Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3731–3735, May 1992
Plant Biology

Low temperature interrupts circadian regulation of transcriptional activity in chilling-sensitive plants

[photosynthesis/ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase/Rubisco small subunit/chlorophyll a/b binding protein]

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Communicated by William L. Ogren, January 21, 1992 (received for review July 3, 1991)

ABSTRACT **Impaired chloroplast function is responsible for nearly two-thirds of the inhibition to net photosynthesis caused by dark chilling in tomato (*Lycopersicon esculentum* Mill.), yet it has not been possible to localize the dysfunction to specific chloroplast reactions. We report here on an effect that low-temperature exposure has in tomato on the expression of certain nuclear-encoded chloroplast proteins, which may be directly related to the chilling sensitivity of photosynthesis. Transcriptional activity of genes for both the chlorophyll a/b binding protein of photosystem II (Cab) as well as for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase was found to be controlled by an endogenous rhythm. For Cab this rhythm was also visible at the level of newly synthesized protein, indicating that the circadian control of transcriptional activity normally ensures that this protein is synthesized only during daylight hours. However, low-temperature treatment suspended the timing of the rhythm in tomato so that, upon rewarming, the circadian control was reestablished but was displaced from the actual time of day by the length of the chilling exposure. In addition, we found that the normal turnover of Cab and Rubisco activase mRNA was suspended during the low-temperature treatment, but, upon rewarming, this stabilized message was not translated into protein. We believe that the low-temperature-induced mistiming of gene expression together with its effect on the translatability of existing transcripts may be an important clue in unraveling the basis for the chilling sensitivity of photosynthesis in tomato.**

Many plant species that are evolutionarily adapted to warm habitats are very susceptible to injury by low-temperature exposure. Brief exposures to low ($0°C < T < 10°C$), but above freezing, temperatures can have profound effects on season-long growth and productivity even though there may be no outward signs of damage. For many chill-sensitive species, an important element of the low temperature-induced injury is an inhibition of photosynthesis.

Low-temperature exposure in combination with high irradiance causes rapid inhibition of photosynthesis in a broad range of plants including tomato (1), cucumber (2), and maize (3). Several elements contributing to the inhibition have been identified. Damage to the reducing side of photosystem II is well documented (4–6) and, for moderately sensitive species such as maize, may be the major cause of impaired whole plant photosynthesis following chilling. However, in the most chill-sensitive species, such as tomato (*Lycopersicon esculentum*), impaired reductive activation of the stromal bisphosphatases appears to be the dominating factor limiting carbon assimilation following chilling in the light (7).

Low temperature at night can also cause severe reductions in CO_2 fixation on the day following the chill. Like the inhibition caused by chilling in the light, it is clear that the primary loss of activity arises due to direct impairment of chloroplast function (8), but in the case of dark chilling it has not been possible to assign the cause to specific reactions. Thus, while the inhibition of net photosynthesis by dark chilling in plants such as tomato can be quite large, the underlying causes are subtle and are likely to involve disruption of the coordination among the component reactions of photosynthesis rather than direct inhibition of the reactions themselves. This paper reports on an effect that low-temperature exposure has on the expression of certain nuclear-encoded chloroplast proteins in tomato, which we feel may be a clue to the underlying basis of the inhibition of photosynthesis following chilling in the dark.

A considerable amount of progress has been made recently in understanding the factors involved in controlling the synthesis of the major chlorophyll a/b binding proteins of photosystem II (Cab). Of particular importance to our study was the discovery of a circadian rhythm in the transcriptional activity of Cab genes (9–13). In the work presented here we show that this rhythm in transcriptional activity is also visible at the level of newly synthesized Cab protein. We further show that the expression of a second nuclear-encoded chloroplast protein, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase (rca), also displays a circadian rhythm in transcriptional activity; however, the control of rca protein synthesis is more complex than for Cab and may involve translational regulation. In tomato, low temperature has two separate effects on the normal pattern of expression of these two proteins: (*i*) progression of the timing of the circadian clock controlling gene transcription is suspended throughout the period of low-temperature exposure, and (*ii*) normal turnover of the existing transcripts is suspended. Upon rewarming, the circadian rhythm of transcriptional and translational activity is reestablished but is out of phase with the actual time of day by the amount of time that the tomato plant was at low temperature. In addition, after rewarming, the messages that were stabilized at low temperature can no longer be translated into protein. We consider the possibility that the mistiming in expression of these and perhaps other chloroplast proteins may be a central factor underlying the chilling sensitivity of photosynthesis in tomato.

