Transcriptional and post-transcriptional processes regulate expression of RNA encoding the small subunit of ribulose-1,5-biphosphate carboxylase differently in petunia and in soybean

Deborah Marin Thompson and Richard B.Meagher*
Department of Genetics, University of Georgia, Athens, GA 30602, USA

Received October 26, 1989; Revised and Accepted May 15, 1990

ABSTRACT

The effects of white light, far-red light and darkness on the *in vitro* transcription and RNA levels of the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*S) were investigated in petunia and in soybean. In petunia plants treated with 48 hours of darkness the *in vitro* transcription rate of two of the *rbc*S subfamilies of petunia, *rbc*S A and *rbc*S C, declined 32- and 8-fold respectively, whereas treatment of dark-adapted plants with light caused the *in vitro* transcription rate of these subfamilies to return to their light-grown levels. Relative RNA levels of *rbc*S A and *rbc*S C declined in parallel with *in vitro* transcription rate changes upon treatment of petunia plants with darkness. However, while relative RNA levels of *rbc*S C changed in parallel with *in vitro* transcription rate under all conditions of far-red light and white light tested, there were differences between the changes in *rbc*S A *in vitro* transcription rate and RNA levels which were consistent with post-transcriptional regulation of *rbc*S A RNA. In addition we observed that nuclei isolated from the leaves of plants which were exposed to darkness for periods of 72 hours or longer were transcriptionally inactive.

Similar experiments on the *in vitro* transcription and relative levels of the *rbc*S RNA in soybean seedlings have lead to the hypothesis that *rbc*S RNA is less stable in light than in darkness. In contrast, small decreases in *rbc*S *in vitro* transcription rate in mature soybean plants treated with darkness were accompanied by large decreases in *rbc*S RNA, suggesting that *rbc*S RNA was degraded more rapidly in darkness than in light in these plants. We have shown that differences in the modulation of *rbc*S RNA levels by post-transcriptional mechanisms exist between plants which belong to different orders, and between different developmental states of the same plant species.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase (Rubisco) is an abundant enzyme that catalyzes competing reactions in photosynthetic CO_2 fixation and photorespiration in vascular plants, green algae, and photosynthetic bacteria [1]. The small subunit (*rbc*S) of the holoenzyme is encoded in the nucleus. The *rbc*S mRNA is translated on free cytoplasmic ribosomes and the protein is transported into the chloroplast via a transit peptide sequence. Expression of the genes encoding *rbc*S is mediated by light through phytochrome, the plant chromoprotein (for reviews see: [2, 3, 4]). In the higher plants examined, levels of *rbc*S RNA increase in response to white light and decrease in response to darkness or far-red light treatment (for example see: [5, 6, 7]). Studies of transcription rates *in vitro* in isolated nuclei have shown that this response is due, at least in part, to changes in transcription [8, 9, 10, 11, 12]. Recent data suggest that light-induced RNA turnover also plays an important role in determining the levels of *rbc*S RNA in soybean seedlings [13].

Ribulose-1,5-bisphosphate carboxylase small subunit polypeptides are encoded by small gene families in all plants investigated [14, 15, 16, 17]. Evolutionary studies have shown that the various members within a *rbc*S gene family are homogenized by mechanisms of concerted evolution to encode similar polypeptides [18, 19, 20]. However, this process can also lead to extreme levels of nucleotide sequence divergence among family members in nucleotide positions which do not affect the encoded amino acids [20]. The effect of these concerted evolutionary events on DNA regulatory sequences which control the expression of various gene family members is unknown. This increased number of nucleotide substitutions could cause diversity in the patterns of expression of individual gene family members within a species and many patterns of expression could be represented in each closely related species. Alternatively, a single novel pattern of expression could be fixed frequently by concerted evolution for all gene family members in a species. In this way, closely related species might exhibit very different patterns of expression.

*rbc*S RNA is encoded by 8 genes in *Petunia hybrida* [21]. These have been shown by differential hybridization [21], nucleotide sequence homology [22], and a quantitative study of *rbc*S molecular evolution [20] to belong to three *rbc*S subfamilies, A, B, and C, representing three ancient lineages (lineages 3, 1, and 2 respectively [20]). Relative levels of the individual RNAs

* To whom correspondence should be addressed

have been measured by Northern blot, slot blot, S1, and primer extension assays [16, 23, 24]. In light-grown plants these RNAs are expressed predominantly in leaves and to a lesser extent in sepal, petal, stem, stigma/anther, and in root tissue [16]. It has also been shown that the absolute levels of RNA expressed by individual gene family members in leaves from light-grown plants vary significantly [16, 23]. However the response of these different genes to light and darkness has not been compared.

In order to determine the contributions of transcription and RNA turnover to the expression of petunia *rbc*S genes, we have compared the changes in *rbc*S *in vitro* transcription rates and RNA levels in *P. hybrida* plants in response to various regimes of white light, far-red light, and darkness. The patterns of expression of two divergent subfamilies, *rbc*S A and *rbc*S C, were compared. The genes from these two subfamilies have different numbers of introns, have not had a common ancestor for approximately 40 million years, and have interacted frequently by gene conversion [20]. Between the two subfamilies there is only a single amino acid difference in the mature protein and yet they have numerous silent nucleotide differences.

We show that with two notable exceptions the *in vitro* transcription and RNA levels of *rbc*S RNA from both subfamilies respond in parallel to changes in light regime. We suggest that changes in levels of *rbc*S RNA from these two subfamilies of *P. hybrida* are due primarily to changes in transcription rates. This is in contrast to results from soybean seedlings where it has been shown that changes in the level of *rbc*S RNA are modulated by changes in the RNA half-life in addition to changes in the transcription rate of the gene [13]. We studied mature soybean plants in order to explore the possibility that this difference in regulation by RNA turnover between mature petunia and soybean seedlings was due to the developmental state of the plants. We show that distinct differences in the post-transcriptional modulation of *rbc*S RNA exist between the three tissue types studied: soybean seedlings, mature soybean plants and mature petunia plants.

MATERIALS AND METHODS

Plant material

Petunia plants used in this study were an F1 hybrid of the V23 and R51 strains of *Petunia hybrida* (a gift from Dr. Anton Gerats, Free University, Amsterdam, the Netherlands), designated VR. Cuttings were rooted directly in soil (without application of rooting hormone) on a mist bench for approximately two weeks and grown thereafter under standard greenhouse conditions for 4 weeks to 6 months. Only young leaves which were not fully expanded (2−3 cm in length) were used. Leaves were harvested at the indicated times (post-treatment), and consisted of both petiole and blade. Seeds of *Glycine max* (var. Pella '86) were soaked in water for 1 hour and then grown in vermiculite at 28°C in darkness (etiolated) or in a 12 hour (6AM/6PM) light/dark cycle (light-grown) for 7 days. Mature soybean plants were grown in soil under greenhouse conditions for approximately 6 weeks.

At least one week before light treatment, mature petunia or soybean plants were placed in a growth room at 28°C under a 12 h (6AM/6PM) light/dark cycle. Light, far-red light, or dark treatments were always started at noon. Far-red light treatments were as described in Berry-Lowe and Meagher [10]. Young leaves which had not fully expanded (mature plants) or primary leaves (soybean seedlings) were harvested directly into liquid nitrogen and stored at −70°C.

