*RI-*Kpn*I fragment derived from *Ccr1*. This 1075-bp cDNA, designated *Ccr2*, was used as a probe to isolate the corresponding genomic sequence from a Col-0 genomic library. Sequence analysis revealed that the *Ccr2* cDNA was identical with the corresponding portion of the *Ccr2* genomic sequence and shared approximately 80% sequence similarity with *Ccr1* cDNA (Fig. 1B). This second gene apparently represents a member of the same gene family as *Ccr1*.

cDNAs corresponding to the *Ccr1* and *Ccr2* genes were recently independently isolated by van Nocker and Vierstra (1993). We noted, as did these authors, the strong similarity to RRM-containing proteins from maize, sorghum, and carrot (see fig. 2 of van Nocker and Vierstra 1993). Comparison of our sequence data for the *Ccr1* and *Ccr2* genes with the

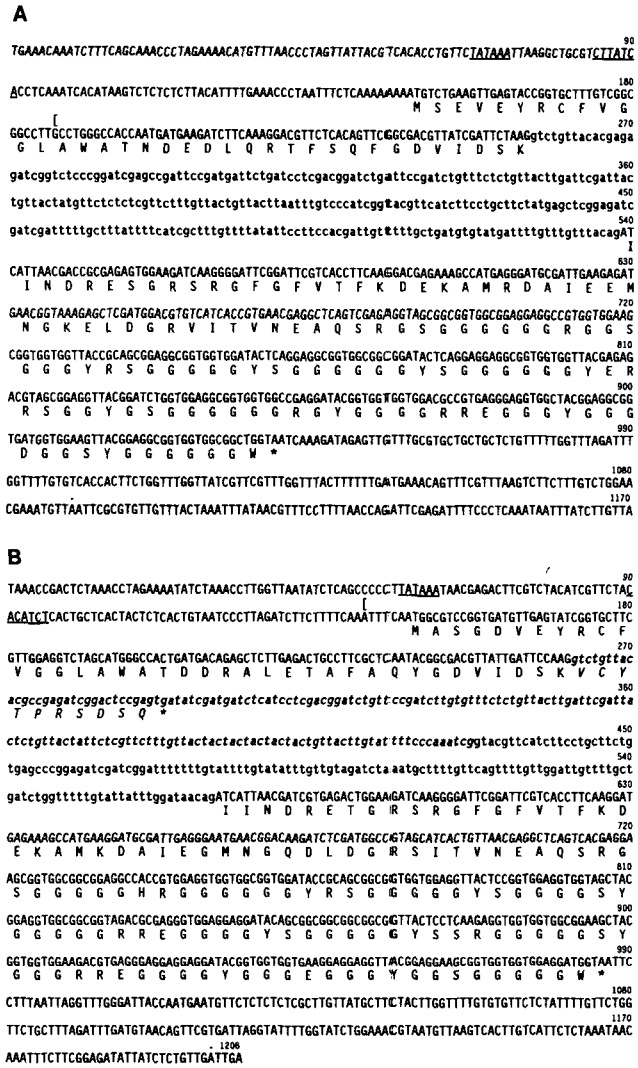

Figure 1. Sequence of the *A. thaliana Ccr1* and *Ccr2* genes. A, Sequence of the *Ccr1* gene. The coding region is interrupted by a single 283-bp intron. A bracket denotes the 5′ end of the cDNA, and a dot indicates the position of the polyadenylation site in the cDNA. The presumptive TATA box and presumptive transcription start site (based on similarities with plant consensus sequences) are underlined. The intervening sequence is in lowercase letters. The deduced polypeptide sequence is given in one-letter amino acid code under the nucleotide sequence. B, Sequence of the *Ccr2* gene. The sequence is presented in the same manner as *Ccr1*. The intron-like sequence found in our cDNA for *Ccr2*, but not in the corresponding cDNA isolated by van Nocker and Veirstra (1993), is in lowercase italics, and the amino acids encoded by the intron-like sequence are also in italics.

cDNAs reported by van Nocker and Vierstra indicated five differences in the 5′ and 3′ untranslated regions of *Ccr1* and two differences in the 5′ and 3′ untranslated regions of *Ccr2*. No differences were observed in the coding sequences of either gene. These sequence differences probably do not represent differences in source material (our *Ccr1* gene was isolated from a Landsberg genomic library, and the *Ccr2* gene, both of our cDNAs, and the cDNAs of van Nocker and Vierstra were derived from Col-0) in that our cDNA sequences matched perfectly with the appropriate regions of our genomic sequences and both differed from the corresponding cDNAs of van Nocker and Vierstra. The intervening sequences in *Ccr1* and *Ccr2* are located within the RRM segment, in the same position as in the genes encoding RRM proteins from tobacco (Li et al., 1991), maize (Gómez et al., 1988), human (Biamonti et al., 1989), and *Xenopus* (Etzerodt et al., 1988).

