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Oat Phytochrome A mRNA Degradation Appears To Occur Via Two Distinct Pathways

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We have identified possible mechanisms for the degradation of oat phytochrome A (*PHYA*) mRNA. The majority of *PHYA* mRNA molecules appeared to be degraded prior to removal of the poly(A) tail, a pathway that differs from that reported for the degradation of other eukaryotic mRNAs. Polyadenylated *PHYA* mRNA contained a pattern of putative degradation products that is consistent with a 5′→3′ exoribonuclease, although the participation of a stochastic endoribonuclease cannot be excluded. The poly(A) tail of *PHYA* mRNA was heterogeneous in size and ranged from ~14 to 220 nucleotides. Early *PHYA* mRNA degradation events did not appear to involve site-specific endoribonucleases. Approximately 25% of the apparently full-length *PHYA* mRNA was poly(A) deficient. Oat H4 histone, β-tubulin, and actin mRNA populations had lower amounts of apparently full-length mRNAs that were poly(A) deficient. Degradation of the poly(A)-deficient *PHYA* mRNA, a second pathway, appeared to be initiated by a 3′→5′ exoribonucleolytic removal of the poly(A) tail followed by both 5′→3′ and 3′→5′ exoribonuclease activities. Polysome-associated RNA contained putative *PHYA* mRNA degradation products and was a mixture of polyadenylated and deadenylated *PHYA* messages, suggesting that the two distinct degradation pathways are polysome associated.

INTRODUCTION

Regulation of mRNA stability is an important mechanism for controlling gene expression post-transcriptionally (Atwater et al., 1990; Peltz et al., 1991; Brawerman, 1993; Sachs, 1993). In both soybean embryos and mouse L cells, there is a poor correlation between the rates of transcription initiation and mRNA abundance for several genes, indicating that post-transcriptional regulation is a common method for modulating eukaryotic gene expression (Carneiro and Schibler, 1984; Walling et al., 1986). The average mRNA half-life in soybean suspension cells is estimated to be 30 hr (Silflow and Key, 1979). Similar values of 10 to 20 hr were estimated for the half-life of an average mammalian mRNA (Hargrove and Schmidt, 1989; Peltz et al., 1991). It is clear that the stability of mRNAs varies greatly depending upon the gene and growth conditions (Brawerman, 1993; Green, 1993). Efforts have been made to identify sequences within mRNAs, *cis* elements, that are involved in regulating stability. Some mammalian mRNAs have been found to contain one or more *cis* elements that have a large impact on mRNA stability (Brewer and Ross, 1988; Klausner and Harford, 1990; Shyu et al., 1991; Theodorakis and Cleveland, 1992; Sachs, 1993). The control of mRNA stability in plants has recently been reviewed by Gallie (1993) and Green (1993). Several *cis* elements that destabilize plant mRNAs have been identified in pea, soybean, and tobacco (Dickey et al., 1992; Newman et al., 1993; Ohme-Takagi et al., 1993). From these studies, it is clear that the regulation of eukaryotic mRNA stability is complex.

Presumably, stability-altering *cis* elements in mRNAs affect the mechanism by which the mRNA is degraded. Currently, little is known about eukaryotic mRNA degradation mechanisms (Savant-Bhonsale and Cleveland, 1992). A common theme in mRNA turnover is first the removal of the poly(A) tail, either by a 3′→5′ exoribonuclease or an endoribonucleolytic cleavage in the 3′ untranslated region, followed by the degradation of the body of the message. Some eukaryotic mRNAs, for example c-*myc* (Brewer and Ross, 1988), apolipoprotein II (Binder et al., 1989), and β-globin (Albrecht et al., 1984), are degraded first by poly(A) tail removal followed by hydrolysis of the body of the message by a 3′→5′ exoribonuclease. The nonpolyadenylated human H4 histone is also degraded by a 3′→5′ exoribonuclease (Peltz et al., 1991). Poly(A) tail removal also appears to be a first step in the degradation of some yeast mRNAs (Vreken and Raué, 1992; Decker and Parker, 1993; Hsu and Stevens, 1993). After deadenylation, these mRNAs are degraded by 5′→3′ exoribonucleases. One degradation mechanism has been reported for a nuclear-encoded plant mRNA. The soybean ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) mRNA is reported to be degraded by a stochastic endoribonuclease (Thompson et al., 1992; Tanzer and Meagher, 1994). Discrete degradation products of the *rbcS* mRNA are observed, indicating that this stochastic endoribonuclease has sequence or structure specificity (Tanzer and

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Meagher, 1994). From these examples, it appears that eukaryotic mRNAs can be degraded by distinct pathways.

Investigations into the role that the poly(A) tail plays in degradation indicate that the length of the tail can affect the stability of mRNAs (Gallie et al., 1989; Baker, 1993). In addition, the coupling of mRNA degradation and translation has been observed for a number of mRNAs (Savant-Bhonsale and Cleveland, 1992; Theodorakis and Cleveland, 1992). Some mRNAs are degraded while associated with polysomes (Brewer and Ross, 1988; Pei and Calame, 1988; Brown and Harland, 1990; Byrne et al., 1993; Pastori and Schoenberg, 1993; Tanzer and Meagher, 1994). Poly(A) shortening was detected with human c-*myc* mRNA in a polysome-based cell-free system (Brewer and Ross, 1988), suggesting that deadenylation of mRNAs can occur on polysomes.

As sessile organisms, plants need to respond quickly to changes in the environment. Rapid environmental responses could be enhanced by utilizing unstable mRNAs (Green, 1993). Phytochrome A (PHYA, etiolated seedling predominant phytochrome) in oats is a light-regulated gene that produces an unstable mRNA. The *PHYA* mRNA encodes the PHYA protein, a plant photoreceptor that regulates plant growth and development in response to the light environment (Furuya, 1993). *PHYA* mRNA is inherently unstable, with an apparent half-life of ~1 hr (Seeley et al., 1992). In 4-day-old dark-grown oat seedlings, the rate of *PHYA* transcription is high, producing an abundant mRNA (Lissemore and Quail, 1988). Within minutes after a red light pulse, transcription of the *PHYA* gene stops and is followed by a rapid decrease in *PHYA* mRNA levels as a result of the short half-life of the *PHYA* mRNA.

Gel blot analysis of total RNA from dark-grown oats reveals the presence of the ~4.2-kb full-length message and low molecular weight *PHYA* RNA fragments. The *PHYA* RNA fragments are distributed throughout a size range from 4.2 kb (full length) to less than 200 nucleotides, with the exception of the positions of the rRNAs where the *PHYA* fragments are displaced by the large mass of rRNA (Seeley et al., 1992; Byrne et al., 1993). These fragments were shown to be produced in vivo by three lines of evidence: (1) four different total RNA isolation methods yield RNA that contains extensive *PHYA* RNA fragments; (2) other mRNAs analyzed in the same oat total RNA samples are intact, lacking detectable fragments of less than full length; and (3) two in vitro–synthesized, radiolabeled RNAs, a 2.7-kb fragment of *PHYA* and a 1.4-kb fragment of β-tubulin, were subjected to RNA isolation with oat total RNA; both remained intact (Seeley et al., 1992). In addition, the RNA fragments that hybridize to *PHYA* probes are observed in RNA isolated from a polysome-based oat in vitro degradation system (Byrne et al., 1993). Because *PHYA* mRNA is highly abundant and unstable, it seems likely that these fragments are in vivo–produced degradation products of the full-length message (Seeley et al., 1992).

