Red Light–Independent Instability of Oat Phytochrome mRNA in Vivo

Kevin A. Seeley, Dennis H. Byrne, and James T. Colbert[1]

Department of Botany, Iowa State University, Ames, Iowa 50011

Phytochrome A (*phyA*) mRNA abundance decreased rapidly in total RNA samples isolated from 4-day-old etiolated oat seedlings following a red light pulse. Putative in vivo *phyA* mRNA degradation products were detectable both before and after red light treatment. Cordycepin-treated coleoptiles were unable to accumulate the chlorophyll *a/b*-binding protein mRNA in response to red light, indicating that cordycepin effectively inhibited mRNA synthesis. In cordycepin-treated coleoptiles, *phyA* mRNA rapidly decreased in abundance, consistent with the hypothesis that *phyA* mRNA is inherently unstable, rather than being destabilized after red light treatment of etiolated oat seedlings.

INTRODUCTION

Phytochrome plays a crucial role in regulating plant development in response to light (Kendrick and Kronenberg, 1986). Considerable evidence has been obtained linking the change in expression of specific light-responsive genes to the photoconversion of phytochrome from the red light–absorbing (Pr) to the far-red-light–absorbing (Pfr) form (for reviews, see Kuhlemeier et al., 1987; Watson, 1989; Smith and Whitelam, 1990). In 4-day-old etiolated oat seedlings, the expression of phytochrome A (*phyA*; the etiolated seedling abundant phytochrome) is decreased at several levels, including selective proteolysis of the Pfr form of the photoreceptor (Shanklin et al., 1987), a decrease in mRNA abundance (Colbert et al., 1985), and a rapid decline in transcription of the *phyA* genes (Lissemore and Quail, 1988). The importance of the downregulation of *phyA* gene expression is clearly demonstrated by the mutant phenotype (dwarf, dark green) observed in transgenic tobacco and tomato plants that overexpress *phyA*-encoded protein (Boylan and Quail, 1989; Keller et al., 1989; Nagatani et al., 1991).

phyA mRNA abundance decreases dramatically when etiolated oat seedlings are given a saturating pulse of red light (Colbert, 1988; Byrne et al., 1990; Colbert, 1991). Previous studies using oat seedlings show a 90% decline in the amount of *phyA* mRNA present in the poly(A)$^+$ RNA fraction within 3 to 5 hr after a red light pulse (Colbert et al., 1985). Studies with isolated nuclei demonstrate that Pfr formation induces a decrease in transcription initiation to ~10% of dark levels within 30 min of a red light pulse (Lissemore and Quail, 1988). The rapid decrease in *phyA* transcription seen in nuclear run-on experiments does not, by itself, explain the rapid decrease in *phyA* mRNA steady-state levels. If *phyA* mRNA

had a half-life of 30 hr in the dark, as estimated for most poly(A)$^+$ RNAs present in soybean suspension culture cells (Silflow and Key, 1979), then *phyA* mRNA would persist at high levels in the cell long after the Pfr-induced decrease in transcription. Kinetic analysis combining the transcription and mRNA abundance data led to the proposal that *phyA* mRNA exhibited a half-life of 16 to 47 hr in etiolated oat seedlings prior to light treatment (Quail et al., 1986; Colbert, 1988).

The available data are consistent with two hypotheses by which the rapid decrease in *phyA* mRNA levels after Pfr production can be explained. First, it is possible that the *phyA* mRNA is inherently unstable and is being rapidly degraded at all times (Kay et al., 1989a). The dramatic decrease in *phyA* transcription induced by red light would then lead directly to the rapid decline in *phyA* mRNA abundance. Second, it is possible that production of Pfr results in both a decrease in transcription of *phyA* genes and a substantial Pfr-induced decrease in the half-life of *phyA* mRNA (Quail et al., 1986; Colbert, 1988). Examples of both possibilities have been well documented (Shapiro et al., 1987; Atwater et al., 1990). Some mRNAs are inherently unstable, e.g., c-*myc* mRNA (Dani et al., 1984; Brewer and Ross, 1989) and c-*fos* mRNA (Wilson and Treisman, 1988; Shyu et al., 1991). Other mRNAs, e.g., β-globin (Shaw and Kamen, 1986), are quite stable. The stability of still other mRNA species has been shown to vary depending on the prevailing conditions. Histone mRNAs are more stable during the S phase of the cell cycle than in other cell cycle phases (Graves et al., 1987; Marzluff and Pandey, 1988), transferrin receptor mRNA is destabilized in the presence of iron (Casey et al., 1988; Müllner and Kuhn, 1988), β-tubulin mRNA is destabilized by microtubule depolymerization (Pachter et al., 1987; Gay et al., 1989), and vitellogenin mRNA is stabilized in the presence of estrogen (Nielsen and Shapiro, 1990).

[1] To whom correspondence should be addressed.