RNA isolation

RNA was isolated by a modification of the DNA extraction procedure of Shure et al. [25]. Briefly: tissue was ground with a polytron for one minute in 8 ml of grinding buffer per g of plant material. An equal volume of a 3:1 mixture of phenol and chloroform (containing 5% isoamyl alcohol) was added. This mixture was shaken for 60 min. The phases were separated by low-speed centrifugation, and phenol-chloroform-isoamyl alcohol extraction of the aqueous phase was repeated. Total nucleic acid was precipitated with ethanol, and the pellet was resuspended in 20 ml of 10 mM Tris (pH 7.4)-10 mM EDTA. The solution was brought to 2 M LiCl and precipitated on ice for 3 hours. Single-strand RNA was recovered by low-speed centrifugation. This pellet was washed once with 2 M LiCl, resuspended in 10 mM Tris (pH 7.4)-200 mM NaCl-1 mM EDTA and ethanol precipitated.

Petunia and Soybean probes

The *in vitro* transcription rates and levels of RNA were examined for two petunia *rbc*S sub-families, and one CAB sub-family. All data were normalized to 18S rRNA expression. In addition, a vector plasmid (pBR322) was used as a negative control for hybridization. CAB gene expression was determined using the plasmid pCAB29 [26], kindly provided by C. Dean (Advanced Genetic Systems Corp.). This is a cDNA clone isolated from *P. hybrida* var. Mitchell (designated Mitchell) which contains sequences beginning 85 nucleotides (nt) upstream of the translational start codon and extends into the polyadenylate tail. In transcription assays, levels of 18S rRNA transcripts were determined using the clone pSR1.2B3 [27] containing a 1071 nt *Bam*HI-*Eco*RI fragment internal to the soybean 18S rRNA gene. A synthetic oligonucleotide probe, complimentary to nt 1764−1803 of the soybean 18S rRNA gene, was used to determine 18S rRNA levels. pBR322 plasmid DNA [28] was used as a negative control for hybridization in both transcription run-on experiments and RNA assays.

It has been shown [22] that the *rbc*S genes of Mitchell belong to 3 subfamilies: the *rbc*S A, B, and C subfamilies (see Table I). *rbc*S A and B contain 1 member each, and *rbc*S C contains six members. Expression of two of the *rbc*S subfamilies from petunia was quantified using genomic clones kindly provided by N. Tumer (Monsanto Corp.). The 3′ *Eco*RI fragment of the petunia *rbc*S gene *rbc*S11A contained in clone pMON9503 [23] was used to probe expression of *rbc*S C. This fragment contains a portion of the second exon, the second intron, third exon, and 128 nt of 3′ untranslated sequences. The 3′ *Hin*dIII fragment of *rbc*S301 contained in clone pMON9559 [23] was used to probe expression of *rbc*S A. This clone contains 32 nt of nontranslated 5′ leader, the entire coding sequence (exons 1−4 and introns 1−3), and 350 nt of downstream non- translated sequence. Southern blots show that these probes detect the same number of genes in both Mitchell and VR genomic DNA (data not shown). Using a dot blot dilution series and hybridization conditions described below pMON9503 hybridizes at least 64-fold more efficiently to DNA fragments from *rbc*S C than to *rbc*S A, and pMON9559 hybridizes at least 16-fold more efficiently to fragments from *rbc*S A than to *rbc*S C in Mitchell and VR.

The transcription rates and levels of RNA were examined for the soybean *rbc*S gene family and for actin. All data were normalized to 18S rRNA expression and pBR322 was included as a negative control for hybridization. Actin gene expression was determined using pSAc6 [29], a genomic clone containing

the entire transcribed region of the soybean actin gene, SAc6, beginning 20 nt upstream of the start of transcription, and extending approximately 200 nt past the termination codon.

The 3′ *Eco*RI fragment of the soybean *rbc*S gene, SRS1, contained in the plasmid pSRS0.8, [14] was used to probe expression of *rbc*S in soybean. This clone includes the second intron, third exon and 300 nt of 3′ untranslated sequence.

When these plasmid clones were used as probes in RNA assays, plasmid inserts were purified on DE 81 paper [30] and labeled with [α-^{32}P] dATP by the random primer method [31]. The oligonucleotide probe for 18S rRNA was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase [32].

Transcription run-on and RNA assays

Isolation and labeling of petunia and soybean nuclei were by the method of Shirley et al. [12]. DNA dot blots were prepared by