MATERIALS AND METHODS

Plant Growth and Chilling Treatment Conditions. Tomato plants (*L. esculentum* Mill. cv. Floramerica) were grown

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Abbreviations: Cab, chlorophyll a/b binding protein of photosystem II; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; rca, Rubisco activase; SSU, small subunit of Rubisco.

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from seed under a 32°C, 14-h light/25°C, 10-h dark regime as described elsewhere (14). Three-week-old plants were chilled in the dark at 4°C and subsequently rewarmed to 25°C under 100% relative humidity. Control plants were treated identically except that the temperature was maintained at 25°C throughout the incubation period.

***In Vivo* Labeling and Analyses of Proteins.** Leaf proteins were *in vivo* labeled with [^{35}S]methionine for 30 min and isolated as described in detail elsewhere (14) followed by separation on 12% acrylamide slab gels using the Laemmli buffer system (15). After electrophoresis, proteins were blotted onto Immobilon-P poly(vinylidene difluoride) paper (Millipore) to obtain an autoradiograph, a Western blot, and a stained image of the leaf proteins from a single gel, thereby avoiding the distortions in size that often accompany the staining/destaining protocols necessary for standard fluorography (16).

Isolation and Analyses of RNA. Total cellular RNA was isolated from tomato leaves by a hot borate extraction method essentially as described by Hall *et al.* (17) or by a small-scale hot phenol procedure (18). The cDNA probes used in this study were (*i*) pea pAB96 (19) for Cab mRNA, (*ii*) spinach pRCA1.9 (20) for rca mRNA, (*iii*) pea SS15 (19) for the small subunit of Rubisco (SSU) mRNA, and (*iv*) a 1.35-kilobase *Eco*RI/*Hin*dIII fragment of flax pBG35 (21) for rRNA. The hybridizations with denatured radiolabeled probes were performed at 42°C overnight. The slot blots were then rinsed twice for 15 min at room temperature in a solution of 360 mM NaCl/20 mM sodium phosphate/2 mM EDTA/0.1% SDS followed by two 10-min washes at 50°C in a solution in which all the ingredients except SDS were diluted 20-fold (22).

Nuclear Run-on Transcription Assays. Intact nuclei were isolated from tomato leaves on a 40%/60%/80% discontinuous Percoll gradient (23) and stored at −80°C by using procedures detailed in ref. 16. Nuclei, isolated from both control and chilled leaf tissue, maintained linear incorporation of radioactivity at 30°C throughout the 30-min duration of the *in vitro* transcription assay (16). Hybridizations of the radioactively labeled RNA with the various cDNA clones were performed as described for the slot blots.

RESULTS

Diurnal and Circadian Patterns Are Evident in the Rate of Net Protein Synthesis and in the Steady-State mRNA Levels for Cab and rca. Patterns of newly synthesized polypeptides in attached tomato leaves were monitored during the plant's normal light/dark cycle (i.e., 14-h light/10-h dark) by *in vivo* pulse-labeling with [^{35}S]methionine. The autoradiograph in Fig. 1*A* shows strong diurnal variation in the rate of net synthesis of both Cab and rca. In contrast, SSU was synthesized at high levels throughout the light/dark cycle, although a low-amplitude diurnal variation was detectable when radioactivity of the bands was determined by scintillation counting (16). Although the synthesis of both Cab and rca is known to be light regulated (24–26), Fig. 1 shows the two had subtly different patterns of induction and timing of maximum expression. Abundant synthesis of rca was evident when the plant was labeled at 6 o'clock in the morning (Fig. 1, lane 06), 2 h before the beginning of the light cycle, whereas radioactively labeled Cab did not begin to accumulate until just before the start of the light cycle (Fig. 1, lane 08). Accumulation of rca declined to low levels by midafternoon, whereas Cab was expressed abundantly throughout the light cycle.