We have analyzed the size distribution of the putative *PHYA* RNA degradation products to investigate the mechanism of *PHYA* mRNA degradation. Possible degradation pathways were identified by hybridizing different *PHYA* RNA probes to total, poly(A)$^+$, poly(A)$^-$, and polysomal RNA from dark-grown oat seedlings. The 5′ and 3′ termini of *PHYA* mRNA were analyzed for possible products of initial degradation events. The *PHYA* poly(A) tail length was determined to see if a correlation existed between tail length and degradation.

RESULTS

PHYA mRNA Appears To Be Degraded, in Part, by a 5′→3′ Exoribonuclease

RNA probes, derived from five different regions of the *PHYA* mRNA, were used to hybridize to RNA gel blots of poly(A)$^+$, total, poly(A)$^-$, and polysomal RNA from 4-day-old dark-grown oat seedlings, as shown in Figure 1. The five probes spanned the majority of the *PHYA* mRNA (Figure 1A). The hybridization pattern of *PHYA* RNA degradation products detected with each probe was used to investigate the mechanism of *PHYA* mRNA degradation. This type of analysis should reveal the activity of exoribonucleases, both 3′→5′ and 5′→3′. Also, a site-specific endoribonuclease activity would be implicated if discrete *PHYA* RNA bands were observed.

The *PHYA* hybridization patterns in poly(A)$^+$ RNA were consistent with a 5′→3′ exoribonuclease activity (Figure 1B). The size of the smallest fragment detected with each probe corresponded to the distance between the 3′ terminus of the full-length *PHYA* mRNA and the 3′ end of the region complementary to that probe. The fact that there was a large amount of *PHYA* RNA fragments significantly smaller than the 4.2-kb full-length mRNA while containing a poly(A) tail long enough to be poly(A$^+$) selected indicates that degradation occurred prior to poly(A) tail removal.

A presumed non-*PHYA* 1.6-kb RNA was detected in poly(A)$^+$ RNA with probe 1 (data not shown). This RNA was too small to be a polyadenylated *PHYA* RNA fragment and to hybridize to probe 1. Higher stringency washes (75°C final wash) preferentially removed the radioactivity associated with this 1.6-kb RNA (Figure 1B), suggesting that this band resulted from a nonspecific interaction of probe 1 with another polyadenylated mRNA.

The *PHYA* hybridization patterns in total RNA differed from that in poly(A)$^+$ RNA (Figure 1C). With the exception of the two regions of little hybridization at ~3.8 kb and ~2.0 kb, corresponding to the positions of the extremely abundant 25S and 18S oat rRNAs, each probe revealed a continuous distribution of *PHYA* fragments that ranged in size from the 4.2-kb full-length transcript to less than 200 nucleotides. The region at ~2.4 kb (Figures 1C, 1D, and 1E) that appears to be a "band" we interpret to be a collection of *PHYA* RNA fragments confined by the rRNAs at both larger (25S) and smaller (18S) sizes. This *PHYA* hybridization pattern is indistinguishable from that previously observed (Seeley et al., 1992) in which the *PHYA* fragments were concluded to be produced in vivo. The

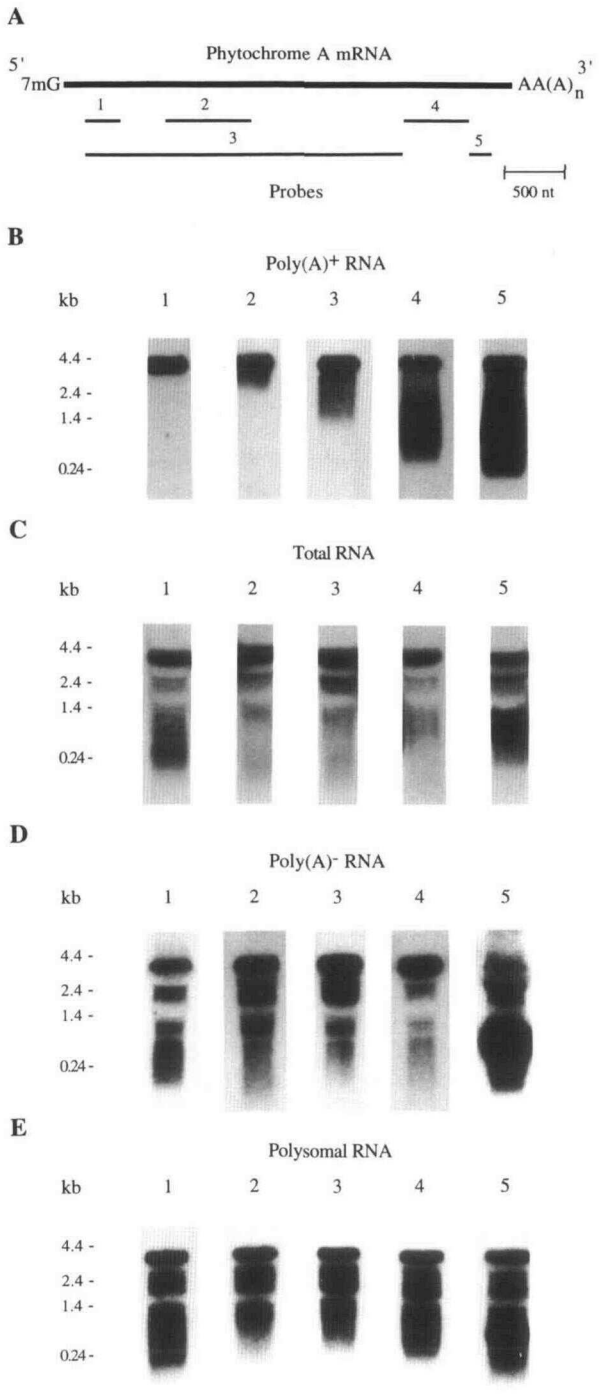

Figure 1. RNA Gel Blot Analyses of *PHYA* RNA Fragments in Dark-Grown Oat Seedlings.

(A) Diagram showing the region of hybridization for each of the five *PHYA* RNA probes. The length corresponding to 500 nucleotides (nt) is indicated. 7mG, 7-methylguanosine cap; $AA(A)_n$, poly(A) tail.
(B) Poly(A)$^+$ RNA, 1 μg per lane. To remove nonspecific hybridization, the blot in lane 1 was washed at 75°C during the final step with no SSC in the wash buffer.
(C) Total RNA, 10 μg per lane.
(D) Poly(A)$^-$ RNA, 10 μg per lane.
(E) Polysomal RNA, 5 μg per lane.
The lane numbers (1 to 5) correspond to the numbered probes in **(A)** used to detect the *PHYA* RNA fragments. Each type of RNA was electrophoresed in the same 1% agarose–3% formaldehyde gel to minimize migration variability. Blots were exposed to film for varying lengths of time (5 to 70 hr) so that the full-length (4.2 kb) band was of approximately equal intensity. RNA molecular length markers are indicated at left. Data represent two independent experiments.