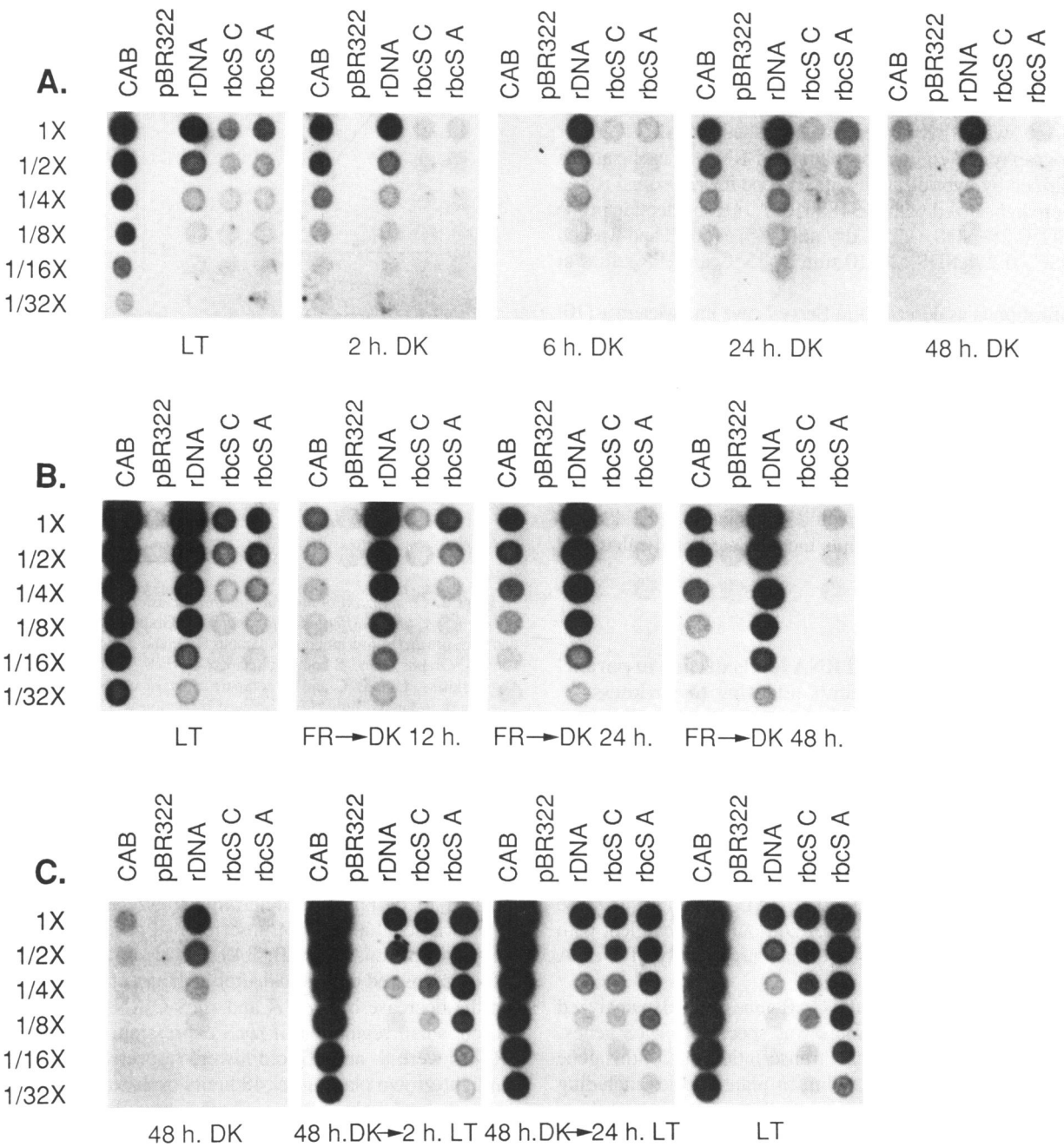

Figure 1. *Run-on transcription assays demonstrate the change in rbcS in vitro transcription rate.* Light-grown plants were treated with darkness for increasing periods of time and leaf samples were collected. Nuclei were isolated, and run-on transcripts were labeled with [α-^{32}P]UTP and used to probe DNA dot blots containing 2-fold dilutions of pCAB29 (CAB), pBR322, pSR1.2B3 (18S rRNA), pMON9503 (*rbc*S C), and pMON9559 (*rbc*S A). Transcription rates for all samples were normalized to rRNA transcription [10]. Quantification is as described in text. A. Decline of *rbcS in vitro* transcription rate after treatment with darkness. Run-on transcripts were prepared from leaves of light-grown petunia plants (LT), or plants treated with darkness (DK) for 2 h, 6 h, 24 h, or 48 h. B. Decline of *rbcS in vitro* transcription rate after far-red light treatment. Run-on transcripts were prepared from leaves of light-grown plants (LT) or plants treated with 15 minutes of far-red light (FR) followed by darkness for 12 h, 24 h, or 48 h. C. Reinduction of *rbcS in vitro* transcription rate upon re-exposure to light. Run-on transcripts were prepared from leaves of dark-adapted petunia plants (48 h DK), dark-adapted plants followed by 2 h or 24 h of light, or light-grown plants.

direct application of denatured DNA (5 μg of *rbc*S, CAB, or actin DNA probes or 1 μg of rDNA probe) onto nitrocellulose as described [29]. (This represents a 4.34:1 ratio of RNA encoding sequence of *rbc*S A:*rbc*S C.) All hybridizations using labeled RNA from these nuclei were carried out at 56°C in 50% formamide, hybridization mix [29]. Filters were washed 4× 10 min each in 0.2×SSC, 0.2% SDS at 56°C.

Relative RNA levels were analyzed on RNA dot blots. RNA was denatured, and applied to Biotrans nylon filters (I.C.N.) by direct application of 5 μg of each RNA sample in a 2-fold dilution series as described in Hightower and Meagher [29] except that RNA samples were individually applied to nitrocellulose filters in 2.5 μl aliquots. RNA blots were hybridized and washed as described for transcription run-on assays except that filters probed with pSAc6 were hybridized and washed at 42°C. After autoradiography the equivalent loading of RNA in each sample was confirmed by hybridization with a probe for ribosomal RNA. Filters were hybridized with the 18S rRNA oligonucleotide probe in 2× SSC, 0.2% SDS, 0.25% dry milk [33] at 60°C and washed in 2× SSC, 0.2% SDS, 2×10 min at 25°C and 1×1 min at 60°C.

Quantification is as described in Berry-Lowe and Meagher [10] and Shirley and Meagher [13]. All experiments were repeated two or more times and the results from a single representative experiment are presented. Of major concern in this study was the normalization of transcription rates in independent preparations of nuclei. Transcription levels were measured relative to rRNA transcription because rRNA represents 40–60% of total transcription under conditions of light or darkness [10]. For most of our data the results would be similar but not identical if measured relative to total counts incorporated or if calculated on a per nucleus basis.

RESULTS

In vitro transcription rate and RNA levels decline in parallel for rbcS A and rbcS C in plants adapting to darkness

Darkness can have a dramatic effect on transcription of *rbc*S genes in many plants including *Lemna* [9], pea [8], and soybean [10]. Therefore the magnitude of the effect of dark treatment on the *in vitro* transcription of two *rbc*S sub-families in petunia, *rbc*S A and *rbc*S C, was examined. After 6 hours of darkness, *in vitro* transcription of *rbc*S A and *rbc*S C was 4-fold lower than in light-grown plants, and decreased to 8- and 16-fold lower, respectively, after 48 hours of treatment (Fig. 1A, 2A). The slight increase in *rbc*S A *in vitro* transcription after 24 hours of treatment with darkness is likely to be due to circadian rhythmicity of *rbc*S A expression [34].

The *in vitro* transcription of the CAB genes was also analyzed to show that the effect of darkness was specific to *rbc*S genes. It has been shown that the *in vitro* transcription rate of this gene family varies with a diurnal rhythm in plant species including tomato seedlings [35] and tobacco seedlings [36]. As in these studies, *in vitro* transcription of CAB RNA decreased at the onset of the dark period, and increased by noon the next day in total darkness. These data confirm that the response of the *rbc*S genes was not due to a general transcriptional inactivation of the petunia nuclei by darkness.

After 3 days of darkness the rate of rDNA transcription was very low, and transcription of the other genes examined was undetectable (data not shown and [12]). Thus, after 3 days in darkness the nuclei were no longer significantly transcriptionally