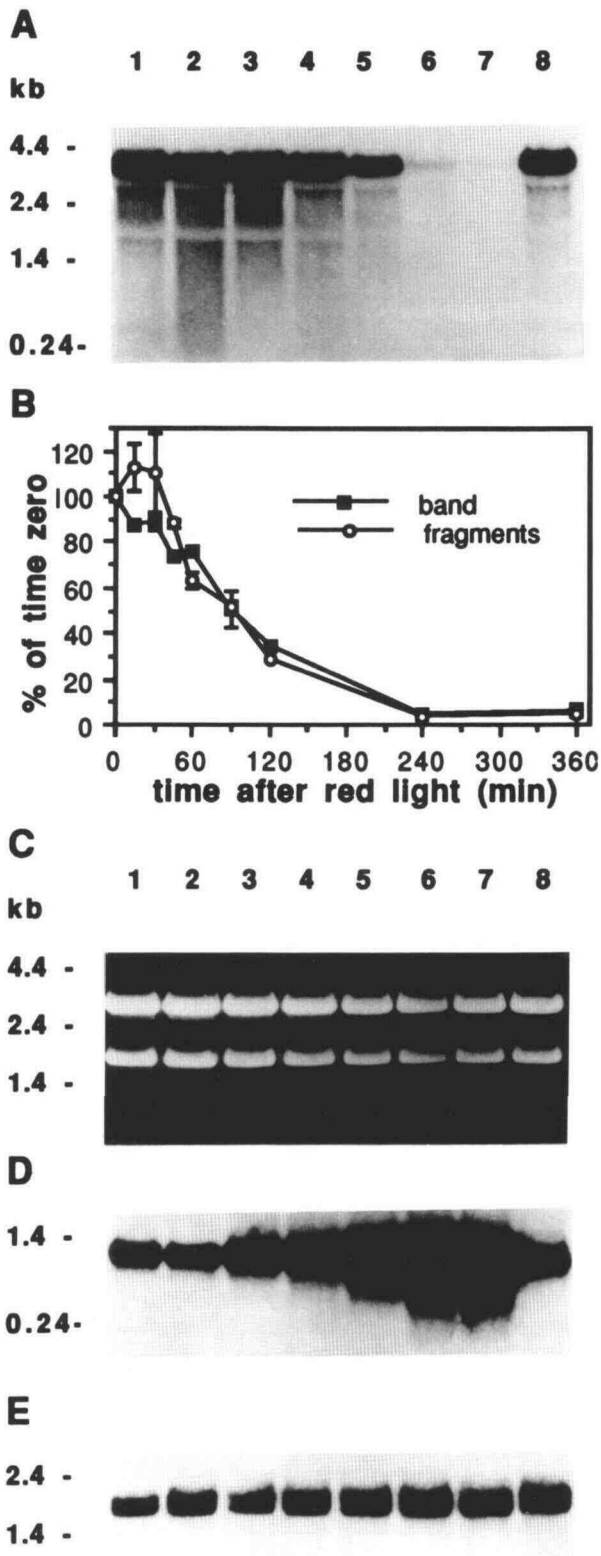## A

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Figure 1. Red Light–Induced Decrease in *phyA* mRNA Abundance and Detection of Putative *phyA* mRNA Degradation Products.

We have attempted to determine whether oat *phyA* mRNA is inherently unstable or is destabilized in response to Pfr by searching for *phyA* mRNA degradation products and by using an mRNA synthesis inhibitor to estimate *phyA* mRNA half-life in dark-grown oat seedlings. We detected putative in vivo *phyA* mRNA degradation products in total RNA samples. However, we observed no substantial increase in these degradation products after red light treatment. We have estimated the half-life of *phyA* mRNA in dark-grown seedlings to be similar to the 1-hr half-life observed after exposure of such seedlings to light. Together, these data lead us to conclude that *phyA* mRNA is inherently unstable.

RESULTS

Effect of Red Light on *phyA* mRNA Abundance in Total RNA

Figure 1 shows that exposure of 4-day-old etiolated oat seedlings to red light resulted in a dramatic decrease in full-length *phyA* mRNA abundance to <10% of the dark level. During this decrease, *phyA* mRNA exhibited an apparent half-life of ~90 min (Figure 1B). In addition, a smear of *phyA* RNA fragments from 4.2 to 0.2 kb in length was present. The *phyA* RNA fragments were detectable in total RNA samples from oat seedlings never exposed to light (Figure 1A, lanes 1 and 8) and

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(A) A representative RNA gel blot of total RNA from 4-day-old etiolated oat seedlings harvested at 15 min (lane 2), 30 min (lane 3), 1 hr (lane 4), 2 hr (lane 5), 4 hr (lane 6), and 6 hr (lane 7) after a saturating red light pulse, harvested prior to the red light pulse (lane 1), or kept in darkness for an additional 6 hr before harvest (lane 8). Samples of 5 μg from each preparation of total RNA were electrophoresed on a 1% agarose/3% formaldehyde gel, blotted to a nylon membrane, hybridized with a ^{32}P-labeled *phyA* antisense RNA probe (pAPSX2.7), and exposed to x-ray film for 12 hr. Positions of the RNA size markers are indicated.

(B) Quantitation of the decrease in *phyA* mRNA abundance after red light. The regions corresponding to full-length *phyA* mRNA and the *phyA* RNA fragments were excised from the blots, and the amount of radioactivity was determined by liquid scintillation spectrometry. The average amount of radioactivity hybridized to the time zero point (100%) was 3733 CPM and 2512 CPM for the band and fragments, respectively. Error bars indicate standard error of the mean of the four independent experiments, including the blot shown in **(A)**. In some instances, the error bars were smaller than the plot symbols.

(C) Ethidium bromide staining of total RNA samples (same RNA samples as in **[A]**). After electrophoresis of the ethidium bromide-stained samples, the agarose-formaldehyde gel was photographed on a UV light–emitting transilluminator.

(D) Replicate RNA gel blot (same RNA samples as in **[A]**) hybridized with the maize *cab* antisense RNA probe (pGAB0.7). This blot was exposed to x-ray film for 6 hr.