hybridization patterns in total RNA were consistent with simultaneous 5′→3′ and 3′→5′ exoribonuclease activities. A discrete 1-kb RNA band was detected with probe 5 (Figure 1C, lane 5). This RNA comigrated with an ethidium bromide–stainable band, assumed to be an organellar rRNA. This band was not detected in the poly(A)$^+$ RNA (Figure 1B). Total RNA from 10-hr light-treated oat seedlings contained an equal amount of this 1-kb RNA as compared to dark-grown oat seedlings (data not shown). A 1-kb *PHYA* RNA fragment that hybridizes to probe 5 would be predicted to also hybridize to probe 4. A discrete 1-kb band was not observed in total, poly(A)$^+$, or poly(A)$^-$ RNA with probe 4 (Figure 1). The radioactive signal in this 1-kb band was also preferentially reduced upon high-stringency washes (data not shown). Together, these data strongly suggest that the 1-kb band was from nonspecific interaction with probe 5 and a highly abundant rRNA and not the detection of a *PHYA* degradation product. The nonspecific interaction of probe 5 with the 1-kb rRNA makes it difficult to be certain of the hybridization pattern of *PHYA* RNA fragments detected with this probe in total RNA. However, probe 4, immediately 5′ of probe 5, detects *PHYA* RNA fragments consistent with 5′→3′ and 3′→5′ exoribonucleases (Figure 1C, lane 4).

Poly(A)$^-$ RNA and polysomal RNA had *PHYA* hybridization patterns (Figures 1D and 1E) similar to those in total RNA (Figure 1C). The apparently full-length *PHYA* mRNA band was detected in poly(A)$^-$ RNA with all five probes. Full-length *PHYA* mRNAs deficient in poly(A) tails would be expected if the poly(A) tail was removed prior to the degradation of the body of the message. Deadenylation prior to degradation would suggest a degradation pathway distinct from the putative 5′→3′ exoribonuclease degradation of polyadenylated *PHYA* mRNA (Figure 1B). Again, the non-*PHYA* 1-kb rRNA was detected with probe 5. In poly(A)$^-$ RNA, the radioactivity at the position of this 1-kb band increased in relation to the *PHYA* mRNA signals, as compared to the signals detected in total RNA (Figure 1C). This observation is consistent with the conclusion that this 1-kb band is a nonpolyadenylated rRNA and not a *PHYA* degradation product. As in total RNA, detection of this rRNA interferes with the analysis of *PHYA* RNA fragments, making it difficult to be certain of the *PHYA* hybridization pattern with probe 5 (Figure 1D, lane 5). The *PHYA* RNA hybridization patterns in polysomal RNA were distinct from those in poly(A)$^+$ RNA (Figure 1B); thus, it seems likely that polysome-associated *PHYA* mRNA is a mixture of poly(A)$^+$ and poly(A)$^-$ messages.

In the polysomal RNA, as compared to total and poly(A)$^-$ RNA, there was an enrichment relative to the full-length *PHYA* mRNA for fragments detected with all five probes. This suggests that *PHYA* mRNA degradation occurred on polysomes. No discrete sizes of *PHYA* degradation products, which would implicate a site-specific endoribonuclease, were observed (*Figure* 1).

In Vitro-Synthesized *GUS* mRNA and β-Tubulin mRNA Are Not Degraded during Poly(A)$^+$ Isolation

The *PHYA* RNA hybridization patterns in poly(A)$^+$ RNA were consistent with a 5′→3′ exoribonuclease activity (Figure 1B). However, these poly(A)$^+$ *PHYA* RNA fragments might have been produced during the poly(A) selection by poly(U) Sephadex column chromatography. Figure 2 shows the results from two experiments that test this possibility. Radioactively labeled 1.9-kb β-glucuronidase (*GUS*) mRNA was synthesized in vitro and added to nonradioactive oat total RNA; the mixture was then poly(A) selected. The in vitro–synthesized *GUS* mRNA had a poly(A) tail with 30 adenylate (A) residues (Higgs and Colbert, 1993). *GUS* mRNA was analyzed with RNA gel blots in samples from the total RNA before selection and in poly(A)$^+$ RNA after selection (Figure 2A). In both the total and the poly(A)$^+$ RNA, *GUS* mRNA was predominantly full length. Poly(A)$^+$ RNA, the identical sample used to analyze *PHYA* mRNA (Figure 1B), was blotted and analyzed for β-tubulin mRNA (Figure 2B). Unlike *PHYA* mRNA, β-tubulin does not contain detectable low molecular weight fragments, indicating that at least one endogenous mRNA species is intact after poly(A) selection. The lack of exogenous *GUS* mRNA and endogenous β-tubulin mRNA degradation suggests that the *PHYA* mRNA was not degraded during poly(A) selection. These observations plus the evidence supporting in vivo production of the *PHYA* fragments in total RNA (Seeley et al., 1992) imply that the polyadenylated *PHYA* RNA fragments (Figure 1B) were produced in vivo.

Analysis of the *PHYA* mRNA 5′ Terminus for an Initial Degradation Event

Determining the initial events in the putative *PHYA* mRNA 5′→ 3′ exoribonuclease degradation is important for characterizing this pathway and how it is regulated. Two likely possibilities exist for an initial step. First, the 5′ cap might be hydrolyzed by either the putative 5′→ 3′ exoribonuclease or by a separate decapping enzyme (Stevens, 1988). Second, an endoribonuclease might cleave *PHYA* mRNA near the 5′ terminus, removing the protective cap and exposing the *PHYA* mRNA to the putative 5′→ 3′ exoribonuclease, similar to the degradation of some yeast mRNAs (Hsu and Stevens, 1993). RNase H was used to more accurately analyze the *PHYA* mRNA 5′ terminus to determine if a site-specific endoribonuclease

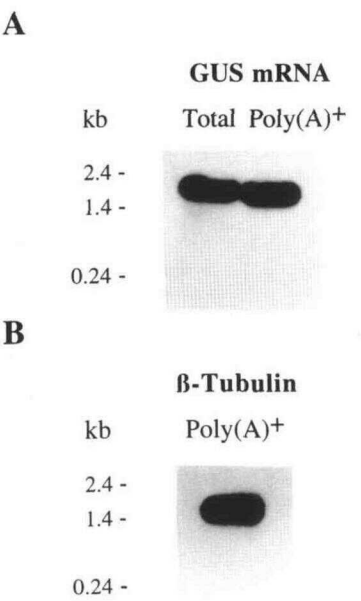

Figure 2. Poly(A)$^+$ Selection Does Not Degrade in Vitro–Synthesized, Labeled *GUS* mRNA or Endogenous β-Tubulin mRNA.

(A) In vitro–synthesized, ^{32}P-labeled *GUS* mRNA (1.9 kb) was analyzed for degradation before and after poly(A) selection. The labeled *GUS* mRNA was added to 5 mg of oat total RNA, and the mixture was poly(A) selected. Equal counts per min (1200) of *GUS* mRNA were loaded in the total and poly(A)$^+$ lanes of a 1% agarose–3% formaldehyde gel, electrophoresed, and then blotted to a nylon membrane. The blot was exposed to film for 24 hr. RNA molecular length markers are indicated at left. Data represent two independent experiments performed before and after the isolation of poly(A)$^+$ *PHYA* mRNA, as shown in **(B)**, with the same poly(U) Sephadex column.
(B) Poly(A)$^+$ RNA (1 μg) was blotted in a 1% agarose–3% formaldehyde gel and hybridized with the 1.4-kb β-tubulin RNA probe. The blot was exposed to film for 1 hr. Data represent two independent experiments.

cleaves *PHYA* mRNA near the 5′ terminus. This method of analysis has previously been used to identify endoribonucleolytic cleavages (Stoeckle and Hanafusa, 1989; Brown and Harland, 1990; Tanzer and Meagher, 1994).