Total cellular RNA was isolated at the same times and from the same plants as the labeled proteins. The slot blots in Fig. 1*B* show that the steady-state Cab mRNA levels oscillated with the same pattern observed at the protein level. Diurnal

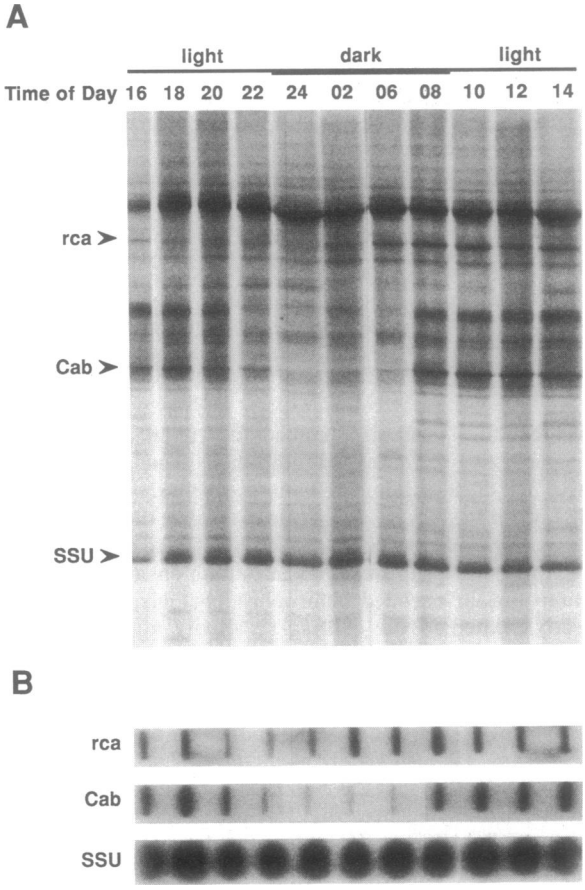

FIG. 1. Diurnal oscillations in the accumulation of newly synthesized proteins and in steady-state mRNA levels in tomato leaves. At 2-h intervals throughout the normal light/dark cycle, fully expanded, attached leaves were gently abraded with 400-grit Carborundum, and 500 μCi (1 Ci = 37 GBq) of [^{35}S]methionine was applied in a 25-μl droplet (14). (*A*) Autoradiograph of an SDS/PAGE gel showing the proteins that were synthesized and accumulated during the 30-min labeling periods. Approximately 5 × 10^5 trichloroacetic-acid-precipitable cpm were loaded per lane. The positions of the bands corresponding to Cab, rca, and SSU, which were identified with specific antibodies, are denoted by arrows. (*B*) Corresponding slot blots of total RNA (2 μg per slot), which were screened with cDNA probes for Cab, rca, and SSU.

oscillations in the level of rca mRNA appeared somewhat weaker than those seen at the protein level, whereas SSU mRNA levels were high throughout the light/dark cycle.

The observation that the net synthesis of Cab and rca protein increased prior to the beginning of the light cycle and began to decline before it ended (Fig. 1) suggested that the light-induced changes were superimposed on an endogenous rhythm. To distinguish between diurnal (i.e., light-induced) and circadian influences on the expression of these two proteins, net protein synthesis and steady-state mRNA levels were measured in plants that were transferred into continuous darkness. The endogenous oscillation of Cab (Fig. 2*A*) and rca (Fig. 2*B*) proteins was followed by cutting the purple bands from an alkaline phosphatase Western blot and determining the radioactivity by scintillation counting. While the net synthesis of both Cab and rca protein showed a strong endogenous rhythm in either continuous dark (Fig. 2) or continuous light (data not shown), the profile for rca expression was much broader than that for Cab. This same pattern of oscillation continued during a second day of continuous darkness, albeit with a reduced amplitude, except that the relatively high level of expression of Cab between 16 and 18 h was absent (16). This is explained by the fact that these