A striking difference between the *Ccr1* and *Ccr2* cDNA sequences is the presence of an insertion of 166 bp in the *Ccr2* cDNA, corresponding to a portion of the *Ccr2* genomic intervening sequence. This 166-bp intervening sequence was not present in the equivalent *Ccr2* cDNA reported by van Nocker and Vierstra (1993). Several lines of evidence suggest that this intron-derived sequence in our *Ccr2* cDNA was not the result of a cloning artifact: (a) the intron segments excised in both *Ccr2* cDNAs have correct splice site junction sequences; (b) northern hybridization analysis (see below) suggests that a second RNA species, slightly larger than the size of fully processed mRNA, is detected using either full-length cDNA or intron-only probes. The presence of this "intron-like" sequence in the *Ccr2* cDNA would have a significant effect on the deduced protein sequence. Protein translated from *Ccr2* transcripts corresponding to our cDNA would be severely truncated because of the presence of an in-frame termination codon near the 5′ end of the intron-derived sequence (Fig. 1B). The resultant protein would have only 48 amino acids with a mol wt of 5268. Distinct forms of RRM-containing proteins have been found in tissues that were generated from alternatively spliced mRNAs (Ge et al., 1991). It is possible that *Ccr2* transcripts may also be conditionally processed in a fashion that would generate a truncated and presumably nonfunctional protein product.

Ccr1 and *Ccr2* Are Members of a Small Gene Family

Genomic Southern blot analysis was performed using either an 883-bp fragment of the *Ccr1* gene or the 1075-bp *Ccr2* cDNA as probes. *Arabidopsis* DNA was digested with restriction enzymes with recognition sites not found within the *Ccr1* and *Ccr2* genomic segments that were sequenced. The results, presented in Figure 2, revealed one strong hybridization signal in each lane along with as many as six weaker signals (visible in the *Ccr2* blot only after a prolonged exposure, data not shown). One of the weaker signals in each lane corresponded precisely to the strong hybridizing signal of the other cloned gene. These results suggest that *Ccr1* and *Ccr2* are members of a small gene family.

Ccr1 Transcripts Are Not Induced in Leaves of Virus-Infected *Arabidopsis*

Since the *Ccr1* gene was isolated based on differential hybridization to cDNA from virus-infected plants, RNA gel blot analysis was performed in an attempt to confirm that *Ccr1* mRNA accumulated during the response of *Arabidopsis* ecotype Col-0 to virus infection. Poly(A)$^+$ RNA was prepared from 4-d postinoculation *A. thaliana* leaves as well as from

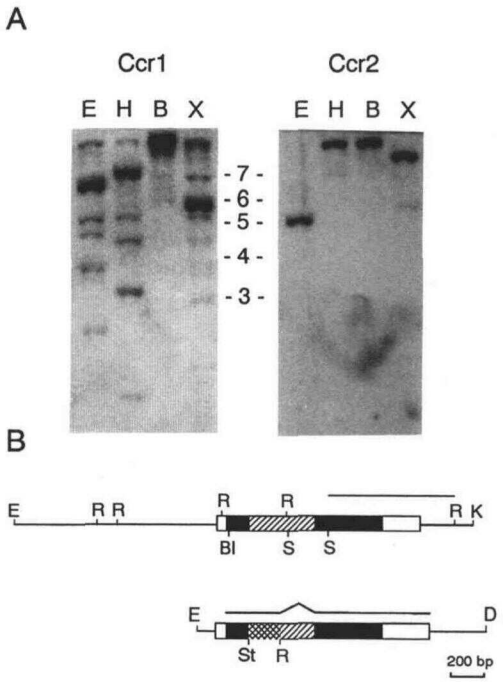

Figure 2. Southern blot analysis of the *Ccr1* and *Ccr2* genes. A, Genomic DNA was isolated from leaves of *A. thaliana*, and 2 μg per lane were digested with the restriction enzymes indicated. The blot was probed first with an 883-bp *Sst*I-*Rsa*I fragment of *Ccr1* as shown in B, stripped, and then probed with the cDNA insert from *Ccr2*. Hybridization conditions were identical for both probes with the final wash at high stringency (68°C, 0.5× SSC, 0.1× SDS). Fragment sizes (in kb), as determined from size marker DNA, are indicated between the blots. B, Diagrams of portions of the *Ccr1* and *Ccr2* genomic clones. Boxes indicate the presumptive transcribed regions oriented 5′ to 3′. The open reading frames are shaded, and the hatched portions indicate intervening sequences. The cross-hatched region in *Ccr2* indicates the intron-like sequence from the cDNA. Probes used in A are indicated by lines over the diagrams. B, *Bam*HI; Bl, *Bgl*II; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; R, *Rsa*I; S, *Sst*I; St, *Sty*I; X, *Xba*I.