RNase H cleaves only the RNA strand in a DNA/RNA hybrid. A DNA oligomer was synthesized to hybridize 890 nucleotides from the 5′ terminus of the *PHYA* mRNA. Cleavage of the *PHYA* mRNA in this hybrid should produce an 890-nucleotide 5′ product and a 3.3-kb 3′ product. The smaller size of the 5′-terminal product and the increased resolution of the 3% agarose gel improved our ability to detect site-specific endoribonuclease cleavages within ~400 nucleotides of the 5′ terminus. In Figure 3, the size of the smaller 5′ product was estimated in an RNA gel blot with probe 1. This probe was used because it would specifically detect the smaller 5′ cleavage product. Any product smaller than the expected size would presumably correspond to an in vivo degradation product. A discrete in vivo degradation product might result from

either a site-specific endoribonuclease or a stalled 5′→3′ exoribonuclease.

The expected *PHYA* RNase H cleavage product was detected only when both RNase H and the oligomer were present with total RNA from dark-grown oats (Figure 3A, lanes 3 to 6). In total, poly(A)$^+$, poly(A)$^-$, and polysomal RNA, a band of 888 ± 11 nucleotides was detected (Figures 3A and 3B). The size of this band corresponded to the expected 5′ cleavage product from the full-length *PHYA* mRNA (890 nucleotides). Smaller, discrete *PHYA* RNA bands were not evident, suggesting that no site-specific endoribonucleolytic cleavages occur at a detectable distance from the 5′ terminus of the *PHYA* mRNA. As a negative control, *PHYA* mRNA–depleted total RNA was analyzed. This RNA was isolated from 4-day-old dark-grown oat seedlings that were illuminated with 10 hr of continuous white light prior to RNA isolation. No RNase H cleavage product was detected in this RNA sample (Figure 3A, lanes 1 and 2).

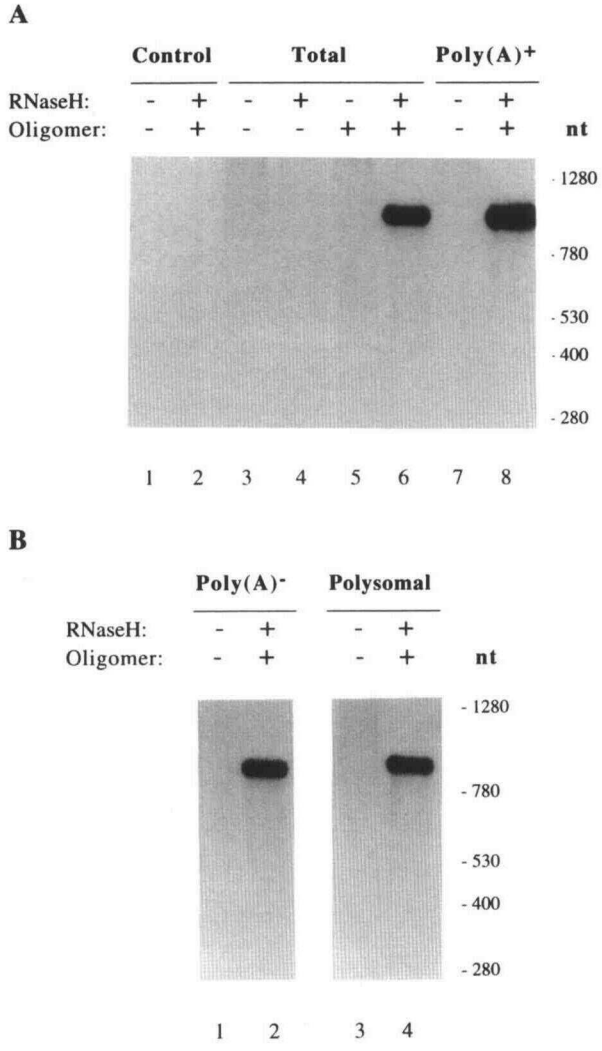

Figure 3. RNase H Analyses of the *PHYA* mRNA 5′ Terminus.

RNase H and a DNA oligomer (22 nucleotides [nt]) that hybridized 890 nucleotides from the full-length *PHYA* mRNA 5′ terminus were added (+) or not added (–) to samples. The conditions for RNase H cleavage are as described in Methods.

(A) Lanes 1 and 2 contain 10 μg of the negative control total RNA (*PHYA* mRNA depleted from 10-hr light-treated oat seedlings); lanes 3 to 6 contain 10 μg of total RNA; and lanes 7 and 8 contain 1 μg of poly(A)$^+$ RNA.

(B) Lanes 1 and 2 contain 30 μg of poly(A)$^-$ RNA and lanes 3 and 4 contain 5 μg of polysomal RNA.

After RNase H cleavage, samples were electrophoresed in a 3% NuSieve agarose–3% formaldehyde gel, blotted, and hybridized with probe 1 (Figure 1A). The blots shown in **(A)** and for poly(A)$^-$ RNA in **(B)** were exposed to film for 22 hr. The polysomal RNA blot in **(B)** was exposed to film for 19 hr. RNA molecular length markers are indicated at right in nucleotides. Data represent two independent experiments.

A Significant Amount of the *PHYA* mRNA Appears To Be Poly(A) Deficient

Further investigations into the presence of the apparently full-length *PHYA* mRNA in the poly(A)$^-$ fraction (Figure 1D) seemed pertinent, because poly(A) tail length and the removal of the poly(A) tail have been suggested as a means of controlling the rate of degradation of some mRNAs (Brewer and Ross, 1988; Gallie et al., 1989; Shyu et al., 1991). To test whether the apparently full-length *PHYA* mRNA in the poly(A)$^-$ fraction was poly(A) deficient (having no poly[A] tail or a poly[A] tail too short to hybridize to poly[U] Sephadex), as opposed to resulting from an inefficient selection of polyadenylated *PHYA* mRNA on the poly(U) Sephadex column, oat total RNA was repeatedly poly(A) selected. The poly(A)$^-$ fraction from the first poly(A) selection was passed through the column three additional times. Each time, RNA from the poly(A)$^-$ fraction was sampled for analysis. Poly(A)$^-$ RNA from each of the four passes was compared to the total RNA in an RNA gel blot with probe 3 as shown in Figure 4A.

After one pass through the poly(A)-selecting column, the poly(A)$^-$ fraction had an apparently full-length *PHYA* band that was 35.0 ± 6.5% of that in total RNA (Figure 4B). By the fourth pass, this value had decreased to 23.6 ± 1.3% of that in total RNA. Most likely, the decrease between the first and fourth pass was a result of the removal of contaminating poly(A)$^+$ *PHYA* mRNA from the poly(A)$^-$ fraction. The number of A residues present in the poly(A)$^-$ *PHYA* mRNA would presumably be less than 30, because an in vitro–synthesized *GUS* mRNA with a poly(A) tail of 30 A residues was efficiently selected and eluted in the poly(A)$^+$ fraction (Figure 2). Because about one-fourth of the total apparently full-length *PHYA* mRNA was poly(A) deficient, it seems likely that deadenylation is a factor in the mechanism of *PHYA* mRNA degradation. These data also imply that ~75% of the apparently full-length *PHYA* mRNA was polyadenylated.