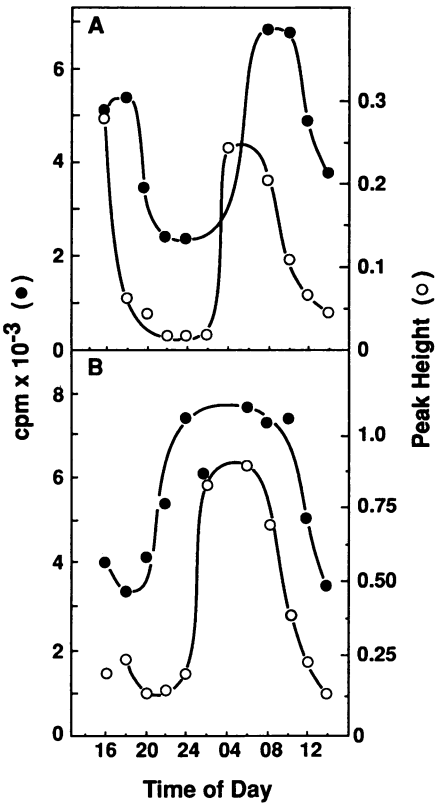

FIG. 2. Oscillations in the accumulation of newly synthesized Cab (*A*) and rca (*B*) proteins (●) and in the steady-state mRNA levels (○) in tomato leaves during continuous dark. Plants were transferred to continuous dark conditions at 14 h, and samples of newly synthesized protein and total RNA were collected at 2-h intervals throughout the next day as described in Fig. 1. The relative amounts of newly synthesized Cab and rca protein were determined from the radioactivity of the purple bands from alkaline phosphatase Western blots by scintillation counting. Comparisons of steady-state levels of mRNA were made based on densitometer scans of slot blots.

plants were transferred to the dark at 14 h, a time in the light cycle when Cab expression was maximal (Fig. 1*A*). Thus, the initially high level of Cab net synthesis shown in Fig. 2 is attributable to lingering effects of light-induced expression, which briefly obscured the true endogenous pattern.

Densitometer scans made on slot blots of total cellular RNA (Fig. 2) show that the steady-state mRNA level for Cab oscillated in phase with the rate of net synthesis of the protein, although the drop in net synthesis of the protein lagged several hours behind the decline in steady-state message level. As was the case with Cab protein, the initially high message level (16 h) was a lingering effect of light-induced expression and was very low at this time point during the second day of continuous darkness (16). While the oscillation pattern of rca mRNA was quite similar to that of Cab, in this case, the increase in the rate of net synthesis preceded the rise in steady-state message level, suggesting that there are translational controls superimposed on the circadian control over transcriptional activity.

In Tomato, Low Temperature Arrests the Natural Progression of the Circadian Rhythm Controlling Cab Expression. Fig. 3 *Upper* is an autoradiograph of newly synthesized proteins from tomato plants that were put in the dark for 16 h at 25°C (control) or 4°C (chill) after which both control and chilled plants were maintained in the dark for an additional 2 h at 25°C, during the final 30 min of which portions of leaves were labeled with [^{35}S]methionine. The dark treatments were started either during active expression of Cab in the afternoon (14 h) or before the beginning of light cycle, when Cab expression is essentially nonexistent (07 h). Protein and mRNA samples were taken 18 h later (i.e., at 08 h and 01 h). The control plants showed the pattern of expression that corresponded to the continuation of the circadian rhythm shown in Fig. 2*A*. However, the chilled plants demonstrated a delay in the expression of Cab protein that was equivalent in time to the length of the low-temperature exposure. For example, when the low-temperature treatment was initiated at 07 h, high levels of newly synthesized Cab protein were detected after the chill even though the sample was taken 2 h after rewarming, at 01 h (i.e., middle of the normal dark period), when the expression of this protein is normally exceedingly low. The clock had advanced only during the 2-h rewarming period and was therefore expressing Cab as if it were 09 h (i.e., early morning). On the other hand, when the dark treatment was initiated at 14 h, Cab protein was strongly expressed 18 h later (08 h) in the control but undetectable in the chilled plants since, because of the 16-h delay in the rhythm, the clock setting was 16 h. Since these plants had been in the dark for 18 h, there was no lingering influence of light-induced expression discussed earlier for Fig. 2*A*, and therefore low Cab expression is expected when the endogenous clock setting is 16 h. Notably, although there was little net Cab protein synthesis in this chilled sample, the slot blots in Fig. 3 *Lower* show that the steady-state level of Cab message remained high; this observation will be considered in more detail below.