leaves of plants treated in an identical fashion with inoculation buffer. Based on the location of the putative transcription initiation signal in the *Ccr1* gene, the expected size of *Ccr1* mRNA is 805 bases plus the poly(A) tail. The *Ccr1* genomic DNA probe hybridized strongly to an RNA of approximately 950 bases and weakly to an RNA of about 3300 bases (Fig. 3A). Since the 950-base mRNA species is approximately the size expected for mRNA transcribed from the *Ccr1* gene, it will be referred to as the *Ccr1* mRNA. To determine whether the 3300-base RNA hybridized to sequences throughout the *Ccr1* coding region, a cDNA clone was constructed that extended from the initiating Met to 11 bp downstream from the termination codon. This cDNA was subdivided into two segments: upstream and downstream from the single *Sst*I site within the coding sequence (Fig. 2B). Both of the 5′ and 3′ open reading frame subclones hybridized to the 3300-base RNA, indicating that this species contains similarities across the entire length of the *Ccr1* coding sequence (data not shown).

The *Ccr1* probe did not hybridize differentially to virus-infected and mock-treated RNA preparations (Fig. 3A). One possible explanation for this result was that *Ccr1* transcripts accumulated differentially within a short time window (e.g. 3.5 or 4.5 d postinoculation) but not precisely when the infected plants were harvested. Therefore, *Arabidopsis* plants were inoculated with TCV or mock treated with buffer, and plants were harvested at 12-h intervals beginning 75 h post-inoculation. The results, presented in Figure 3B, again revealed the lack of differential hybridization to RNA from mock-treated and virus-infected plants. However, *Ccr1* transcript levels fluctuated depending on the time of day that the plants were harvested. Both infected and mock-treated plants harvested at 7:00 AM, 87 or 111 h postinoculation, accumulated substantially less *Ccr1* mRNA than plants harvested at 7:00 PM, 75 or 99 h postinoculation. Unlike the 950-base RNA species, the steady-state level of the 3300-base species remained constant in these preparations (determined upon overexposure of the autoradiogram, data not shown).

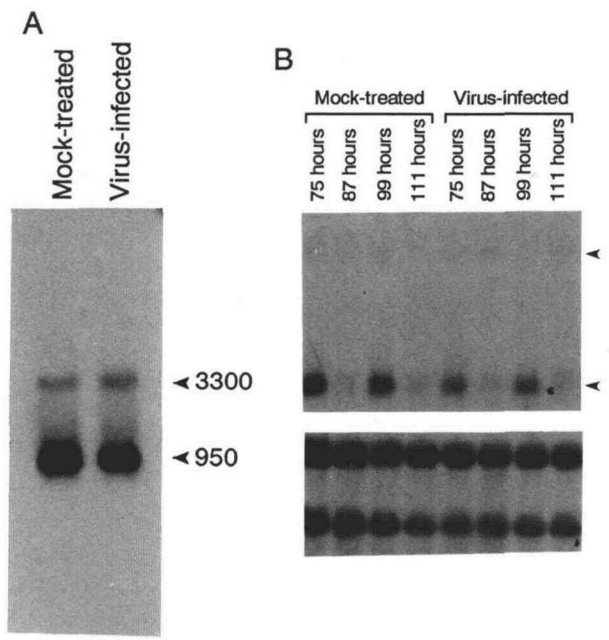

Figure 3. RNA gel blot analysis of *Ccr1* mRNA levels in response to virus infection. A, Poly(A)$^+$ RNA was isolated from *A. thaliana* 4 d postinoculation with TCV (Virus-infected) or treated in an identical fashion with inoculation buffer (Mock-treated). Eight micrograms of RNA per lane were hybridized with the genomic DNA-derived probe described in Figure 2. The approximate size of the two hybridizing species (in bases) was determined by comparison with ethidium bromide-stained rRNA and viral RNA species. B, Total RNA was prepared from plants treated as in A but harvested at the times shown above each lane. Four micrograms of RNA per lane were hybridized first with the *Ccr1* genomic DNA probe (top) and then with a pea rDNA probe (Jorgensen et al., 1987; bottom). Arrowheads indicate the positions of the 950- and 3300-base RNA species. The 3300-base species was clearly visible with longer exposure.