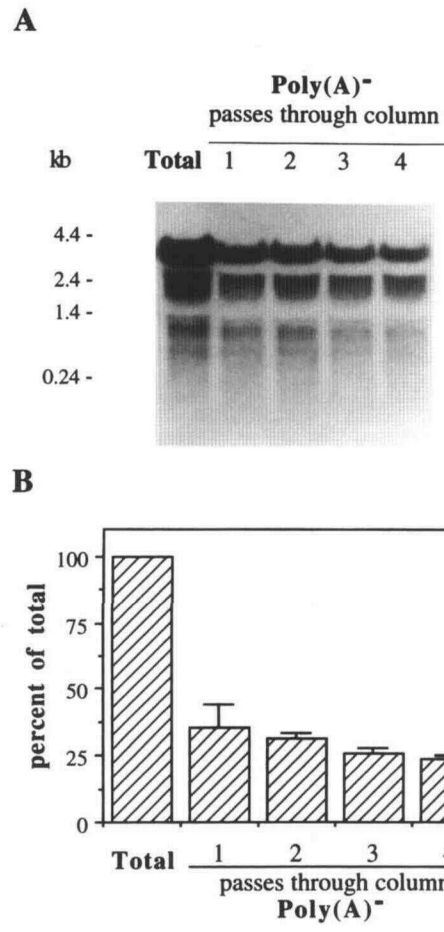

Figure 4. Estimating the Amount of Apparently Full-Length *PHYA* mRNA in the Poly(A)$^-$ Fraction.

(A) Total RNA (5 mg) was poly(A) selected with a poly(U) Sephadex column. The poly(A)$^-$ fraction was collected (one pass), and poly(A) was reselected. Again, the poly(A)$^-$ fraction was collected (two passes). This was repeated two more times to obtain the third- and fourth-pass poly(A)$^-$ fractions. Ten micrograms from the original total RNA and from each of the poly(A)$^-$ fractions (one to four passes) was analyzed in a blot and hybridized with probe 3 (Figure 1A). The blot was exposed to film for 15 hr. Molecular length markers are indicated at left.

(B) The amount of *PHYA* full-length band in each lane was quantified by excising it from the blot in **(A)** and determining the amount of radioactivity by scintillation spectroscopy. The amount of radioactivity is indicated as a percentage of the total (2323 counts per min for the blot in **[A]**). The mean of two independent experiments was determined, and error bars indicate the standard deviation.

To determine if other oat mRNAs have large amounts of poly(A)-deficient full-length messages, H4 histone, β-tubulin, and actin mRNAs were also analyzed as shown in Figure 5. The abundance of each mRNA in total and poly(A)$^-$ RNA, after four passes through a poly(U) Sephadex column (Figure 4A), was determined in RNA gel blots (Figure 5A). The percentage of total mRNA that is poly(A) deficient for each mRNA species was calculated (Figure 5B). Unlike histone mRNAs in mammalian cells, the oat H4 histone mRNA is polyadenylated (D.H. Byrne and J.T. Colbert, unpublished data) as are histone mRNAs in other plant species (Chaubet et al., 1988). All three of these mRNAs have a significantly lower percentage of poly(A)-deficient apparently full-length molecules than does the *PHYA* mRNA.

Analysis of the *PHYA* mRNA Poly(A) Tail Length and 3′ Terminus

RNase H was used to estimate the length of poly(A) tails and to determine if any site-specific endoribonucleolytic cleavages occurred in the *PHYA* mRNA 3′ untranslated region. A 24-

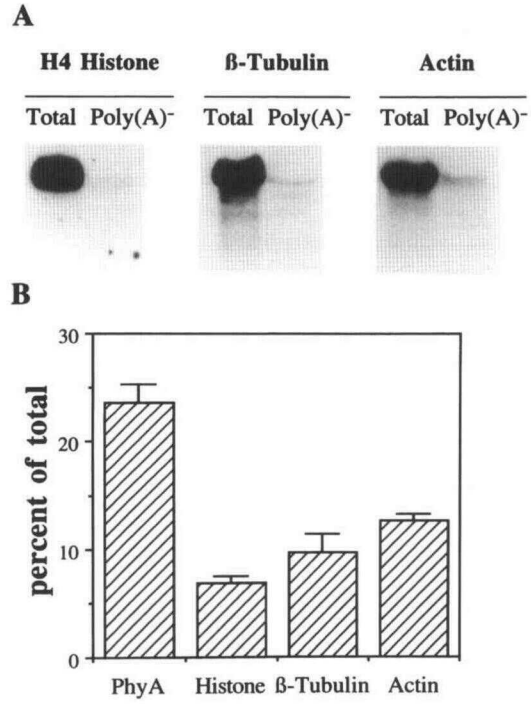

Figure 5. Estimating the Amount of Apparently Full-Length Message in the Poly(A)$^-$ Fraction for Additional Oat mRNAs.

(A) Total RNA (10 μg) and poly(A)$^-$ RNA (10 μg) after four passes through a poly(U) Sephadex column were blotted; hybridized with RNA probes for H4 histone, β-tubulin, and actin; and exposed to film for 48 (H4 histone), 10 (β-tubulin), and 4 hr (actin).

(B) The amount of each mRNA in the total and poly(A)$^-$ lanes in **(A)** was quantified by excising the full-length band and determining the amount of radioactivity by scintillation spectroscopy. The amount of poly(A)-deficient mRNA for each species is presented as a percentage of the respective total RNA. From the blot in **(A)**, the counts per min in total RNA were 305, 1260, and 3424 for H4 histone, β-tubulin, and actin, respectively. The data for *PHYA* mRNA were taken from Figure 4, fourth pass. The data are the means from two independent experiments, and error bars indicate the standard deviation.

nucleotide DNA oligomer was synthesized to hybridize 390-nucleotide 5′ of the start of the *PHYA* mRNA poly(A) tail. Cleavage of the mRNA with RNase H would produce a 3.6-kb 5′ product and smaller 3′ product, variable in size (~390 to 600 nucleotides) depending upon the length of the poly(A) tail. The size of the 3′ cleavage product was determined by RNA gel blot analysis with probe 5. This probe was chosen because it would specifically detect the small 3′ cleavage product. Because the poly(A) tail would be a significant portion of the small 3′ product, the length of the tail could be accurately estimated. To further confirm the presence of the poly(A) tail and to identify the size of the deadenylated *PHYA* RNA 3′ product, oligo(dT) was included in some of the RNase H digestions to remove the poly(A) tail. The expected sizes of the polyadenylated 3′ products would be 390 nucleotides plus the length of the poly(A) tail, and the deadenylated 3′ product would be 390 nucleotides.

Total, poly(A)$^+$, poly(A)$^-$, and polysomal RNA from 4-day-old dark-grown oat seedlings were subjected to RNase H cleavage and analysis, as shown in Figure 6. In addition, the same negative control (*PHYA* mRNA depleted total RNA) was used in this analysis as was used in the RNase H analysis of the *PHYA* mRNA 5′ terminus (Figure 3). Two bands of ~475 and ~580 nucleotides were detected in the negative control RNA (Figure 6A, lanes 1 and 2). These bands are presumed to be non-*PHYA* RNA. This result was not surprising because probe 5 repeatedly bound to a 1-kb rRNA (Figure 1). Detection of these non-*PHYA* bands was dependent upon RNase H digestion, indicating that the 3′ *PHYA* oligomer also bound to this rRNA (data not shown). These same non-*PHYA* bands were observed in total RNA (Figure 6A, lanes 6 and 7) and poly(A)$^-$ RNA (Figure 6B, lanes 2 and 3) from dark-grown oats.