(E) Replicate RNA gel blot (same RNA samples as in **[A]**) hybridized with the antisense soybean actin RNA probe (pAct24). This blot was exposed to x-ray film for 48 hr.
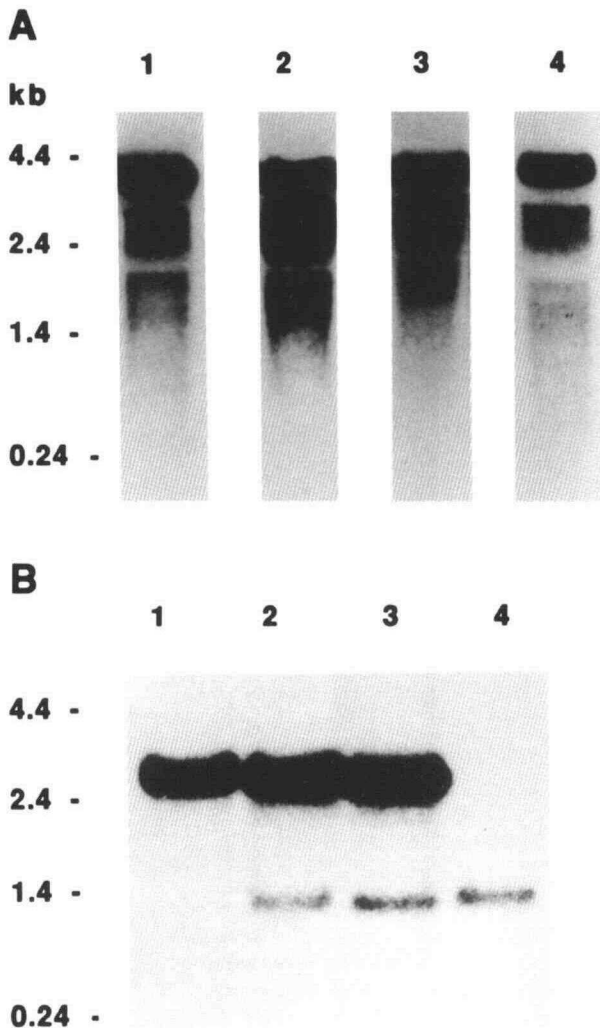
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Figure 2. Visualization of *phyA* mRNA Fragments in RNA Samples Isolated by Various Methods and Absence of Ribonuclease Activity during the Small-Scale RNA Isolation Procedure.

(A) Total RNA was isolated from 4-day-old etiolated oat seedlings by the small-scale RNA isolation method (lane 1), by phenol/chloroform extraction of purified polysomes (lane 2), or by large-scale RNA isolations using either guanidinium thiocyanate followed by centrifugation through CsCl (lane 3) or a phenol/SDS extraction procedure (lane 4). Total RNA was loaded at 10 μg per lane, then samples were electrophoresed, blotted onto nylon membranes, and hybridized with a ^{32}P-labeled *phyA* probe (pGAP1.7 or pAPSX2.7). Positions of the RNA size markers are indicated.

(B) Representative gel showing the protection of RNAs synthesized in vitro from general ribonuclease activity during the isolation of total RNA from 4-day-old etiolated oat seedlings. ^{32}P-labeled sense strand RNAs were synthesized from an oat *phyA* cDNA subclone (pAPSX2.7) and from an oat β-tubulin cDNA subclone (pGβ1). The predicted lengths of the in vitro transcripts were 2.65 kb (*phyA*) and 1.2 kb (β-tubulin). The transcripts lacked both a poly(A) tail and a 5′,7-methylguanosine cap. To visualize the labeled RNAs, they were electrophoresed, blotted onto a nylon membrane, and autoradiographed.

from those irradiated with red light (Figure 1A, lanes 2 to 7). The *phyA* RNA fragments disappeared at approximately the same rate as the mature mRNA band (Figure 1B), supporting the interpretation that the hybridization was *phyA* specific. The regions of little hybridization at ∼3.8 and 2.0 kb in the *phyA* RNA fragment smears coincided with the 25S and 18S ribosomal RNAs (Figure 1C), demonstrating that the *phyA* probe was not binding to the 25S and 18S rRNAs under our hybridization conditions.

phyA mRNA Fragments Are Produced in Vivo

There are several possible explanations for the presence of these *phyA* RNA fragments. First, they could be the product of general degradation that occurred during the RNA isolation procedure. Second, they could arise as products of premature transcription termination or degradation of the *phyA* heterogeneous nuclear RNA (hnRNA) in the nucleus. Third, the *phyA* RNA fragments might be degradation products of the mature cytosolic *phyA* mRNA.

To address the possibility of mRNA degradation during total RNA isolation, several control experiments were performed. First, replicate RNA gel blots of total RNA samples from the red light time course experiment (Figure 1A) were hybridized with RNA probes complementary to chlorophyll *a/b*-binding protein (*cab*) or actin mRNA. *cab* mRNA abundance increased in response to red light as expected (Figure 1D), whereas the actin mRNA levels remained constant following red light treatment (Figure 1E). The actin data, taken together with the ethidium bromide staining of rRNA (Figure 1C), demonstrate that approximately equal amounts of mRNA and total RNA were electrophoresed in each lane. Actin mRNA showed none of the RNA fragments seen on the *phyA* blots, indicating that at least one poly(A)$^+$ mRNA species in these total RNA samples was intact after isolation and RNA gel analysis (Figure 1E).

Figure 2A shows that the *phyA* RNA fragments were present in total RNA samples isolated using a variety of different RNA isolation techniques. These techniques included the use of a small-scale RNA isolation procedure containing the ribonuclease inhibitor aurintricarboxylic acid, polysomal preparations, and two large-scale preparations employing either guanidinium thiocyanate or phenol/SDS to inhibit ribonuclease activity. All of these techniques yielded RNA that

The expected length of the RNAs synthesized in vitro was observed either with (lane 3) or without (lane 2) 5 μg of dark-grown oat total RNA added to the labeled RNA samples. The labeled RNAs were then added separately to etiolated oat seedlings at the time of RNA extraction by the small-scale RNA isolation method. The labeled RNAs were coisolated with the oat RNA present in each sample of etiolated oat seedlings. Samples of 5 μg of total RNA containing 275 cpm of *phyA* probe or 75 cpm of β-tubulin probe were electrophoresed in lanes 1 and 4, respectively.

gave an extensive smear of *phyA* RNA fragments in RNA gel blot analysis.