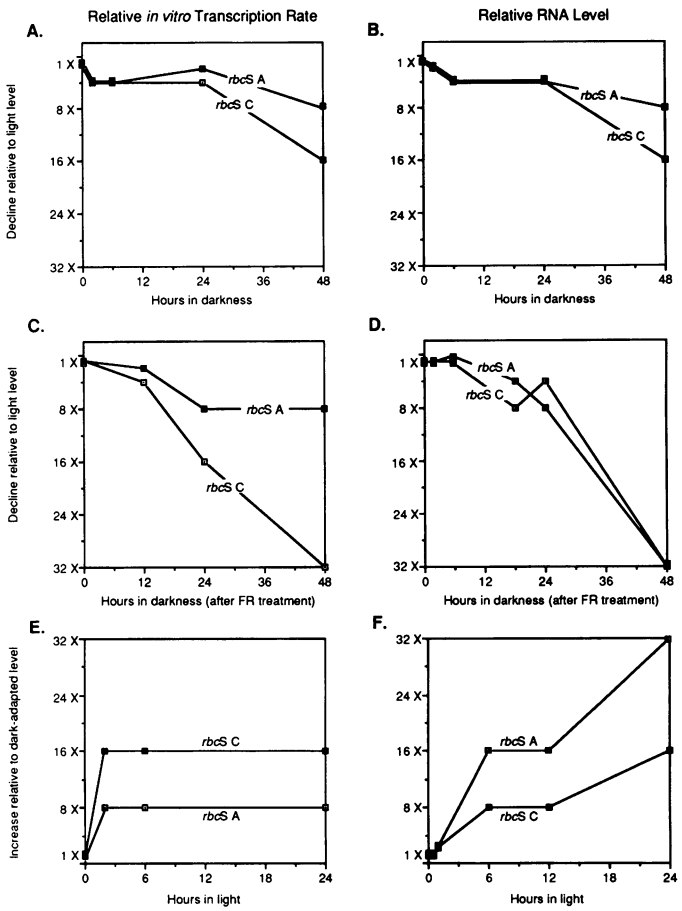

Figure 2. *Comparison of the changes in expression of two subfamilies, rbcS C and rbcS A, under different light conditions.* Graphs A,C, and E show changes in *rbcS in vitro* transcription rate. Graphs B,D, and F show changes in *rbcS* RNA levels. Graphs A and B compare changes in *rbcS* A and *rbcS* C upon exposure to darkness. Graphs C and D compare changes in *rbcS* A and *rbcS* C upon exposure to far-red light (FR) followed by darkness. Graphs E and F compare changes in *rbcS* A and *rbcS* C in plants dark-adapted for two days followed by exposure to white light.

active. This may reflect an overall low energy state or senescence of the plant resulting from the long dark treatment.

Relative levels of *rbc*S RNA were also measured in plants shifted to darkness to determine the relative contribution of transcriptional and post-transcriptional processes to *rbc*S RNA levels in petunia (Fig 2B, 3A). Levels of *rbc*S A and *rbc*S C RNA decreased only 4-fold after 24 hours of darkness. The delay in the decrease of *rbc*S A and *rbc*S C RNAs may also be due to circadian regulation of *rbc*S expression. The levels of these RNAs were 8- and 16-fold lower, respectively, than the levels in light-grown plants after 48 hours of darkness. After 72 hours of darkness levels of RNA of both subfamilies were consistently 16-fold lower than they were in a light-grown plant.

CAB RNA levels in petunia [37] and other species [34, 36, 38] vary on a diurnal rhythm. Therefore, levels of CAB RNA were determined in petunia plants treated with darkness in order to show that decreases in RNA levels were specific to *rbc*S. In agreement with published reports, CAB RNA levels varied on a diurnal rhythm.

Changes in *in vitro* transcription rates and relative RNA levels were correlated throughout various dark treatments. At individual

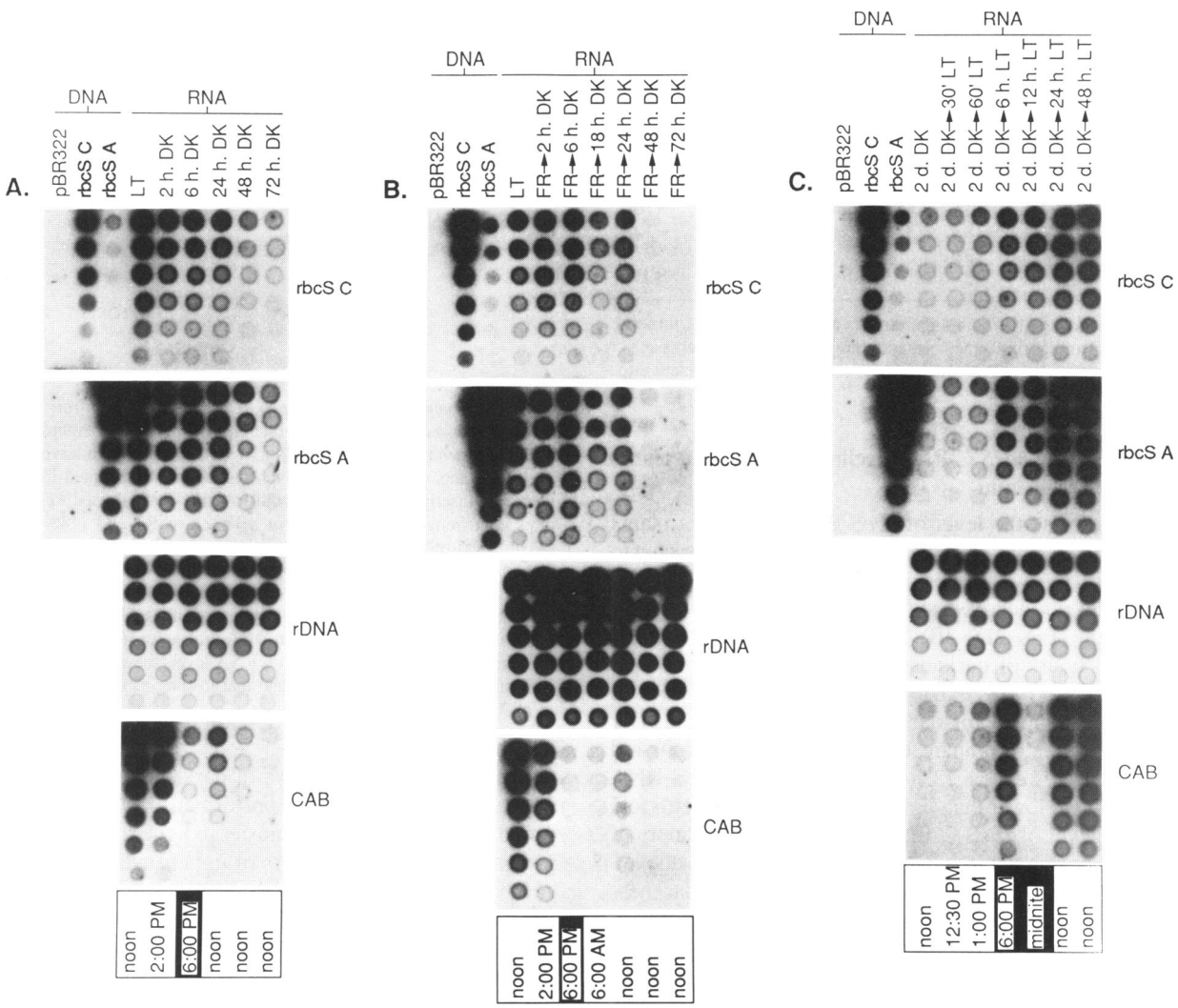

Figure 3. *RNA dot blots demonstrate the change in rbcS RNA level.* RNA prepared from leaves collected at the designated times was applied to filters in a 2-fold dilution series (2.5 μg RNA in the first dot) and hybridized with DNA probes for *rbc*S C, *rbc*S A, or CAB. After filters were used to measure *rbc*S and CAB steady state RNA levels these dot blots were probed with an rDNA probe to demonstrate that loading of total RNA was equivalent in all treatments. The legend at the bottom of each panel indicates the time of day when leaves were harvested. The dark box indicates the beginning of the dark period of the day/night cycle. A. Decline of RNA in darkness. Total RNA was isolated from light-grown petunia plants (LT), or plants treated with 2 h, 6 h, 24 h, 48 h, or 72 h of darkness (DK). B. Decline of RNA after far-red light treatment. Total RNA was isolated from light-grown petunia plants, or plants treated with 15 minutes of far-red light (FR) followed by 2 h, 6 h, 24 h, 48 h, or 72 h of darkness. C. Re-induction of RNA in light. Total RNA was isolated from dark-adapted petunia plants (2 d DK), or dark-adapted plants treated with 0.5 h, 1 h, 6 h, 12 h, 24 h, or 48 h of light.

time points during the 48 hours in darkness, there was no more than a 2-fold difference between the changes in *in vitro* transcription rate and the changes in the levels of RNA for both *rbc*S subfamilies examined (compare Fig. 2A and 2B) and for the CAB gene family (Table I).