PHYA RNase H cleavage products of the expected size range (~390 to 600 nucleotides) were observed only when the oligomer and RNase H were both included in the digestion reaction with total RNA (Figure 6A, lanes 3 to 7). The addition of oligo(dT) to the reaction reduced the signal intensity in the region between ~400 to 600 nucleotides and increased the signal intensity at a deadenylated band of 386 ± 6 nucleotides, as was expected. In total, poly(A)$^+$, and polysomal RNA, the *PHYA* mRNA poly(A) tails were heterogeneous in size (Figures 6A, lanes 6 and 9 and 6B, lane 4). The poly(A)$^-$ RNA contained a *PHYA* band at 395 ± 9 nucleotides and no detectable poly(A) tail, as expected (Figure 6B, lanes 2 and 3).

The length of the poly(A) tails was estimated by subtracting the size of the deadenylated cleavage product, as was determined in samples that included oligo(dT) for each type of RNA, from the size of the adenylated cleavage products, as was determined in samples without oligo(dT). The estimated size ranges (minimum to maximum sizes) for the *PHYA* tails are as follows: total RNA, 14 ± 1 to 193 ± 8 nucleotides; poly(A)$^+$ RNA, 24 ± 4 to 219 ± 7 nucleotides; and polysomal RNA, 3 ± 3 to 210 ± 25 nucleotides. The minimum length of the poly(A) tail in polysomal RNA (~0 nucleotides) is significantly shorter than the minimum lengths estimated for total and poly(A)$^+$ RNA, whereas the maximum length in polysomal RNA is similar to the length in poly(A)$^+$ RNA, suggesting that polysomal RNA contains both adenylated and deadenylated *PHYA* messages.

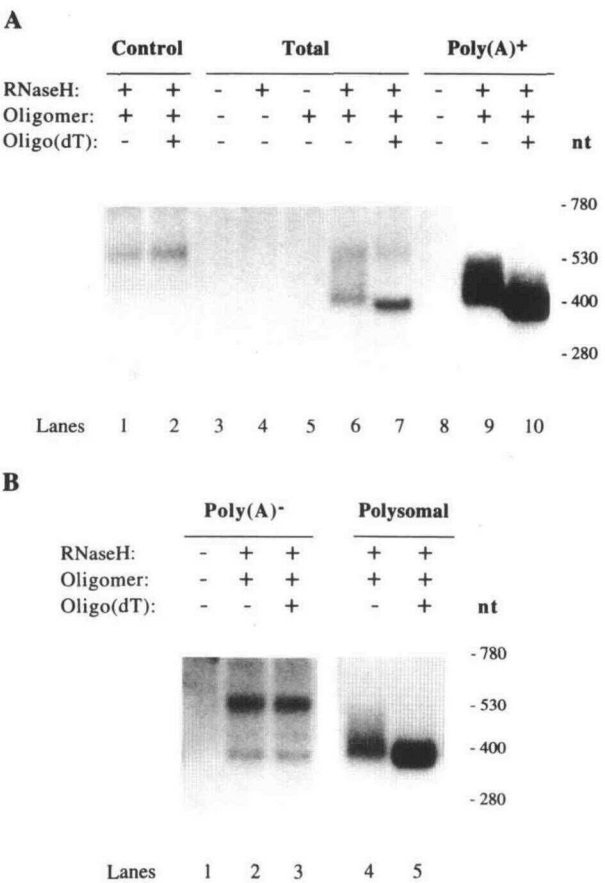

Figure 6. RNase H Analyses of the *PHYA* mRNA 3′ Terminus.

RNase H, the 24-nucleotide (nt) DNA oligomer (that hybridized 390 nucleotides from the start of the poly[A] tail), and oligo(dT)$_{12\text{-}18}$ were added (+) or not added (–) to the samples. The conditions for RNase H cleavage are as described in Methods.

(A) Lanes 1 and 2 contain 10 μg of the negative control total RNA (*PHYA* mRNA depleted from 10-hr light-treated oat seedlings); lanes 3 to 7 contain 10 μg of total RNA; and lanes 8 to 10 contain 1 μg of poly(A)$^+$ RNA.

(B) Lanes 1 to 3 contain poly(A)$^-$ RNA (30 μg) after four passes through a poly(U) Sephadex column, and lanes 4 and 5 contain polysomal RNA.

After RNase H cleavage, samples were electrophoresed in a 3% NuSieve agarose–3% formaldehyde gel, blotted, and hybridized with probe 5 (Figure 1A). The blots in both **(A)** and **(B)** were exposed to film for 16 hr. RNA molecular length markers are indicated at right in nucleotides. The data represent two independent experiments.

In all of these RNase H analyses, the *PHYA* RNA 3′ untranslated region appeared to be intact. Aside from the variable length of the poly(A) tail, no additional *PHYA* RNA fragments were detected. If *PHYA* mRNA was cleaved by a 3′ site-specific endoribonuclease, then discrete degradation intermediates

less than 390 nucleotides in size might have been detected in these RNase H experiments. No such intermediates were evident.

DISCUSSION

The majority (~75%) of the apparently full-length *PHYA* mRNA was polyadenylated. A comparison of the putative degradation products observed when poly(A)$^+$ RNA was hybridized with RNA probes, derived from five different regions of the *PHYA* mRNA, indicated that polyadenylated *PHYA* mRNA was extensively degraded prior to removal of the poly(A) tail. The hybridization patterns of polyadenylated *PHYA* RNAs were consistent with a 5′→3′ exoribonuclease activity (Figure 1B). Probes that hybridized toward the 5′ terminus of the *PHYA* mRNA detected full-length and near full-length *PHYA* RNA fragments. Probes that hybridized toward the 3′ terminus detected *PHYA* RNA fragments that ranged in size from full length to extensively degraded. The smallest detected fragment with each probe corresponded to the distance between the 3′ end of the region complementary to the probe and the 3′ terminus of the *PHYA* mRNA. The polyadenylated *PHYA* fragments appeared to be produced in vivo.

A 5′→3′ exoribonuclease activity would be expected to produce a continuous distribution of *PHYA* fragments that would correspond to the 890-nucleotide and smaller fragments in the 5′ terminus RNase H analysis (Figures 3A, lanes 6 and 8 and 3B, lane 4). Of these fragments, only those greater than ~400 nucleotides could be detected with probe 1, as a result of its small size and region of hybridization. Such degradation intermediates were not evident in the RNase H analysis of the 5′ terminus. One possible explanation for the inability to detect these fragments could be the higher resolution of the 3% agarose gels used in the 5′-terminal RNase H analyses (Figure 3), as compared to the 1% agarose gels used in the RNA gel blot analyses (Figure 1). Spreading the *PHYA* fragments over a larger region of nylon membrane would decrease probe hybridization per unit area of membrane and limit detection.