Finally, assays were performed to determine whether exogenously added RNA was degraded during RNA extraction. pAPSX2.7, a clone containing 2.7 kb of the *phyA* coding region, was transcribed in vitro to produce a radiolabeled 2.7-kb sense strand *phyA* RNA fragment. This *phyA* RNA fragment was added to frozen oat seedlings at the start of the small-scale RNA extraction. The RNA sense strand synthesized in vitro had no 5′ end cap or 3′ end poly(A) tail and would presumably have been degraded quickly by any general ribonuclease activity present during the isolation procedure. As shown in Figure 2B (lanes 1 to 3), the *phyA* RNA fragment was not degraded during the RNA isolation procedure. In addition to the *phyA* RNA, a smaller β-tubulin in vitro–synthesized radiolabeled sense strand RNA fragment was added to a separate small-scale RNA isolation. The β-tubulin RNA also remained intact during RNA isolation (Figure 2B, lanes 2 to 4).

These data suggest that the *phyA* RNA fragments observed in RNA gel blot analysis represent *phyA* mRNA degradation products produced in vivo. The high abundance of these degradation products in light-treated and dark-grown seedlings, relative to the full-length *phyA* mRNA band (Figure 1A), suggests that *phyA* mRNA is rapidly degraded both before and after Pfr production.

Phytochrome mRNA Has a Short Half-Life Prior to Pfr Production

To test the prediction that *phyA* mRNA is rapidly degraded both before and after a light treatment, we used an RNA synthesis inhibitor to estimate the half-life of *phyA* mRNA in etiolated oat seedlings never exposed to light. An infrared (IR) viewing system was used to allow manipulation of the oat seedlings in otherwise total darkness.

We used cordycepin, a chain-terminating adenosine analog (Cline and Rhem, 1974; Schopfer, 1989), to inhibit RNA synthesis. Preliminary studies revealed that virtually all of the *phyA* mRNA hybridizing to the pAPSX2.7 antisense RNA probe was localized in the coleoptile of 4-day-old etiolated oat seedlings (K. Seeley and J. Colbert, manuscript in preparation). We also determined that whereas most of the *cab* mRNA is in the leaves enclosed by the coleoptiles, detectable quantities of *cab* mRNA are induced by light in the coleoptile, as shown in Figure 3. To aid in the uptake of cordycepin by the coleoptile, we excised oat seedlings, using the IR viewer, and abraded off the cuticle using a polishing cloth (Edelmann and Schopfer, 1989; Schopfer, 1989) prior to immersion in incubation buffer. This treatment did not substantially affect either the light-induced decrease in *phyA* mRNA abundance or the increase in *cab* mRNA abundance (Figures 3A and 3B, lanes 1 to 3). However, when 100 μg/mL cordycepin was included in the incubation buffer, beginning 30 min before the light treatment, the induction of *cab* mRNA accumulation by light was completely inhibited (Figure 3B, lane 4). In the presence of cordycepin, *phyA* mRNA abundance decreased after
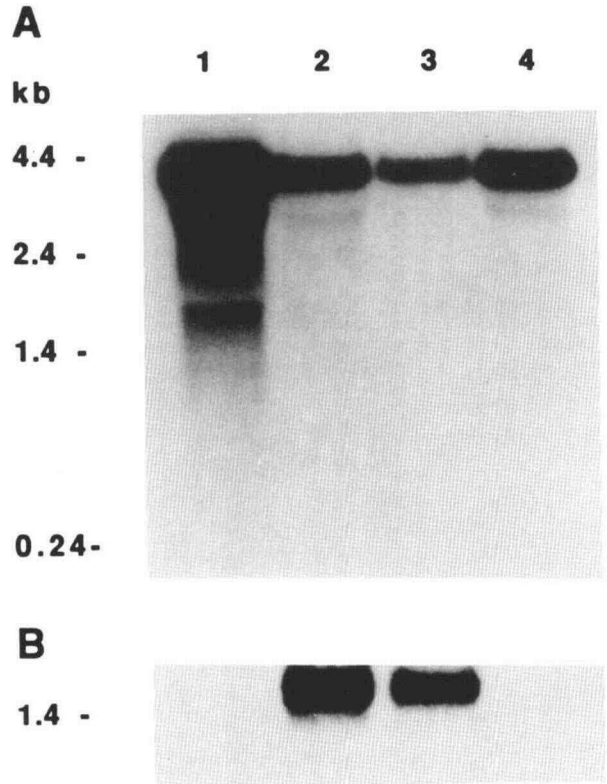

Figure 3. Effect of Cordycepin on Light Regulation of *phyA* and *cab* mRNA Levels.

(A) Representative RNA gel blot hybridized to *phyA* antisense probe from pAPSX2.7 with total RNA (10 μg) isolated using the small-scale technique. Intact etiolated oat seedlings were either harvested in the dark (lane 1) or exposed to a red light pulse and harvested 3.5 hr later (lane 2). Other seedlings (using the IR viewer) were excised, abraded, and floated in buffer without (lane 3) or with (lane 4) 100 μg/mL cordycepin for 30 min. After the 30-min pretreatment, the excised abraded seedlings were exposed to a red light pulse and incubated an additional 3.5 hr in the dark. At harvest, the coleoptiles were separated from the enclosed leaves and total RNA was isolated from the coleoptiles. The hybridized blot was exposed to x-ray film for 2 hr.