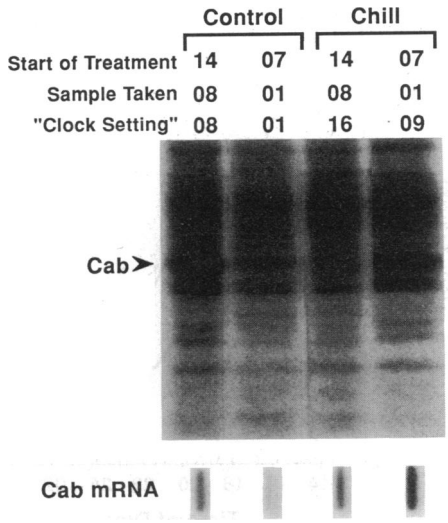

FIG. 3. Effect of low temperature on the circadian regulation of Cab protein synthesis and mRNA levels in tomato. The 18-h dark treatments were started at two times during the circadian cycle (14 h and 07 h), such that samples were taken in the morning (08 h) and in the middle of the night (01 h). After 16 h in the dark at either 25°C (control) or 4°C (chill), both control and chill plants were maintained in the dark for an additional 2 h at 25°C, during the last 30 min of which the leaves were labeled as described in Fig. 1. (*Upper*) Autoradiograph showing newly synthesized proteins. (*Lower*) Corresponding slot blots of total RNA screened with a Cab cDNA probe. Comparison of the "clock setting" between control and chill treatments shows the 16-h delay in the circadian rhythm caused by the 16-h chill treatment.

The nearly one-to-one dependence of the length of the delay in Cab expression on the length of the chill treatment is shown in Fig. 4. On the basis of densitometric scans of autoradiographs, Fig. 4 shows that the decline in the rate of net Cab synthesis that normally occurs in continuous darkness between 14 and 18 h was shifted by ≈4 h following a 4-h chill. Thus low temperature prevented the advance in the circadian rhythm controlling the expression of Cab protein, but, upon rewarming, the rhythm was reinitiated from where

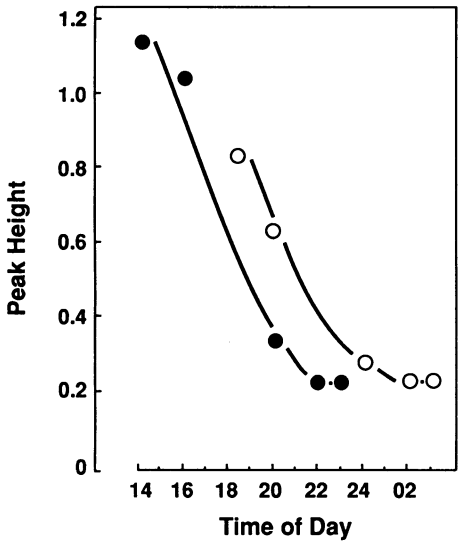

FIG. 4. Correspondence between the length of the chill treatment and the length of the delay induced in circadian rhythm of Cab expression. The relative rate of Cab protein accumulation was determined from densitometric scans of autoradiographs of *in vivo* labeled plants. ●, Normal circadian pattern of Cab expression for plants held under continuous dark conditions at 25°C showing net synthesis falling from maximum levels in early afternoon to a barely detectable level in early evening; ○, shift in the Cab expression pattern when the plants were held at 4°C from 12 to 16 h. These plants had been maintained in the dark for 16 h prior to the beginning of the experimental treatments, and therefore the endogenous rhythm was fully visible and not obscured by any residual effects of light-induced expression that were seen in Fig. 2 (see text).

it had stalled and followed the normal pattern of expression except that it was offset from the actual time of day by the length of the chill treatment.