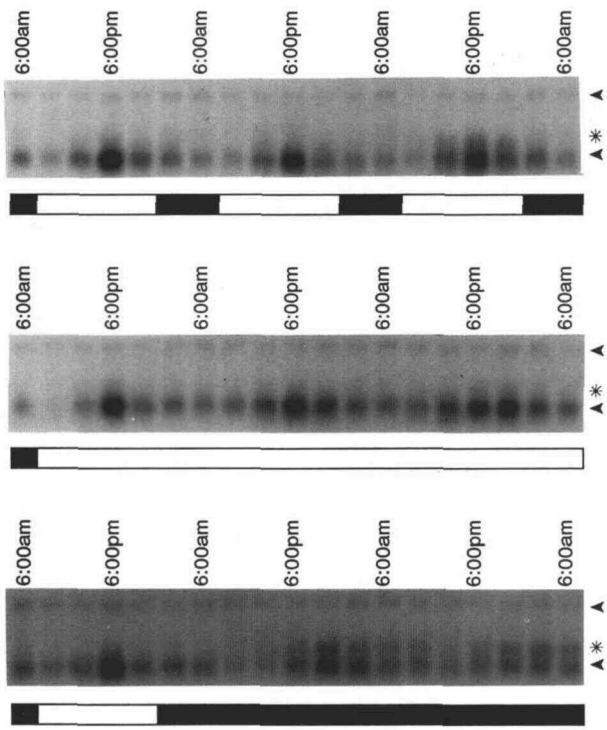

Figure 4. *Ccr1* transcript levels varied according to a circadian rhythm. *A. thaliana* plants were grown for 3 d in a light/dark cycle (top), maintained in continuous light after the first light period (middle), or maintained in continuous dark after the first light period (bottom). Four to six plants were harvested every 4 h. Four micrograms of total RNA per lane were hybridized to a full-length *Ccr1* cDNA fragment. Uniformity of loading and transfer of the RNA was confirmed by reprobing the blots with pea rDNA (Jorgensen et al., 1987). White and black bars below each blot denote periods of light and dark, respectively. Arrowheads indicate the positions of the 950- and 3300-base RNA species. An asterisk (*) denotes the position of an additional hybridizing species present mainly in plants grown under conditions of continuous darkness.

Ccr1 and *Ccr2* Transcript Levels Are Influenced by a Plant Circadian Rhythm

To examine in greater detail the pattern of *Ccr1* mRNA accumulation over time, untreated *Arabidopsis* plants were grown under our standard photoperiod of 16 h of light/8 h of dark, at a constant temperature of 20°C, and plants were harvested at 4-h intervals beginning at 6:00 AM (the light period begins at 7:00 AM). *Ccr1* mRNA displayed a clear pattern of accumulation, with minimal levels at 10:00 AM and maximal levels at 6:00 PM (Fig. 4, top). The periodic increase and decrease in the level of *Ccr1* transcripts suggested the involvement of a circadian rhythm. One of the defining characteristics of genes whose patterns of expression are under control of a circadian clock is the persistence of the rhythm in the absence of external cues such as a light/dark cycle (Feldman, 1989). To determine whether the variation observed for the accumulation of *Ccr1* mRNA was influenced by a circadian rhythm, plants were grown under a 16-h light/8-h dark photoperiod and then transferred to conditions of either continuous light or continuous dark. As shown in Figure 4 (middle), plants shifted to conditions of continuous light amassed *Ccr1* mRNA with a periodicity similar to plants grown under a light/dark cycle. A damping of the fluctuation occurred, beginning in the second cycle in the absence of environmental cues, and the timing of maximal accumulation was shifted in the third cycle (from 6:00 PM to 10:00 PM). Damping of the fluctuations and shifting of the phase of the rhythm was more evident in the dark-grown plants and began during the first constant dark cycle (Fig. 4, bottom). Similar fluctuations in transcript accumulation were also found for *Ccr2* (Fig. 5).

Intron Sequence from *Ccr2* Hybridizes to a Constitutively Expressed RNA

RNA from dark-grown plants contained additional species that hybridized to the *Ccr1* and *Ccr2* probes. These RNA transcripts, which migrated slightly slower than the 950-base RNA species, were also found in several other RNA preparations but not to the same extent as in dark-grown plants. It was possible, based on our isolation of a *Ccr2* cDNA containing 166 bp of intervening sequence, that the larger RNA species represented an alternatively spliced product analogous to those found associated with transcripts for other RRM-containing proteins such as *Drosophila* sex-lethal (Bell et al., 1988) and transformer (Nagoshi et al., 1988). To test this, the intervening sequence found in the *Ccr2* cDNA was used to probe the northern blot of RNA from dark-grown plants (Fig. 5, bottom). The result is shown in Figure 6. The probe hybridized to an RNA that migrated slower than the fully processed message and co-migrated with the higher-mol wt species detected with the full-length probe. However, the level of the intron-hybridizing species did not fluctuate during the circadian cycle, and the exposure time required to detect this partially spliced RNA was 3 times longer than for the dark-abundant RNA. These results suggest that the prom-