In vitro transcription rates of rbcS A and rbcS C are affected differentially by far-red light

Far-red light reverses the effects of white light during the induction of phytochrome regulated genes (including *rbc*S and CAB) and represses transcription of *rbc*S in light-grown plants, including soybean seedlings [10] and *P. hybrida* plants [12]. In order to determine the subfamily specific responses of *rbc*S gene expression to far-red light treatment, VR plants were treated with 15 minutes of far-red light followed by treatment with darkness. After 24 and 48 hours of treatment *rbc*S C *in vitro* transcription decreased 16-fold and 32-fold, respectively, relative to the level in light-grown plants (Fig. 1B, 2C). This decrease in *rbc*S C *in vitro* transcription in response to far-red light was consistently greater than for plants simply adapted to darkness (compare Fig. 2A to 2C). In contrast, far-red light had a less dramatic affect on the *in vitro* transcription of the *rbc*S A subfamily. The *in vitro* transcription rate of *rbc*S A decreased only 8-fold after far-red light treatment followed by 24 or 48 hours of darkness. This is equivalent to the change in *rbc*S A *in vitro* transcription in dark-adapted plants. *In vitro* transcription of the CAB gene family continued to be regulated on a diurnal cycle after far-red light treatment, again demonstrating that there had not been a general transcriptional inactivation of the nuclei during the treatment with far-red light. The decrease in CAB *in vitro* transcription rate after far-red light treatment was no greater than the decrease observed in plants treated with darkness alone.

Table I. Relative change in CAB *in vitro* transcription rate (Tc) and RNA level (RNA).

	Time of Treatment (hours)				
	0 (noon)	2 (2PM)	6 (6 PM)	24 (noon)	48 (noon)
DK Tc	1	1	−128	−8	−64
RNA	1	−2	−64	−16	−64
FR Tc	1	−32	−256	−16	−16
RNA	1	−8	−128	−64	−128
LT Tc	1	32	32	32	32
RNA	1	2	64	>128	>128

Plants were grown under a 12 hour light/dark cycle, with the lights turned on at 6:00 AM and off at 6:00 PM. Negative numbers indicate decreases relative to light-grown levels. Positive numbers indicate increases relative to light-grown levels.

Levels of rbcS A and rbcS C RNA decline in parallel in plants treated with far-red light

In order to determine the level of *rbc*S RNA in petunia plants treated with far-red light followed by darkness, a time course of the decline of RNA was performed. Levels of *rbc*S RNA (*rbc*S A or *rbc*S C) did not decrease significantly until 18 hours after far-red treatment (Fig. 2D, 3B). After far-red light treatment followed by 48 hours of darkness, levels of *rbc*S A and *rbc*S C RNA were 32-fold lower than in the light. This was lower than the level determined for dark-shifted plants without prior far-red light treatment (compare Fig. 2B to 3D). After far-red light treatment followed by 72 hours of darkness, *rbc*S RNA levels were 128-fold lower than light-grown levels. A brief 2-fold increase in the level of *rbc*S C RNA at noon, after 24 hours of treatment, may be due to circadian control, as described above.

At the time points examined, up to 24 hours of darkness following far-red treatment, there was a good correlation between the change in *in vitro* transcription rate and the change in the levels of RNA for both *rbc*S subfamilies examined (see Fig. 2C, 2D). In contrast, after 48 hours of treatment, the level of *rbc*S A RNA was much lower than would be predicted based on the change in *rbc*S A *in vitro* transcription rate alone. Apparently the changes in *in vitro* transcription rate can not account entirely for the changes in *rbc*S A RNA after far-red light treatment followed by long periods of darkness.

CAB RNA levels varied on a diurnal rhythm following far-red light treatment, as they did after dark treatment. However, after 48 hours of treatment CAB RNA decreased rapidly, reaching levels lower than would be predicted based only on the observed changes in the *in vitro* transcription rate of CAB. Furthermore, far-red light treatment prior to dark-adaptation caused a greater drop in CAB RNA than dark-adaptation alone.

rbcS in vitro transcription rate and RNA levels are rapidly re-induced by light

In order to determine how rapidly *rbc*S RNA was re-induced from dark-adapted levels upon transfer to light, plants were treated with darkness for two days without prior far-red light treatment (dark-adapted) to depress *rbc*S transcription and RNA levels. These plants were then treated with light for increasing times. *In vitro* transcription rates of both *rbc*S subfamilies increased to light-grown levels within 2 hours of transfer to light (Fig. 1C), but did not increase further even after an additional 22 hours of light exposure. The *in vitro* transcription rate of CAB also increased rapidly upon re-induction by light, increasing to light-grown levels within 2 hours.

In order to determine the change in *rbc*S RNA in petunia plants

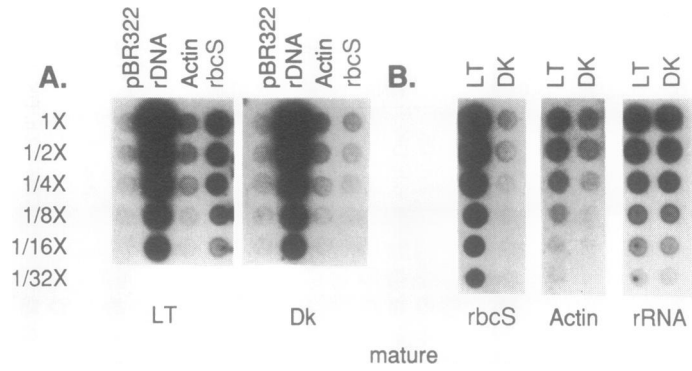

Figure 4. *Comparison of changes in in vitro transcription rate and RNA levels in mature soybean plants.* A. Run-on transcription assays demonstrate the change in *rbcS in vitro* transcription rate. Nuclei were isolated from leaves of light-grown soybean plants (LT) or plants which had been dark-adapted for 2 days (DK). Run-on transcripts were labeled with [α-^{32}P]UTP and used to probe DNA dot blots containing 2-fold dilutions of pBR322, pSR1.2B3 (18S rRNA), pSAc6 (Actin), and pSRS1 (*rbc*S). Quantification is as described in text. B. RNA dot blot analysis demonstrates the change in *rbc*S RNA level. Total RNA was isolated from light-grown soybean plants or plants which had been treated with 2 days of darkness. RNA was applied to filters in a 2-fold dilution series (2.5 μg RNA in the first dot) and hybridized with DNA probes for *rbc*S and actin. After filters were used to measure *rbc*S and actin RNA levels these dot blots were re-probed with an rDNA probe to demonstrate that loading of total RNA was equivalent in all treatments.

returned to light after 2 days of darkness, a time course of the increase in *rbc*S RNA was performed. *rbc*S C RNA increased 4-fold within 6 hours after transfer to light, and increased to light-grown levels within 24 hours of light treatment (Fig. 3C). *rbc*S A RNA also increased to light-grown levels rapidly after light treatment, reaching a level 4-fold higher than light-grown within 24 hours of light treatment. These data are summarized in Fig. 2E and 2F.