(B) Replicate RNA gel blot hybridized with *cab* antisense probes from pGAB0.7. The hybridized blot was exposed to x-ray film for 24 hr.

exposure to red light, but to a lesser extent than in the absence of cordycepin (Figure 3A, lane 4). In Figure 4A, the abraded dark control (lane 8) demonstrates that the experimental manipulation of the seedlings, including IR exposure, did not cause changes in *phyA* mRNA levels during the 4-hr experiment.

Having demonstrated that 100 μg/mL cordycepin is effective in preventing *cab* mRNA synthesis in the coleoptile, we used the same experimental conditions to estimate the half-life of *phyA* mRNA both before and after red light treatment. Following the 30-min immersion in treatment buffer with or without cordycepin, the excised abraded seedlings were either allowed to remain in total darkness or given a red light
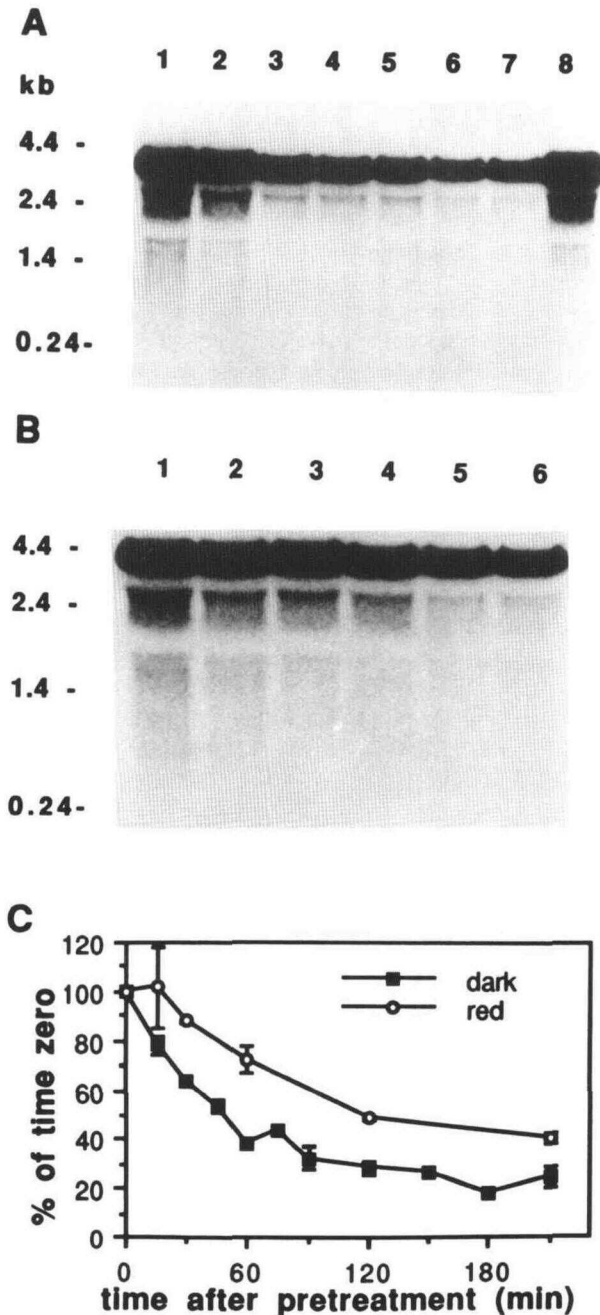
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Figure 4. Effect of Cordycepin on *phyA* mRNA Abundance.

(A) Etiolated oat seedlings were excised, abraded, placed in control or cordycepin-containing buffers, and harvested at various times following a 30-min pretreatment in darkness. All procedures were done using the IR viewer in otherwise total darkness. Total RNA (10 μg) was probed for *phyA* mRNA and exposed to x-ray film for 3 hr. Lanes 1 and 8 are abrasion and buffer controls (without cordycepin) at time zero (100%) and 3.5 hr of incubation, respectively. For this experiment, seedlings exposed to cordycepin (lanes 2 to 7) were harvested at 30, 60, 120, 150, 180, and 210 min after pretreatment.

(B) Etiolated seedlings were treated, and RNA was isolated as described above except that after the 30 min cordycepin pretreatment

treatment, returned to darkness, and harvested at times up to 3.5 hr after pretreatment. Figure 4 shows that in those coleoptiles maintained in darkness, *phyA* mRNA rapidly decreased in abundance, exhibiting a half-life of ∼50 min (Figures 4A and 4C). *phyA* mRNA abundance reached the minimum level of ∼30% of the time zero control by 120 min after pretreatment (Figure 4C). In separate experiments in which coleoptiles were given a red light pulse after the cordycepin treatment, the half-life of *phyA* mRNA was estimated to be somewhat longer (120 min) than in those coleoptiles maintained in darkness (Figures 4B and 4C). However, both sets of experiments are consistent with a Pfr-independent, short (1 to 2 hr) half-life for *phyA* mRNA.

Experiments employing metabolic inhibitors are susceptible to artifacts resulting from unintended secondary effects of the inhibitors. We attempted to minimize the potential for such artifacts by directly demonstrating that mRNA synthesis (i.e., *cab* mRNA synthesis) was inhibited in the coleoptile and by measuring *phyA* mRNA abundance at relatively short times after exposure of the seedlings to cordycepin (Figure 4). Another strategy to minimize secondary effects is to use inhibitors at the minimum required concentration. Therefore, we investigated whether or not 100 μg/mL cordycepin was near the minimum concentration needed to cause the maximum decline in *phyA* mRNA abundance. Excised and abraded seedlings were exposed to various concentrations of cordycepin for 4 hr. As shown in Figure 5, saturation was observed between 50 and 100 μg/mL cordycepin, indicating that the cordycepin concentration used in the half-life estimation experiments was not far in excess of the required concentration.