In addition to a 5′→3′ exoribonuclease, cleavage by a stochastic endoribonuclease and the subsequent selection of the polyadenylated 3′ products might also be consistent with the *PHYA* hybridization patterns observed in poly(A)$^+$ RNA (Figure 1B). A stochastic endoribonuclease activity might also explain the *PHYA* hybridization patterns detected in total and polysomal RNA (Figures 1C and 1E). Recently, a novel mRNA degradation mechanism that is proposed to involve a stochastic endoribonuclease has been reported for soybean *rbcS* mRNA (Tanzer and Meagher, 1994).

Discrete *rbcS* mRNA degradation products produced by the stochastic endoribonuclease are detected in RNA gel blots and RNase H analyses, indicating that this endoribonuclease has some sequence or structure specificity (Thompson et al., 1992; Tanzer and Meagher, 1994). Discrete *PHYA* degradation products that might have been produced by a stochastic endoribonuclease, similar to that reported for *rbcS* mRNA, were not detected in RNA gel blots or the 5′ and 3′ termini RNase H analyses of *PHYA*. In addition, RNA gel blots of *PHYA* mRNA with high resolution NuSieve agarose gels, similar to those used in the RNase H analyses (Figures 3 and 6), detected the continuous distribution of *PHYA* fragments but failed to detect discrete degradation products (data not shown). This does not rule out the possibility of a stochastic endoribonuclease activity, but a 5′→3′ exoribonuclease seems more likely to degrade *PHYA* mRNA because similar enzymes are reported to be involved in the degradation of plant mRNAs (Shimotohno et al., 1977) and other eukaryotic mRNAs (Furuichi et al., 1977; Stevens, 1980; Stevens and Maupin, 1987; Vreken and Raué, 1992).

The involvement of 5′→3′ exoribonucleases in mRNA turnover has recently been reviewed (Stevens, 1993). Such ribonuclease activities are reported in Xenopus oocytes and mouse L cells (Furuichi et al., 1977), yeast cells (Stevens, 1980; Vreken and Raué, 1992), human placental nuclei (Stevens and Maupin, 1987), and wheat germ extracts (Shimotohno et al., 1977). Two 5′→3′ exoribonucleases (*XRN1* and *HKE1/RAT1*) have been cloned from yeast cells (Larimer et al., 1992; Kenna et al., 1993). Both appear to be involved in mRNA degradation. The *XRN1* 5′→3′ exoribonuclease is reported to degrade yeast mRNAs after deadenylation and cap removal (Stevens and Maupin, 1987; Hsu and Stevens, 1993). In oat seedlings, the apparent 5′→3′ exoribonucleolytic degradation and the presence of a poly(A)-deficient *PHYA* mRNA are consistent with the results in the yeast study. However, *PHYA* mRNA degradation appeared to differ from that in yeast because significant amounts of *PHYA* mRNA appeared to be degraded prior to poly(A) tail removal (Figure 1B). This type of degradation pathway differs from other eukaryotic mRNAs that are reported to be deadenylated prior to degradation (Albrecht et al., 1984; Peltz et al., 1987; Brewer and Ross, 1988; Binder et al., 1989; Shyu et al., 1991; Larimer et al., 1992; Thompson et al., 1992; Vreken and Raué, 1992; Decker and Parker, 1993; Hsu and Stevens, 1993).

Discrete degradation products have been detected in eukaryotic mRNA populations and have been shown to be products of endoribonuclease cleavages (Albrecht et al., 1984; Binder et al., 1989; Stoeckle and Hanafusa, 1989; Brown and Harland, 1990; Vreken and Raué, 1992). Because discrete degradation products were not detected with RNase H analyses of the 5′ terminus, there is no evidence for a 5′-specific endoribonuclease in the degradation of *PHYA* mRNA. However, an endoribonuclease digestion, either within a few nucleotides of the cap or that produces a rapidly degraded cleavage product, cannot be ruled out. Ribonucleases that hydrolyze only the cap structure have been isolated from plants (Shinshi et al., 1976; Bartkiewicz et al., 1984). Once the cap is removed, then translation initiation should not occur on that message (Gallie et al., 1989). Such an event would render the apparently full-length *PHYA* mRNA nontranslatable, causing a more rapid reduction in protein synthesis after a light treatment than would occur if *PHYA* mRNA was degraded solely by a 3′→5′

exoribonuclease. Thus, there could be a regulatory advantage to the early degradation of the 5′ terminus of *PHYA* mRNA.

RNase H experiments were used to determine that the *PHYA* mRNA poly(A) tails were heterogeneous in size. *PHYA* mRNAs in poly(A)$^+$ RNA had poly(A) tails that ranged in size from ~24 to 220 nucleotides. In total RNA, the tails ranged in size from ~14 to 190 nucleotides, similar to poly(A)$^+$ RNA. Finally, in polysomal RNA, the poly(A) tails ranged in size from ~0 to 210 nucleotides. Poly(A) tails of other mRNAs have also been shown to be heterogeneous in size (Brewer and Ross, 1988; Baker et al., 1989; Baker, 1993). Interestingly, the minimum *PHYA* tail length in poly(A)$^+$ RNA (~24 nucleotides) was similar to the minimum size to which the poly(A) binding protein (PABP) will associate (Baker, 1993). Previously, the oat full-length *PHYA* mRNA was estimated to be 4.2 kb in size (Hershey et al., 1984). However, from the RNase H poly(A) tail data and the known length of *PHYA* cDNA clones (Hershey et al., 1985), the size of the polyadenylated full-length *PHYA* mRNA is calculated to be 4030 nucleotides.

RNase H and RNA gel blot experiments indicated that polysome-associated *PHYA* mRNA was a mixture of adenylated and deadenylated messages. Possibly, adenylated *PHYA* messages are recruited to ribosomes where deadenylation occurs, a pathway that has been reported for c-*myc* mRNA (Brewer and Ross, 1988). Oat polysomes, from which RNA was extracted for use in analyses presented here, have previously been used for cell-free in vitro mRNA degradation (Byrne et al., 1993). In addition, this polysome-based in vitro degradation system produces *PHYA* RNA fragments similar to those seen in vivo (Seeley et al., 1992). Thus, it seems likely that *PHYA* mRNA degradation is ribosome associated, as has been proposed for other eukaryotic mRNAs (Brewer and Ross, 1988; Pei and Calame, 1988; Savant-Bhonsale and Cleveland, 1992; Theodorakis and Cleveland, 1992; Pastori and Schoenberg, 1993; Tanzer and Meagher, 1994).

Some mRNAs in plants and yeast are thought to be destabilized because they include rare codons that cause the ribosome to stall during translation (Hoekema et al., 1987; Murray et al., 1991). A comparison of *PHYA* mRNA codon usage to the average codon usage of 207 plant genes (Murray et al., 1991) indicated that *PHYA* mRNA contains a distribution of codons that closely fits the average plant codon usage distribution (data not shown), suggesting that *PHYA* mRNA is not destabilized by this mechanism.