Upon re-induction with light, plants were returned to a 12 hour light/dark cycle. We therefore expected that CAB RNA levels would re-enter a diurnal cycle. CAB RNA increased 32-fold within 6 hours of re-induction by light (Fig. 3C). At midnight (12 hours of re-induction, but in the median of the dark portion of the cycle) CAB RNA decreased dramatically, consistent with diurnal cycle and/or phytochrome control of CAB expression.

At the time points examined, there was no more than a 2-fold difference between the change in *in vitro* transcription rate and the change in levels of RNA for *rbc*S C and for the CAB gene family during re-induction. However, there was a 4-fold difference between increases in *in vitro* transcription rate and relative RNA level of *rbc*S A upon reinduction by light.

rbcS RNA accumulation is regulated developmentally

It has been shown [13] that transcriptional and post-transcriptional modulation of *rbc*S RNA expression are regulated by light in soybean seedlings. In contrast, it appeared from the experiments described above, that *rbc*S RNA level in mature petunia plants was regulated predominantly by changes in transcription, its RNA accumulation paralleling *in vitro* transcription. We wished to determine if the differences observed between the regulation of *rbc*S RNA in soybean seedlings and mature petunia plants were due to differences in the developmental state of the plants assayed, or to differences in the light regulation of *rbc*S RNA stability between soybean and petunia, or both. Mature soybean plants were grown in light, or dark-adapted for two days, and transcription assays and RNA determinations were performed

(Fig. 4A and 4B). In mature soybean plants (Fig. 4A) which had been dark-adapted for two days, the *in vitro* transcription rate was approximately 16-fold lower than in light-grown plants relative to ribosomal RNA transcription. In contrast to the relatively small decline in *rbc*S RNA in soybean seedlings, *rbc*S RNA (Fig. 4B) decreased 64-fold in mature dark-adapted soybean plants relative to mature light-grown plants. Thus there was 4-fold less *rbc*S RNA in dark-adapted mature soybean plants than would be expected based on *in vitro* transcription rate changes. Transcription and RNA levels of a control gene, actin, decreased 4-fold under the same conditions, with no discrepancy between changes in *in vitro* transcription and RNA level. These data were compared to the changes observed in soybean seedlings [13]. In contrast to the results in mature soybean plants, there was 4-fold more *rbc*S RNA in dark-grown soybean seedlings (32-fold increase in *in vitro* transcription as compared to an 8-fold increase in RNA) than would be expected based on *in vitro* transcription rate changes alone. These data are summarized in figure 5 and show that there was a significant difference in the regulation of *rbc*S RNA expression between soybean seedlings, mature soybean plants, and mature petunia plants.

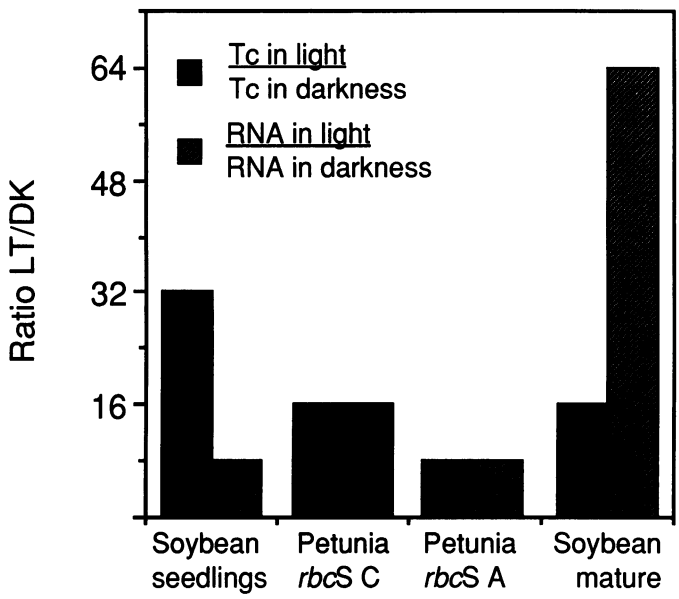

Figure 5. *A comparison of relative in vitro transcription rates and relative RNA levels of rbcS genes in soybean seedlings, mature soybean plants, and petunia plants.* Dark bars show the change in *in vitro* transcription rate (Tc) upon treatment of light-grown plants (LT) with 48 hours of darkness (DK). Hatched bars show the change in relative RNA levels (RNA) upon treatment with darkness.

DISCUSSION

In many plants, phytochrome has been shown to control expression of *rbc*S and CAB RNAs (for example: [3]). Studies in transgenic plants have shown that the region upstream of the genes is responsible, at least in part, for light-regulated changes in transcription rate and RNA levels (for example: [12, 39, 40, 41]). In addition to the known effects on *in vitro* transcription rate, it has recently been shown [13] that light regulates *rbc*S RNA levels by modulating stability of the *rbc*S RNA. In this manuscript we have examined the contribution of *in vitro* transcription rate changes to changes in the RNA levels of two *rbc*S subfamilies in mature petunia. Our results indicate that relative levels of individual *rbc*S RNAs in petunia leaves may vary based on growth conditions (including light intensity and spectrum of illumination) of the plants. We have also examined the relative contribution of transcriptional and post-transcriptional processes to *rbc*S RNA levels in soybean seedlings [13] and mature soybean plants. Independent studies have shown that developmental differences exist in the expression of one of the genes encoding glutamine synthase (GS_2, [42]) and one gene encoding *rbc*S (*rbcs*-3A, [41]) in pea. Fluhr et al. showed that in addition to the known red/far-red light receptor there exists a developmentally regulated blue light receptor acting in mature plants to regulate RNA levels of some genes, including *rbc*S [41]. Our results indicate that although *rbc*S RNA is less stable in light than in darkness in soybean seedlings [13], *rbc*S RNA is more stable in light than in darkness in mature soybean plants. We suggest that the developmental state of these plants determined the mode of post-transcriptional regulation of *rbc*S gene expression and ultimately the level of *rbc*S mRNA.

rbcS A and rbcS C are affected differentially by darkness, far-red light, and white light

The *rbc*S gene family consists of 3 subfamilies in petunia: *rbc*S A, B, and C. We wished to determine if the subfamilies *rbc*S A and *rbc*S C reacted equally to far-red light. *In vitro* transcription of *rbc*S C was depressed more by treatment with far-red light than with darkness alone, decreasing 16-fold after far-red light treatment as compared with a 4-fold decrease after 24 hours of treatment with darkness alone. In contrast, *in vitro* transcription of *rbc*S A was not affected by far-red light, decreasing only 8-fold in 48 hours (with or without prior far-red light treatment).