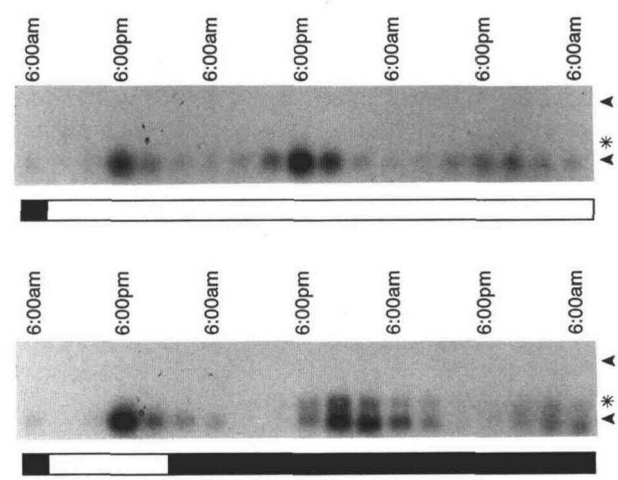

Figure 5. *Ccr2* transcript levels also varied according to a circadian rhythm. The blots shown in the middle and bottom panels of Figure 4 were reprobed with *Ccr2* cDNA. The arrowheads and asterisks (*) are as described in the legend to Figure 4. Hybridization of the *Ccr2* probe to the 3300-base species was only detectable after prolonged exposure of the autoradiogram.

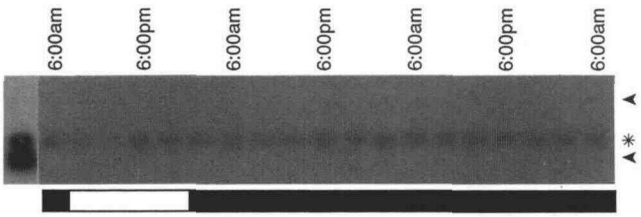

Figure 6. *Ccr2* intron hybridizes to a larger than full-length RNA. The northern blot of RNA from dark-grown plants used in Figure 5 (bottom) was stripped and reprobed with the 170-bp *Sty*I-*Rsa*I fragment from the *Ccr2* genomic clone. The second 10:00 PM lane from the bottom panel in Figure 5 is reproduced at the left to show the relative location of the intron-containing band. The hybridization conditions used were identical with those from Figures 4 and 5. The arrows and asterisk are as described in the legend to Figure 4.

inent, larger RNA species detected as increasing in abundance in dark-grown tissue is not due to alternative splicing of the *Ccr2* transcript.

Effect of Circadian Rhythm on the Levels of CCR1 Protein

To determine whether the levels of CCR1 protein also varied in a rhythmic fashion, CCR1 protein was purified as a GSH *S*-transferase fusion protein produced in *E. coli*, and antibodies to the fusion protein were generated in rabbits. Anti-CCR1 antibodies cross-reacted with a single polypeptide of 16 kD, the expected mass of an unprocessed protein encoded by the *Ccr1* fully processed transcript (Fig. 7). Unlike *Ccr1* transcripts, CCR1 protein levels did not fluctuate significantly or reproducibly during a 24-h period.

Effect of Different Stress Conditions on the Levels of *Ccr1* and *Ccr2* Transcripts

The steady-state levels of transcripts specifying RRM proteins from maize and carrot increase during conditions of water stress, ABA treatment, or wounding (Gómez et al., 1988; Sturm, 1992). To monitor the accumulation of *Ccr1* and *Ccr2* mRNAs in response to stress, 3-week-old plants were subjected to wounding, drought, ABA, or cold. Plants exposed to stress conditions were grown under the same light/dark cycle as control plants, and all plants were harvested at 10:00 AM, the low point in the circadian cycle. The results are presented in Figure 8A. *Ccr1* and *Ccr2* mRNAs did not accumulate differentially in response to wounding. It should be noted that harvesting the plants at the low point in the circadian cycle might preclude observing an induction if the circadian regulation overrides a stress-induction signal. However, we did observe responses to other stresses at 10:00 AM (see below), suggesting that the circadian regulation of the *Ccr* genes does not prevent responding to other stimuli. *Ccr1* mRNA levels decreased in plants treated with ABA or subjected to water stress, whereas *Ccr2* transcript levels increased in water-stressed plants. Plants exposed to cold stress showed a marked increase in the amounts of both *Ccr1* and *Ccr2* transcripts. As a control, the blot in Figure 8A was also probed with pHH29, which had previously been shown to hybridize to an RNA species that accumulated in response to drought, ABA, and cold (Hajela et al., 1990); these results were repeated under our stress conditions.