DISCUSSION

We have estimated the apparent half-life of *phyA* mRNA in total RNA to be ∼90 min in seedlings treated with red light. This

a red light pulse was given and then excised seedlings were incubated for various times in darkness. Total RNA (10 μg) was probed for *phyA* mRNA and exposed to x-ray film for 3 hr. For this experiment, seedlings exposed to cordycepin (lanes 1 to 6) were harvested at 0, 15, 30, 60, 120, and 210 min after pretreatment.

(C) The bands corresponding to full-length *phyA* mRNA were excised from RNA gel blots, and the amount of radioactivity was determined by liquid scintillation spectrometry. The data for dark-maintained seedlings (solid squares) from **(A)** show the means from four independent experiments for the 0-, 30-, and 60-min time points, three independent experiments for the 90- and 120-min time points, and two independent experiments for all others. These data include the blot shown in **(A)**. The data for red light–treated seedlings (open circles) are derived from two independent experiments at all time points tested including the blot shown in **(B)**. The average amount of radioactivity hybridized to time zero (100%) was 4159 CPM and 8827 CPM for the dark and red time courses, respectively. Error bars indicate standard error of the mean of the independent experiments. (In some instances, the error bars were smaller than the plot symbols.)
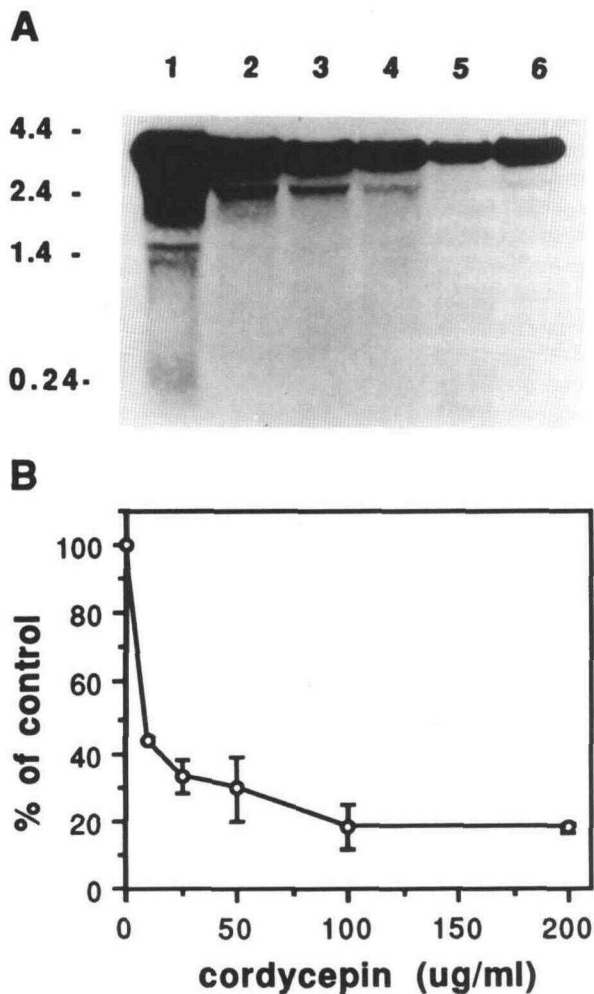
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Figure 5. Effect of Cordycepin Concentration on *phyA* mRNA Abundance.

Etiolated oat seedlings were excised, abraded, placed in control buffer or buffer with cordycepin at various concentrations, and harvested after 4 hr. All procedures were done using the IR viewer in otherwise total darkness.

(A) Representative blot of cordycepin concentration experiment. Total RNA (10 μg) was probed for *phyA* mRNA and the blot exposed to x-ray film for 3 hr.

(B) The bands corresponding to the full-length *phyA* mRNA were excised, and the amount of radioactivity was determined by liquid scintillation spectrometry. The average amount of radioactivity hybridized to the zero cordycepin point (100%) was 4859 CPM. Data show the mean and the standard error of the mean of two independent experiments, including the blot shown in **(A)**.

estimate is similar to the 1-hr half-life previously estimated for *phyA* mRNA in the poly(A)$^+$ fraction (Colbert, 1988, 1991). We conclude that the consistently observed *phyA* RNA fragments in total RNA samples are not an artifact of the isolation of

RNA from oat seedlings but are present in vivo. Similar *phyA* RNA fragments have been observed in cucumber (Tirimanne and Colbert, 1991), suggesting that the presence of *phyA* RNA fragments is not exclusive to oats. It is noteworthy that similar *phyA* RNA fragments were evident in RNA gel blots from transgenic tobacco plants possessing a rice *phyA* gene that was under the control of a constitutive promoter (Kay et al., 1989b).

There are a number of possible sources of the *phyA* mRNA fragments produced in vivo, including (1) premature transcription termination products in the nucleus, (2) degradation of hnRNA, or (3) rapid turnover of mature *phyA* mRNA in the cytoplasm. A kinetic argument suggests that the *phyA* RNA fragments are not due to premature transcription termination. Because *phyA* transcription drops to minimal levels within 15 to 30 min after red light treatment (Lissemore and Quail, 1988), we would expect to see the abundance of premature transcription termination fragments to decrease over the same time course as the downregulation of transcription. In fact, the *phyA* RNA fragments are still readily detectable at 2 hr postirradiation. In addition, we detect no apparent splicing intermediates, suggesting that hnRNA does not represent a substantial fraction of the total RNA and, therefore, that the observed fragments are unlikely to be degradation products of hnRNA. Finally, the presence of *phyA* RNA fragments in total RNA samples prepared from isolated polysomes supports a cytosolic origin for the *phyA* RNA fragments.