We were interested in determining the relative contribution of *rbc*S A and *rbc*S C to the total *rbc*S RNA in a light-grown petunia plant. We estimate that *rbc*S C represented approximately twice as much *rbc*S RNA as *rbc*S A in light-grown petunia (see Table II). These data did not resolve the discrepancy reported regarding the relative RNA levels of *rbc*S301 (the sole member of the *rbc*S A subfamily) and *rbc*S11A (one of 6 members of the *rbc*S C subfamily) in light-grown plants. Dean et al. [16, 24] determined that *rbc*S A accounts for 47% and *rbc*S C accounts for greater than 30% of petunia *rbc*S RNA. In contrast, Tumer et al. [23] found that *rbc*S301 RNA is one-eighth as prevalent as *rbc*S11A (see Table II). We also compared the relative *in vitro* transcription rates of *rbc*S A and *rbc*S C in the light. When a correction was made for the amount of transcribed DNA in each dilution series, we estimate that *rbc*S C was transcribed at a rate 2-fold higher than *rbc*S A in light-grown plants. This was consistent with *rbc*S C RNA being 2-fold more prevalent than *rbc*S A in total petunia RNA, a result intermediate between those reported by Dean [16, 24] and Tumer [23].

When light-grown petunia plants were transferred to darkness this ratio of *in vitro* transcription of *rbc*S C to *rbc*S A was also maintained. However *in vitro* transcription of *rbc*S C was more sensitive to far-red light than *rbc*S A, decreased more rapidly and became undetectable after 48 hours of darkness. Thus, after far-red light treatment, the ratio of *rbc*S C to *rbc*S A *in vitro* transcription decreased dramatically. This differential sensitivity of the *in vitro* transcription of *rbc*S C and *rbc*S A subfamilies to far-red light suggested that differences in seasonal growth conditions, day length, light intensity and illumination spectrum may explain the discrepancies between the results of Dean [16, 24], Tumer [23], and those presented herein.

Table II. The relative contribution of the *rbc*S family members to *rbc*S RNA levels in the light.

Subfamily	A		B		C	
# of members	1		1		6	
relative expression (individual genes) (Dean, 1985, 1987)	*rbc*S301[a]	47%	*rbc*S611	23.2%	*rbc*S11A[b] + *rbc*S231 =	15.2%
					*rbc*S491	7%
					*rbc*S112	5.4%
					*rbc*S211	1.9%
					*rbc*S911	1%
(subfamilies)	*rbc*S A	47%	*rbc*S B	23.2%	*rbc*S C	30.5%
relative expression (Tumer, 1986)	*rbc*S301	5%	----------	------	*rbc*S11A	40%
relative expression (this manuscript)	*rbc*S A	1×	----------	------	*rbc*S C	2×

a. also designated *rbc*S8 [23]
b. also designated *rbc*S511 [16]

CAB in vitro transcription rate and RNA levels vary on a diurnal rhythm

It has been shown that CAB RNA levels are regulated on a circadian rhythm in several plants, including pea seedlings [34], developing tomato fruits [38], and mature petunia plants [37]. This circadian rhythmicity is also seen at the transcriptional level, as has been shown in tomato seedlings [35], tobacco seedlings [36] and seedling and mature leaves of maize [43]. In plants grown under a light/dark cycle CAB *in vitro* transcription rate or RNA levels decreased at or before the onset of the dark period, and increased at or before the onset of the light period. When plants which have been entrained to a light/dark cycle are treated with either continual light or continual darkness, this pattern of expression is maintained for at least 24 hours.

CAB expression was studied in order to confirm that decreases in *rbc*S *in vitro* transcription rate and RNA levels were not due to a general affect of the various light treatments on all petunia RNAs or even on all phytochrome regulated RNAs. When petunia plants were transferred to darkness *rbc*S and CAB *in vitro* transcription rates and RNA levels decreased by 6 PM (the onset of the dark portion of the day/night cycle). Although *rbc*S *in vitro* transcription and RNA did not increase significantly in the absence of re-induction by white light, CAB *in vitro* transcription and RNA increased by noon the next day, in the absence of any external light signal. This pattern also existed when plants were treated with far-red light followed by darkness, although the magnitude of the increase during the second and third days of treatment (24 and 48 hours) was less. These results are consistent with the changes in RNA reported for *cab*91R from petunia [37]. Further, these data show that the diurnal pattern of expression occurs at the level of transcription.

RNA processing and the control of rbcS RNA levels

It has been shown [13] that *rbc*S RNA levels in soybean seedlings are regulated by light which not only effects changes in *in vitro* transcription rate, but also causes changes in post-transcriptional regulation, specifically the rate of *rbc*S RNA degradation. Discrepancies between *in vtiro* transcription rate changes and changes in *rbc*S RNA levels led Shirley and Meagher [13] to propose that *rbc*S RNA is less stable in light than in darkness in soybean seedlings.

In mature petunia plants changes in the *in vitro* transcription rates of *rbc*S and CAB were compared with changes in levels of these RNAs. Changes in levels of *rbc*S and CAB RNA reflected *in vitro* transcription rate changes in these genes under most conditions of light and darkness. Thus, in general, RNA levels appeared to be modulated by changes in the transcription rate of these genes, and with two exceptions, little differential turnover of these RNAs was observed.

rbcS A and CAB RNAs may be regulated by RNA processing after treatment with far-red light

Treatment of petunia plants with far-red light followed by darkness appeared to affect the degradation rate of *rbc*S A. Under these conditions *rbc*S C *in vitro* transcription decreased 32-fold after 48 hours, with a concommitent 32-fold decrease in *rbc*S C RNA. In contrast, *rbc*S A *in vitro* transcription rate was not altered by far-red light treatment, decreasing only 8-fold after 48 hours. However, *rbc*S A RNA decreased 32-fold after the same treatment. This represents a 4-fold discrepancy between *in vitro* transcription rate changes and RNA changes, and we suggest that *rbc*S A RNA is selectively destablilized after treatment of plants with far-red light.

Under the same conditions of treatment with far-red light, CAB *in vitro* transcription (compared at noon, its diurnal peak of expression) decreased only 16-fold after two days. However, CAB RNA decreased 64-fold after 24 hours, and 128-fold after 48 hours of treatment. Thus accumulation of CAB RNA was also affected by far-red light. This may be due to a decrease in CAB RNA stability after far-red light treatment.

Light may control the processing of rbcS A RNA upon reinduction of dark-adapted plants by light

Treatment of petunia plants with darkness for two days followed by reinduction of *rbc*S transcription by light appeared to affect the turnover of *rbc*S A RNA but not *rbc*S C or CAB RNAs. Under these conditions *rbc*S C and CAB *in vitro* transcription and RNA are increased rapidly to light-grown levels. In contrast, although *rbc*S A *in vitro* transcription increased rapidly to light-grown levels, *rbc*S A RNA increased 32-fold in 24 hours, a level 4-fold higher than normal light-grown levels. This represents a 4-fold difference between *in vitro* transcription rate changes and RNA changes, and we suggest that *rbc*S A RNA is stabilized upon light treatment of dark-adapted petunia plants.