To assess the kinetics of *Ccr1* and *Ccr2* mRNA accumulation over time due to cold treatment, plants were placed at 4°C at 6:00 AM and harvested at 4-h intervals (Fig. 8B). As expected, *Ccr1* and *Ccr2* mRNAs from control plants accumulated higher levels over time because of the circadian fluctuations described above. However, *Ccr1* and *Ccr2* transcripts in leaves from plants incubated at 4°C for 12 and 16 h were more abundant than in control plants. After we normalized for the amount of RNA loaded and subtracted the signal due to circadian effects observed in the controls, transcript levels for *Ccr1* and *Ccr2* increased approximately 4-fold in plants incubated for 16 h at 4°C. The timing of the increased accumulation of *Ccr1* and *Ccr2* mRNAs was less rapid when compared with mRNA hybridizing to pHH29, which began accruing after 4 h of cold treatment (Fig. 8B). The RNA levels for pHH29 may be regulated by a circadian rhythm (Fig. 8B), but confirmation will require further analysis. Computer analysis of the upstream sequences from *Ccr1* and *Ccr2* did not yield any strong similarities with consensus sequences of other promoters that are known to be inducible (as defined by Cattivelli and Bartels, 1992; Olsen et al., 1992; Schindler et al., 1992).

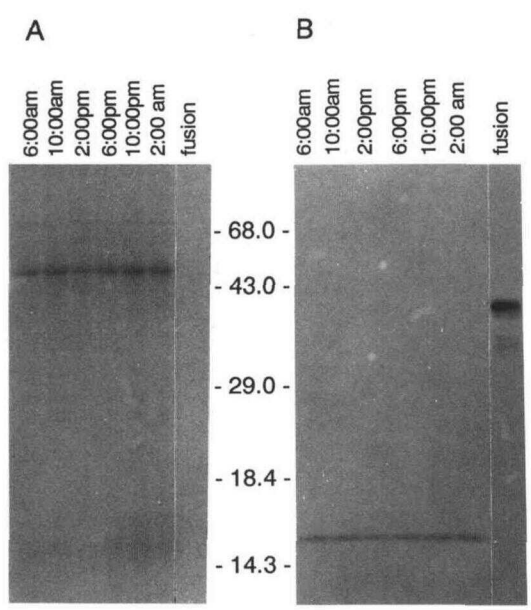

Figure 7. Western blot analysis of CCR1 protein levels. Protein extracts were made from plants harvested at 4-h intervals (times are given above the lanes). Protein (20 μg) was subjected to electrophoresis on a 12% SDS-polyacrylamide gel; one set of two duplicate samples was stained with Coomassie blue to determine loading uniformity (A), and the second set was used to prepare a western blot (B). Numbers on the sides denote mol wts of protein markers ($\times 10^{-3}$). CCR1 protein was detected using rabbit polyclonal antisera generated using recombinant CCR1 fused to GSH *S*-transferase. A sample of the original antigen was included as a positive control (fusion; expected mol wt of 42×10^3).

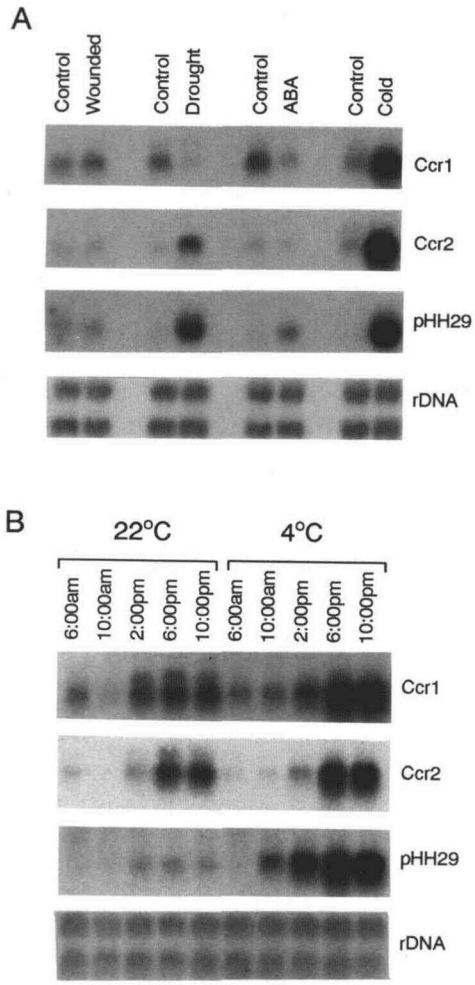

Figure 8. Effects of wounding, drought, ABA, and cold on *Ccr1* and *Ccr2* transcript levels. Stress treatments are described in "Materials and Methods." A, A single blot was probed in succession with *Ccr1* genomic DNA, *Ccr2* cDNA, pHH29 (Hajela et al., 1990), and pea rDNA. pHH29 is a cDNA clone from *A. thaliana* whose transcript levels have previously been shown to increase following treatment with cold, ABA, or drought (Hajela et al., 1990). Plant treatments with ABA were repeated three times with very similar results. B, Time course for the accumulation of *Ccr1* and *Ccr2* transcripts in response to cold. Plants were grown under constant light (beginning at 6:00 AM), and leaves were harvested at the times indicated.