If we accept that the *phyA* RNA fragments are produced in vivo, in the cytosol, then the most likely explanation for their presence is that they are intermediates of *phyA* mRNA degradation and that the conversion of *phyA* degradation intermediates to nucleotides is slow relative to the initial step in *phyA* mRNA degradation. If *phyA* mRNA were destabilized after Pfr production, then more of these degradation products might be observed after light treatment. However, we detect similar amounts of the degradation products in dark control and light-treated seedlings, suggesting that the rate of *phyA* mRNA degradation does not dramatically increase after Pfr production.

A more direct way to address the question of whether *phyA* mRNA exhibits inherent instability or Pfr-induced instability is to estimate the half-life of the *phyA* message in etiolated oat seedlings. We have estimated the half-life of *phyA* mRNA using the RNA synthesis inhibitor cordycepin (Cline and Rhem, 1974; Schopfer, 1989). These experiments show that the half-life of *phyA* mRNA is ~1 hr before red light treatment in etiolated oat seedlings. This value is quite similar to the estimated half-life of *phyA* mRNA in both total RNA (Figure 1) and poly(A)$^+$ RNA after Pfr production (Colbert et al., 1985). Production of Pfr in the presence of cordycepin did not result in a more rapid loss of *phyA* mRNA, suggesting that Pfr only affects *phyA* gene transcription, not the stability of the *phyA* mRNA.

A half-life of ~1 hr is substantially shorter than the average half-life of 30 hr reported for most poly(A)$^+$ RNAs in soybean

suspension culture cells (Silflow and Key, 1979) and the average half-life of 16 hr reported for most poly(A)$^+$ RNAs in Xenopus liver cells (Brock and Shapiro, 1983). A 1-hr half-life is also much shorter than the 16- to 47-hr half-life previously predicted for *phyA* mRNA in etiolated seedlings before light treatment (Quail et al., 1986; Colbert, 1988). The possibility of artifacts resulting from the use of a metabolic inhibitor was minimized by using a cordycepin concentration near that required for maximal inhibition and limiting the treatment with cordycepin to 4 hr or less. Furthermore, use of an RNA synthesis inhibitor is more likely to lead to an overestimation, rather than an underestimation, of the half-life of an mRNA due to potentially incomplete inhibition of RNA synthesis. We conclude, therefore, that *phyA* mRNA is inherently unstable, with a half-life of ~1 hr, similar to that reported for histone (Graves et al., 1987; Marzluff and Pandey, 1988), c-*myc* (Dani et al., 1984), and c-*fos* (Wilson and Treisman, 1988; Shyu et al., 1991) mRNAs. In addition, we conclude that, unlike earlier proposals (Quail et al., 1986; Colbert, 1988), the instability of *phyA* mRNA is Pfr independent.

Although the half-life of *phyA* mRNA is similar after cordycepin treatment or Pfr production, cordycepin does not lead to as great a decrease in *phyA* mRNA abundance as does red light. This could be due to incomplete inhibition of *phyA* mRNA synthesis by cordycepin. However, *cab* mRNA synthesis appears to be completely inhibited under the same conditions. Other experiments (Figures 3 and 4) have shown that the production of Pfr in the presence of cordycepin leads to a smaller decrease in *phyA* mRNA abundance than in the absence of cordycepin. These observations suggest that a labile factor required for *phyA* mRNA degradation is depleted during cordycepin treatment, inhibiting degradation of *phyA* mRNA. This factor could either be a protein or an RNA molecule and could be envisioned to be either a *phyA*-specific ribonuclease or a molecule that targets *phyA* mRNA for degradation by a more general nuclease. Exposure of oat seedlings to cycloheximide before light treatment resulted in higher levels of *phyA* mRNA at 2 hr postirradiation than in the absence of cycloheximide (Colbert et al., 1991). Together with the cordycepin data, these data are consistent with the possibility that an unstable protein factor plays an essential role in *phyA* mRNA degradation.

The significance of the rapid degradation of *phyA* mRNA to plant development is still uncertain. The possibility that oat seedlings need to decrease their capacity to synthesize *phyA* mRNA after light treatment to properly induce photomorphogenesis is supported by the observation that overexpression of *phyA* protein in transgenic tobacco (Keller et al., 1989; Nagatani et al., 1991) and tomato (Boylan and Quail, 1989) leads to developmental abnormalities. Light-induced decreases in *phyA* expression occur, in part, at the protein level because the Pfr form of the protein is rapidly degraded by way of the ubiquitin pathway (Shanklin et al., 1987). An inherently unstable mRNA would further decrease the abundance of the *phyA* protein by rapidly leading to a diminished amount of *phyA*

mRNA available for translation after the Pfr-induced decrease in transcription.

The data presented here support the conclusion that *phyA* mRNA is inherently unstable. Understanding the role that mRNA degradation plays in the maintenance of steady-state levels of *phyA* mRNA will, hopefully, lead to increased understanding of the regulation of gene expression in plants. Currently, little is known about how plant cells recognize mRNAs that are to be rapidly degraded (Okamuro and Goldberg, 1989). Further work is being performed to determine which primary sequences or secondary structures are required to target oat *phyA* mRNA for rapid degradation.

METHODS

Growth and Light Treatment of Plants

Four-day-old dark-grown oat (*Avena sativa* cv Garry) seedlings were harvested by excision just below the mesocotyl node. Harvest of etiolated seedlings was performed under dim green light, except in those experiments involving cordycepin treatment (see below). After harvest, the plant tissues were frozen in liquid nitrogen and stored at −80°C until the isolation of RNA. Saturating red light treatments (10 to 20 sec in duration) were provided as described previously (Colbert et al., 1983).