Developmental control of rbcS expression

It has been shown that *rbc*S RNA and RNA encoded by GS_2, another phytochrome regulated gene, accumulate more rapidly

in mature pea plants than in pea seedlings [41, 42]. These results suggest that the age of a plant may affect its regulation, and that differences between our results in mature petunia leaves and soybean seedling leaves may have been due to developmental differences. The difference in the ratio of *in vitro* transcription to *rbc*S RNA level was therefore compared between soybean seedlings and mature soybean plants (Fig. 4). The *in vitro* transcription of *rbc*S decreased 16-fold upon dark treatment of mature soybean plants. The level of *rbc*S RNA decreased dramatically upon dark-adaptation, to 64-fold lower than light-grown levels. Thus, whereas the stability of *rbc*S RNA is decreased in light in soybean seedlings, it is increased in the light in mature soybean plants. These data are summarized in figure 5. Thus, not only do petunia and soybean regulate their *rbc*S RNA levels differently, but, in addition, developmental differences in *rbc*S RNA stability exist between soybean seedlings and mature soybean plants.

The common patterns of light regulated expression observed for *rbc*S subfamilies A and C in petunia suggest that either the regulatory machinery has been selectively conserved for both gene subfamilies or that regulatory sequences among gene family members are frequently homogenized along with amino acid encoding sequences. The distinctly different patterns of regulation observed among mature petunia plants, mature soybean plants and soybean seedlings emphasizes the possibility that gene conversion could elevate the rate of sequence change resulting in changes in regulation for *rbc*S gene families.

ACKNOWLEDGEMENTS

We would like to thank Nilgun Tumer and Dilip Shah for the generous gift of the *rbc*S A and *rbc*S C probes. We also thank Brenda Shirley, Julie Senecoff, and Judy Strommer for constructive criticism and editing of this manuscript. This work was supported by D.O.E. Grants DE-AS09-83ER13077 and DE-FG09-86ER13543 and by a N.I.H. Predoctoral Training Grant to D.M.T. This work serves as partial fulfillment of a Ph.D. Degree in Genetics for D.M.T.

REFERENCES

1. Miziorko, H.M. and Lorimer, G.H. (1983) Ann. Rev. Biochem. **52**, 507–535.
2. Tobin, E.M. and Silverthorne, J. (1985) Ann. Rev. Plant Physiol. **36**, 569–593.
3. Silverthorne, J. and Tobin, E.M. (1987) Bioessays. **7**, 18–23.
4. Watson, J.C. (1989) In S. D. Kung and C. J. Arntzen (ed.) Plant Biotechnology. Butterworths. Stoneham, MA. 161–205.
5. Thompson, W.F., Everett, M., Polans, N.O., Jorgensen, R.A. and Palmer, J.D. (1983) Planta. **158**, 487–500.
6. Fluhr, R. and Chua, N.-H. (1986) Proc. Natl. Acad. Sci. USA. **83**, 2358–2362.
7. Fluhr, R., Moses, P., Morelli, G., Coruzzi, G. and Chua, N.-H. (1986) The EMBO J. **5**, 2063–2071.
8. Gallagher, T.F. and Ellis, R.J. (1982) The EMBO J. **1**, 1493–1498.
9. Silverthorne, J. and Tobin, E.M. (1984) Proc. Natl. Acad. Sci. USA. **81**, 1112–1116.
10. Berry-Lowe, S.L. and Meagher, R.B. (1985) Mol. and Cell. Biol. **5**, 1910–1917.
11. Ernst, D., Pfeiffer, F., Schefbeck, K., Weyrauch, C. and Oesterhelt, D. (1987) Plant Mol. Biol. **10**, 21–33.
12. Shirley, B.W., Berry-Lowe, S.L., Rogers, S.G., Flick, J.S., Horsch, R., Fraley, R.T. and Meagher, R.B. (1987) Nucl. Acids Res. **15**, 6501–6514.
13. Shirley, B.W. and Meagher, R.B. Nucl. Acids Res. in press.
14. Berry-Lowe, S.L. and Meagher, R.B. (1982) J. Mol. Appl. Genet. **1**, 483–498.
15. Stiekema, W.J., Wimpee, C.F., Silverthorne, J. and Tobin, E.M. (1983) Plant Physiol. **72**, 717–724.
16. Dean, C., van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedbrook, J. (1985) The EMBO J. **4**, 3055–3061.
17. Krebbers, E., Seurinck, J., Herdies, L., Cashmore, A. and Timko, M.P. (1988) Plant Mol. Biol. **11**, 745–759.
18. Pichersky, E., Bernatzky, R., Tanksley, S.D. and Cashmore, A.R. (1986) Proc. Natl. Acad. Sci. USA. **83**, 3880–3884.
19. Sugita, M., Manzara, T., Pichersky, E., Cashmore, A. and Gruissem, W. (1987) Mol. Gen. Genet. **209**, 247–256.
20. Meagher, R.B., Berry-Lowe, S. and Rice, K. (1989) Genetics. **123**, 845–863.
21. Dean, C., van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedbrook, J. (1985) Proc. Natl. Acad. Sci. USA. **82**, 4964–4968.
22. Dean, C., van den Elzen, P., Tamaki, S., Black, M., Dunsmuir, P. and Bedbrook, J. (1987) Mol. Gen. Genet. **206**, 465–474.
23. Tumer, N.E., Clark, W.G., Tabor, G.J., Hironaka, C.M., Fraley, R.T. and Shah, D.M. (1986) Nucl. Acids Res. **14**, 3325–3342.
24. Dean, C., Favreau, M., Dunsmuir, P. and Bedbrook, J. (1987) Nucl. Acids Res. **15**, 4655–4669.
25. Shure, M., Wessler, S. and Fedoroff, N. (1983) Cell. **35**, 225–233.
26. Dunsmuir, P., Smith, S.M. and Bedbrook, J. (1983) J. Mol. App. Genet. **2**, 285–300.
27. Eckenrode, V.K., Arnold, J. and Meagher, R.B. (1985) J. Mol. Evol. **21**, 259–269.
28. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Gene. **2**, 95–113.
29. Hightower, R.C. and Meagher, R.B. (1985) The EMBO J. **4**, 1–8.
30. Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P. (1981) Anal. Biochem. **112**, 295–298.
31. Feinberg, A.P. and Volgstein, B. (1983) Anal. Biochem. **132**, 6–13 and Addendum.
32. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: A laboratory manual., Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
33. Johnson, D.A., Gautsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) Gene Anal. Techn. **1**, 3–8.
34. Kloppstech, K. (1985) Planta. **165**, 502–506.
35. Giuliano, G., Hoffman, N.E., Ko, K., Scolnik, P.A. and Cashmore, A.R. (1988) The EMBO J. **7**, 3635–3642.
36. Paulsen, H. and Bogorad, L. (1988) Plant Physiol. **88**, 1104–1109.
37. Stayton, M.M., Brosio, P. and Dunsmuir, P. (1989) Plant Physiol. **89**, 776–782.
38. Piechulla, B. and Gruissem, W. (1987) The EMBO J. **6**, 3593–3599.
39. Facciotti, D., O'Neal, J.K., Lee, S. and Shewmaker, C.K. (1985) Bio/Technology. **3**, 241–246.
40. Nagy, F., Morelli, G., Rogers, S.G. and Chua, N.-H. (1985) The EMBO J. **4**, 3063–3068.
41. Fluhr, R., Kuhlemeier, C., Nagy, F. and Chua, N.-H. (1986) Science. **232**, 1106–1112.
42. Edwards, J.W. and Coruzzi, G.M. (1989) The Plant Cell. **1**, 241–248.
43. Taylor, W.C. (1989) The Plant Cell. **1**, 259–264.