Accumulation of *Ccr1* and *Ccr2* Transcripts in Different *Arabidopsis* Tissues

To determine whether *Ccr1* and *Ccr2* are expressed in a tissue-specific manner, RNA was extracted from *Arabidopsis* leaves, flowers, siliques, and stems at both 10:00 AM and 6:00 PM (low and high transcript accumulation points in the circadian cycle). The results, presented in Figure 9, indicate that *Ccr1* and *Ccr2* are expressed at comparable levels in all plant tissues tested, and both are still regulated by the circadian rhythm. Curiously, the transcript that migrated slightly slower than the 950-base transcript was not detected in RNA isolated from silique tissue probed with either *Ccr1* or *Ccr2*. Rather, a new larger transcript that hybridized to the *Ccr1* probe was found in silique RNA preparations at both 10:00 AM and 6:00 PM. This transcript could represent an additional family member or an alternatively processed form of *Ccr1* mRNA.

DISCUSSION

van Nocker and Vierstra (1993) recently reported the sequences of two *A. thaliana* cDNAs that contained RRMs. We report here the independent isolation and characterization of two very similar, and possibly identical, *A. thaliana* genes that encode the cDNAs. Based on the regulation of expression of these genes, we have named them *Ccr1* and *Ccr2* (Cold, Circadian, RNA binding). *Ccr1* and *Ccr2* mRNA levels exhibited cyclic variation that was maintained under constant environmental conditions. This pattern of expression indicated that the *Ccr1* and *Ccr2* genes are under control of an endogenous circadian clock. A number of processes in plants are affected by a biological timer, including photosynthesis, leaf positioning, and sap exudation (reviewed by Feldman, 1989). *Cab*, encoding the major light-harvesting Chl-binding protein of the chloroplast, is expressed at maximal levels soon after the onset of light in wheat (Nagy et al., 1988), tomato

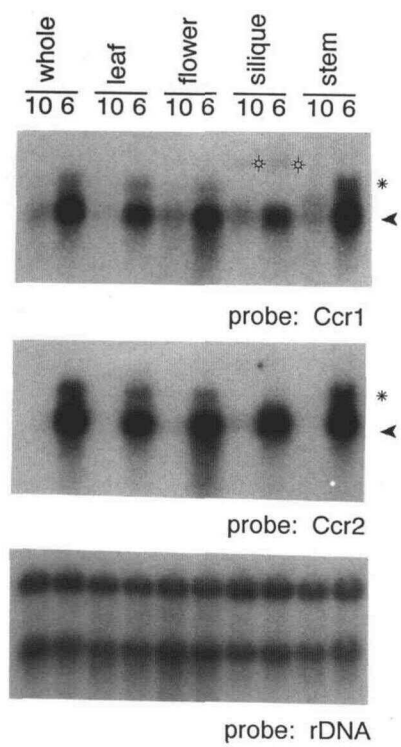

Figure 9. *Ccr1* and *Ccr2* transcript levels in different tissues. RNA was isolated from various tissues (listed above the lanes; whole = whole plants). Tissues were harvested at the low (10:00 AM) and high points (6:00 PM) in the circadian cycle of *Ccr1* and *Ccr2* (times listed above each lane). A northern blot was produced, probed with the *Ccr1* genomic probe (see Fig. 2B), stripped, probed with the cDNA clone for *Ccr2*, stripped, and reprobed with the pea rDNA gene. Arrow and asterisk symbols are defined in the legend to Figure 4. The open asterisks denote a new *Ccr1*-hybridizing species found only in RNA prepared from siliques.

(Piechulla, 1988), and *Arabidopsis* (Millar and Kay, 1991). The pattern of accumulation of *Ccr1* and *Ccr2* mRNAs, with minimal levels occurring 3 h following illumination and maximal levels 11 h following illumination, is distinctly different from the expression pattern of *Cab*. The circadian rhythm for the *Ccr* genes is also different from that observed for the nitrate reductase genes in *Arabidopsis* (Cheng et al., 1991) in which the RNA levels peaked in the early morning and decreased during the day to reach a low point at the end of the light period. It is interesting that the cyclic expression pattern of the *Ccr1* and *Ccr2* genes is very similar to the pattern of expression of the maize catalase 3 gene (Redinbaugh et al., 1990).