RNA Isolation and Quantification

The principal method for RNA isolation was a modification of the rapid small-scale RNA isolation described by Wadsworth et al. (1988). Frozen tissue samples (100 to 200 mg) were ground in liquid nitrogen with a mortar and pestle and thawed in the presence of aurintricarboxylic acid (Sigma A-1895) and phenol chloroform. After phenol chloroform extraction, the RNA was separated from DNA by differential precipitation with 3 M LiCl. Total RNA yields using this technique ranged from 300 to 800 μg/g fresh weight for whole seedlings and ~50 μg/g fresh weight for isolated coleoptiles. Other techniques for RNA isolation included large-scale isolations using phenol/SDS (Dean et al., 1985), or guanidinium thiocyanate/CsCl (Colbert et al., 1983), and polysome isolation (Larkins and Hurkman, 1978; Taliercio and Chourey, 1989).

Total RNA (5 to 10 μg) was electrophoresed in 1% agarose/3% formaldehyde gels, blotted onto nylon membranes (GeneScreen, Du Pont), and hybridized (50% deionized formamide [nucleic acid grade, Bethesda Research Laboratories], 1.0 M NaCl, 10% dextran sulfate, and 1% SDS [Bio-Rad]) at 65°C with specific ^{32}P-labeled antisense RNA probes synthesized in vitro (Riboprobe, Promega) added to the hybridization mixture at 500,000 cpm/mL (Cotton et al., 1990). Autoradiographic exposures were made at −80°C on XAR5 (Kodak) x-ray film with intensifying screens (Du Pont). Quantitation of hybridization was accomplished by overlaying the autoradiogram on the blot, carefully cutting out the desired region, and determining the amount of radioactivity bound to the excised membrane piece using liquid scintillation spectroscopy. Adjustment for background hybridization was done by subtracting the amount of radioactivity bound to

a similarly sized membrane piece from a region on the same blot in which no RNA was bound.

Plasmid DNA and in Vitro RNA Synthesis

DNA isolation and modification, in vitro transcription template preparation, agarose gel electrophoresis, and subcloning were performed using standard techniques or by following the manufacturer's instructions (Sambrook et al., 1989; Bio 101, La Jolla, CA; Promega; Stratagene; Qiagen, Chatsworth, CA). Fragments from different parent clones were subcloned into pGEM3 (Promega) or pBluescript SK+ (Stratagene) transcription vectors. Plasmid pGAP1.7 (Edwards and Colbert, 1990) contains an internal 1.7-kb PstI fragment of the oat *phyA* cDNA clone pAP3.2 (Hershey et al., 1985) cloned into pGEM3. This fragment includes the chromophore binding region sequence. In vitro transcription from the T7 polymerase promoter of NcoI-linearized pGAP1.7 gives antisense (complementary to *phyA* mRNA) *phyA* RNA. Plasmid pAPSX2.7 is a 2.7-kb SacI-XbaI fragment from the oat *phyA* subclone pFY122 (Boylan and Quail, 1989) cloned into pGEM3. This fragment includes the chromophore binding region and 1.2 kb of the *phyA* gene 3' to the end of pGAP1.7. Probe synthesis using SP6 polymerase on EcoRI-linearized pAPSX2.7 gives antisense *phyA* mRNA. Sense strand (identical to the *phyA* mRNA) RNA was transcribed at low specific activity (50 mM concentration of all four unlabeled nucleotides) using T7 polymerase on an XbaI-linearized pAPSX2.7 template. The construct pGβ1 contains a 1.2-kb fragment in pGEM3 from the 3' end of the pβ1 β-tubulin oat cDNA clone (Colbert et al., 1990). Low specific activity sense strand RNA was transcribed from the SP6 promoter on a HindIII-linearized pGβ1 template. Plasmid pAct24 is a 0.14-kb XhoI-EcoRI subclone from the third exon of the pSAc3 (Shah et al., 1982) soybean actin gene in pBluescript SK+. Antisense probes were transcribed from the T3 polymerase promoter on an XhoI-linearized pAct24. The subclone pGAB0.7 (Edwards and Colbert, 1990) contains a 0.7-kb PstI fragment from pAB1084 in pGEM3; it is a corn *cab* cDNA clone (Sullivan et al., 1989) from the 3' end of the *cab* cDNA, including the 3' untranslated region. Antisense probes were synthesized from the T7 polymerase promoter using HindIII-linearized pGAB0.7.

Coleoptile Abrasion and Inhibitor Treatment

Four-day-old etiolated seedlings were excised 5 to 10 mm below the mesocotyl node and passed four to six times through a loop of dry polishing cloth as described previously (Edelmann and Schopfer, 1989; Schopfer, 1989). Control experiments were performed using Evans blue (0.5 µg/mL) or neutral red (0.5 µg/mL) dyes as described (Edelmann and Schopfer, 1989; Schopfer, 1989) to determine the amount of abrasion needed to remove the waxy cuticle but not damage the underlying tissue. For all experiments, an IR viewer (FJW Optical Systems, Palatine, IL) was used to allow manipulation of the seedlings in the absence of phytochrome-active radiation. After abrasion, the seedlings (~10 per dish) were placed in incubation buffer (1 mM Pipes, pH 6.25, 1 mM sodium citrate, 1 mM KCl, 15 mM sucrose) in a 60 × 15 mm plastic dish, with or without inhibitor (cordycepin, Sigma C-3394). This mixture was then swirled at 75 rpm, either in total darkness for the desired time or irradiated with red light and returned to darkness. At harvest, excised abraded seedlings were blotted dry on paper towels, frozen in liquid nitrogen, and stored at −80°C. RNA was isolated using the small-scale procedure.

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