Low Temperature Affects both Transcriptional and Post-transcriptional Events in the Expression of Cab and rca in Tomato. Information about the transcriptional activity of specific genes can be obtained from assays with isolated nuclei that, when primed with ribonucleotides, complete elongation of already engaged RNA polymerase II molecules. It is reasoned that the strength of the hybridization of these run-on transcripts with a given cDNA reflects the *in vivo* activity of that gene at the time the nuclei were isolated (23, 27, 28). Fig. 5 shows *in vitro* run-on transcription assays that correspond to the treatments and time points presented in Fig. 3, which demonstrates that the transcriptional activity of the Cab and rca genes was high during the midmorning (08 h) when mRNA and the rate of net protein synthesis were at their highest levels (Fig. 3). Unfortunately, for the time point taken in the middle of the night (01 h), the yield of nuclei from control plants was very low as reflected by the faint rRNA hybridization. The low yield and reduced competence of the nuclei may be due to the fact that these plants had been in the dark for 27 consecutive hours. The nighttime isolation was somewhat more successful from the chilled plants, in which 16 of the 27 h in the dark was at 4°C. In spite of this problem, it is clear that the run-on transcriptional activity in nuclei isolated from chilled tomato was delayed in comparison to the control by the period of time that the plant was held at low temperature. This delay in transcriptional activity was almost certainly the origin of the low-temperature-induced shift in the pattern of Cab mRNA levels and net protein synthesis rates depicted in Fig. 3.

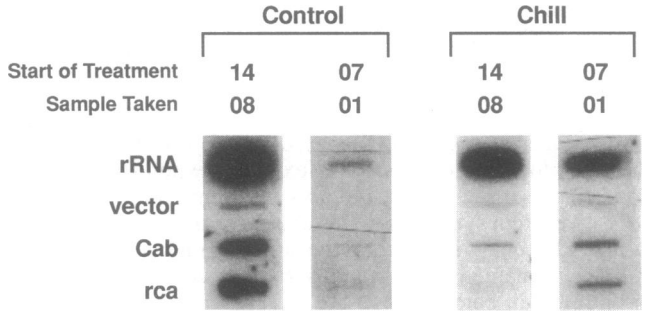

FIG. 5. Nuclear run-on transcription assays after low-temperature treatment. Nuclei were isolated from tomato plants that were put in the dark for 16 h at 25°C (control) or 4°C (chill) plus, as in Fig. 3, an additional 2 h at 25°C. The dark treatments were started at two points in the diurnal cycle (14 h and 07 h) such that samples for nuclei isolation were taken in the morning (08 h) and in the middle of the night (01 h). The RNA labeled *in vitro* during the 30-min reaction was isolated and used to probe a blot containing cDNA clones specific for rRNA, vector without insert, Cab, and rca.

Evidence that a posttranscriptional dysfunction was also induced by chilling in tomato is found in the high levels of Cab message that were present in the morning (08 h) following a 16-h chill treatment when no net synthesis of Cab protein occurred (Fig. 3). Fig. 6*A* shows that the strong oscillation of steady-state Cab mRNA levels that occurs in continuous darkness at 25°C (Fig. 2) is absent throughout the period that the plant was at 4°C during which time the Cab message level remained stable. Furthermore, the Cab cDNA probe hybridized strongly with run-on transcripts made (at 30°C) by nuclei isolated immediately after, or even 2 h after (data not shown), the plants had been placed at low temperature (Fig. 6*B*). In spite of the strength of the hybridization, it is expected that the *in vivo* rate of transcription would be low at 4°C because of the strong thermodynamic effect of temperature on enzymatic processes. After 16 h at low temperature, the strength of the hybridization was weaker, which indicates that the transcripts initiated prior to the 4°C treatment were eventually aborted. It follows that the maintenance of the high steady-state Cab message levels depicted in Fig. 6*A* was due to a lowered rate of mRNA degradation while the plants were at 4°C. Upon rewarming, this residual message was degraded at least as rapidly as newly transcribed Cab message [apparent half-time of about 3 h (16)].