Ccr1 and *Ccr2* transcript levels also appeared to be influenced by cold treatment. Plants incubated at 4°C for 24 h beginning at 10:00 AM amassed significantly more *Ccr1* and *Ccr2* transcripts than plants grown at 22°C (Fig. 8A). Caution must be used, however, when interpreting these results. Martino-Catt and Ort (1992) found that the circadian rhythm responsible for fluctuations in expression of *Cab* was suspended during cold treatment in tomato, a highly chill-sensitive plant. Furthermore, the turnover of *Cab* mRNA was suspended during the cold treatment. However, changes in environmental conditions have been shown to transiently affect circadian regulation in the short term, followed by a resumption of normal cycling after prolonged exposure (Pittenrigh, 1993, and refs. therein). We are in the process of determining *Ccr* RNA levels in cold-treated plants after several days of exposure to the reduced temperature. It should be noted that the RNA levels for *Ccr1* and *Ccr2* increased during the cold treatment above the levels for the corresponding times in the control tissues. These results suggest either a cold responsiveness in the control of transcription or a cold-sensitive RNA degradation component of the circadian rhythm regulation.

Southern blot analysis revealed that *Ccr1* and *Ccr2* are members of a small gene family. The 3300-base RNA species that hybridized to fragments from both 5′ and 3′ end-coding sequences derived from *Ccr1* did not accumulate differentially in response to cold or the circadian cycle and may represent an additional family member. In dark-grown plants and in the circadian cycle when maximal levels of *Ccr* transcripts are produced, an additional RNA species was observed that hybridized strongly to both *Ccr1* and *Ccr2* probes and migrated slightly slower than the 950-base transcript. A second RNA species, which co-migrated with the dark-abundant RNA species, was detected when the northern blot of RNA from dark-grown plants was probed with a fragment of the *Ccr2* intron that was found in the *Ccr2* cDNA. However, based on the low abundance of this intron-containing sequence and its lack of circadian regulation, this transcript is unlikely to be the same as the larger transcript detected with the full-length *Ccr2* probe. Other explanations for the identity of the dark-abundant transcript include another member of the *Ccr* gene family or a different transcription start site. We are currently working to identify the additional cycling RNA species that accumulates in dark-grown plants.

The *Ccr1* gene was isolated from a genomic library by differential screening using cDNAs generated from RNA isolated from *A. thaliana* that had been either inoculated 4 d previously with TCV or mock treated with buffer. RNA gel blot analysis using RNA preparations differing from those used in the initial library screenings revealed that *Ccr1* mRNA did not accumulate in response to virus infection as originally thought. What then was the difference between the original mock-treated and virus-infected plants? One possible explanation was the way in which the plants used to prepare RNA for the first screen were harvested. The several thousand mock-treated plants required for the generation of the cDNA probes used in the initial differential screening of the genomic library were harvested in the late morning and an equal number of virus-infected plants were harvested in the early afternoon. As shown in Figure 4, levels of *Ccr1* transcripts fluctuated according to a plant circadian rhythm with minimal amounts present at 10:00 AM and maximal amounts at 6:00 PM. Plants harvested in the late morning would, therefore, contain less *Ccr1* mRNA than plants harvested in the afternoon.

Although this explanation seems promising, it should be noted that a second genomic clone isolated in the same screen did not accumulate in response to the virus or the plant circadian rhythm. This clone has been determined to encode a novel Gly-rich protein with sequence similarity to group 2 late embryogenesis abundant proteins (C.D. Carpenter and A.E. Simon, unpublished data). Therefore, it is possible that the two populations of mRNA from virus-infected and mock-treated plants differed in another, yet undefined, parameter.

The cellular role(s) of this class of plant Gly-rich RRM proteins remains a mystery. Many of the RRM proteins from other organisms have a binding affinity for specific target sequences such as the 70K U1 snRNP protein, which binds with high affinity to the 5′ half of U1 RNA (Query et al., 1989). Other RRM proteins, such as the poly(A)-binding protein, bind to mRNAs in general (Adam et al., 1986). One possible role for CCR1 may be to stabilize mRNAs, either specific species or, in general, during conditions of cold or other, as yet undetermined, environmental stresses. We have recently demonstrated that CCR1 can indeed bind RNA (C.D. Carpenter and A.E. Simon, unpublished data) and are in the process of determining whether CCR1 preferentially binds to specific RNAs and/or sequences as a prerequisite to ascertaining a functional role for the protein. It is interesting that the increase in mRNA levels for other *A. thaliana* cold-inducible proteins involves a posttranscriptional mechanism (Hajela et al., 1990) that could be mediated by binding to a protein such as CCR1. A second possible role for CCR1 may be in activating mRNAs that are translationally suppressed because of secondary structure or other constraints. This speculation is based on early reports that RRM-containing proteins can function as helix-unwinding proteins (Herrick and Alberts, 1976). Work is currently under way to determine the RNA-binding capabilities of CCR1 and to distinguish between these and other possible functions for the protein.

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