DISCUSSION

Several years ago we found that dark chilling had numerous obvious effects on protein synthesis in tomato including a dramatic, but puzzling, change in Cab synthesis (14). On the

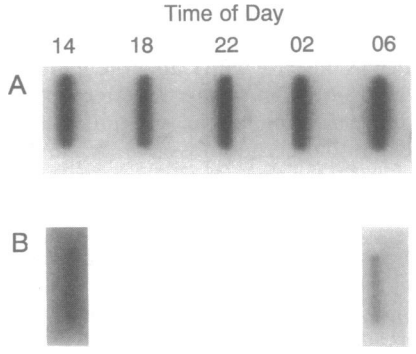

FIG. 6. Analysis of the effect of low-temperature treatment on Cab mRNA stability and on the maintenance of transcriptional competence. (*A*) At 4°C, the circadian oscillation in steady-state Cab message level seen in Fig. 2 at 25°C is suspended, and the steady-state Cab message level remains constant. (*B*) Effect of prolonged low temperature on the ability of isolated nuclei to complete run-on transcription of Cab transcript.

basis of the data presented here, it is now clear that chilling affects Cab synthesis by suspending the progression of the circadian rhythm controlling the expression of these genes. We found that, while the delay was visible at the level of net protein synthesis (Fig. 3), the underlying basis of the delay could be traced to an effect of low temperature directly on the transcriptional activity of the gene(s) (Fig. 5). Although it has been shown that the circadian rhythm in Cab gene expression in tomato is temperature insensitive over a relatively wide range (29), at some critical temperature below 10°C, the mechanism for temperature compensation fails and the circadian rhythm stalls. The stoppage was fully reversible since, upon returning the plants to permissive temperatures, the endogenous rhythm in Cab expression continued forward from the point it had been just prior to the chilling treatment.

There were also posttranscriptional dysfunctions in tomato induced by the low-temperature exposure. During the chilling treatment, the normal oscillations in steady-state mRNA levels of Cab (Fig. 6) and rca (16) ceased because low temperature prevented both production of new mRNA, as well as the degradation of transcripts already present. A surprising additional feature of the Cab message stabilized by low temperature is that, upon rewarming, it was not translated into protein (Fig. 3). In contrast, newly transcribed message that coexisted with the carryover was fully competent. It remains to be determined why the message stabilized at low temperature and present at the time of rewarming is apparently not translated. It also remains to be determined how general this effect of low temperature may be in tomato since, for SSU and other genes that do not show large daily oscillations in expression, the presence of untranslatable message would not have been detected.

These low-temperature-induced changes in the timing of Cab and rca expression may be an important clue in unraveling the basis of the low-temperature sensitivity of photosynthesis in chilling-sensitive plants. Each of these gene products plays a crucial role in the overall ability of a plant to perform efficient photosynthesis from the standpoint of light absorption and activation of a central enzyme in the carbon reduction cycle. However, it is doubtful that the changes observed in the expression of Cab and rca portend any direct detrimental effects on photosynthetic performance, since this transient perturbation is small in comparison to the total amount of these proteins already present in chloroplasts of mature leaves. Indeed, we showed in earlier work on tomato that overnight chilling caused no measurable change in chlorophyll content, a/b ratio (8), or in the ability of illuminated leaves to activate Rubisco (30). The situation may be different in developing photosynthetic tissue since the level of accumulated Cab protein is low enough that diurnal oscillations in the chlorophyll a/b ratio are detectable (31). The responses of Cab and rca transcriptional activity in tomato to low temperature may be most significant as models for the behavior of other, much less abundantly expressed genes for which the interruption or mistiming in expression could have serious consequences for photosynthesis. For example, low-temperature-induced mistiming in the expression of genes normally functional only at night could cause futile biochemical cycles or disrupt metabolite-based regulatory controls if expressed in the chloroplast during the day. The effect of low temperature on the stability and translatability of Cab and rca message may be of secondary importance in regard to the low-temperature sensitivity of photosynthesis, since only those transcripts present at the time of the chill are involved. However, if this effect of chilling in tomato turns out to involve a broad spectrum mRNA, and not just that produced by genes under circadian regulation, its contribution to chilling sensitivity could then be much more significant.

This work was supported in part by a grant from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (91-37100-6620) and by an award from the McKnight Foundation.

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