The observation of apparently full-length *PHYA* mRNAs lacking poly(A) tails was in agreement with a pathway of degradation in which mRNAs are deadenylated prior to degradation. Other oat mRNAs (H4 histone, β-tubulin, and actin) also had apparently full-length messages in the poly(A)$^-$ fraction, but the percentages were significantly lower than that for *PHYA* mRNA. Similar percentages of mRNA in the poly(A)$^-$ fraction have been reported for yeast (Hsu and Stevens, 1993). There appeared to be no correlation between the percentage of poly(A)-deficient messages and the mRNA half-life in the oat seedlings. *PHYA* mRNA has a reported half-life of ~1 hr (Seeley et al., 1992), and 23.6% of the apparently full-length mRNA was poly(A) deficient (Figure 4B). H4 histone has a half-life of ~1.5 hr (D.H. Byrne and J.T. Colbert, unpublished data), yet only 6.9% of the apparently full-length mRNA was poly(A) deficient. β-Tubulin and actin have reported half-lives of ~2 and ~3.3 hr, respectively (Byrne et al., 1993), and the percentage of poly(A)-deficient and apparently full-length mRNA was 9.7 and 13.6%, respectively. RNase H experiments confirmed that the *PHYA* mRNA in the poly(A)$^-$ fraction lacked a poly(A) tail of any detectable size. These experiments also indicated that the poly(A)-deficient *PHYA* 3′ terminus was relatively uniform in length. Because no evidence implicates a site-specific endoribonuclease cleavage of *PHYA* mRNA in the 3′ untranslated region, it seems more likely that an enzyme similar to poly(A) nuclease might exoribonucleolytically deadenylate *PHYA* mRNA, as occurs with yeast mRNAs (Baker, 1993; Sachs, 1993; Stevens, 1993). The high amount (23.6%) of apparently full-length poly(A)-deficient *PHYA* RNA would not be predicted to result from a stochastic endoribonuclease activity because a cleavage at the junction of the 3′ untranslated region and the poly(A) tail should be random and relatively infrequent. The observed *PHYA* poly(A) tails of heterogeneous sizes are consistent with a poly(A) nuclease-like activity.

We propose two tentative models for the degradation of *PHYA* mRNA. Both models include two pathways of *PHYA* mRNA degradation, one for polyadenylated messages (~75% of the population) and a second for poly(A)-deficient messages (~25% of the population). Model a involves processive exoribonucleases and model b involves stochastic endoribonucleases. Both models are diagrammed in Figure 7. In model a, the polyadenylated *PHYA* messages appear to be degraded by a 5′→3′ exoribonuclease. This degradation would be initiated by a decapping enzyme or an endoribonuclease cleavage within a few nucleotides of the 5′ cap. Subsequently, the 5′→3′ exoribonuclease would degrade the *PHYA* message by the hydrolysis of mononucleotides. The abundance of the *PHYA* RNA fragments in poly(A)$^+$ RNA was high, suggesting that a high percentage of *PHYA* messages are degraded by this pathway. This pathway appears capable of degrading the entire *PHYA* mRNA molecule to mononucleotides.

The second pathway of model a is involved in degrading the poly(A)-deficient *PHYA* mRNA. Given the heterogeneous size of the poly(A) tails detected in RNase H experiments, it seems likely that the 3′→5′ exoribonucleolytic removal of the poly(A) tail by a poly(A) nuclease-like enzyme initiates this pathway. The poly(A)-deficient *PHYA* mRNA appears to be completely degraded by a combination of a 5′→3′ exoribonuclease and a 3′→5′ exoribonuclease. From the *PHYA* hybridization patterns in total, poly(A)$^-$, and polysomal RNA, a single, unidirectional exoribonuclease (5′→3′ or 3′→5′) can be ruled out, because a continuous distribution of fragments is detected with each of the five probes (Figure 1). These exoribonucleases could conceivably degrade the same molecule or different molecules within the deadenylated pool. The method of cap removal and the 5′→3′ exoribonuclease are assumed in this model to be the same as that proposed for the degradation of polyadenylated *PHYA* mRNA.

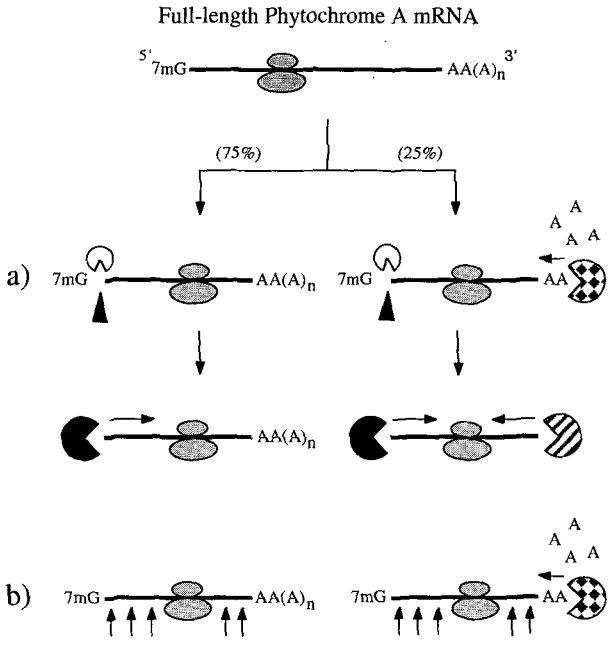

Figure 7. Two Tentative Models for the Degradation of *PHYA* mRNA in Oat Seedlings.

Ribosomal subunits are indicated by filled ovals. 7mG indicates the position of the 7-methylguanosine cap, and $AA(A)_n$ indicates the poly(A) tail. Both models a and b have a polyadenylated degradation pathway and a deadenylated degradation pathway. Distinct ribonuclease activities are indicated with the following patterns: decapping enzyme, open; 5′-terminal specific endoribonuclease, filled arrowhead; 3′→5′ poly(A) nuclease-like exoribonuclease, checkered; 5′→3′ exoribonuclease, filled; 3′→5′ exoribonuclease, diagonally hatched; stochastic endoribonuclease, vertical arrows.

Currently, there is only indirect evidence for a 3′→5′ exoribonuclease degradation of poly(A)-deficient *PHYA* mRNA. Frequently, deadenylation of an mRNA precedes degradation by a 3′→5′ exoribonuclease (Albrecht et al., 1984; Peltz et al., 1987; Brewer and Ross, 1988; Binder et al., 1989; Shyu et al., 1991; Theodorakis and Cleveland, 1992). It seems feasible that the poly(A)-deficient *PHYA* messages are degraded, in part, by a pathway similar to that reported for these mRNAs. Both pathways appear to be able to degrade *PHYA* mRNA while it is associated with ribosomes.

In model b, both polyadenylated and poly(A)-deficient *PHYA* mRNAs are cleaved by stochastic endoribonucleases while polysome associated. Degradation of polyadenylated *PHYA* mRNAs would be initiated by a stochastic endoribonuclease. The products of this initial cleavage would be subsequently degraded by combinations of stochastic endoribonucleases and exoribonucleases, eventually producing mononucleotides. The observed continuous distribution of *PHYA* RNA fragments might be explained by two mechanisms: (1) a stochastic endoribonuclease with very little or no specificity to internal *PHYA* mRNA sequence or structure; or (2) a stochastic endoribonuclease with some specificity quickly followed by rapid exoribonucleases, such that the immediate products of a stochastic endoribonuclease would be a very minor portion of the *PHYA* fragments.

We have identified possible mechanisms for the degradation of oat *PHYA* mRNA. Our data do not unequivocally distinguish between the two presented models of *PHYA* mRNA degradation. In our opinion, model a, the 5′→3′ exoribonucleolytic degradation, seems more likely because of the continuous distribution of *PHYA* fragments and the prevalence of reported exoribonucleases in the degradation of eukaryotic mRNAs (Stevens, 1993). In each model, *PHYA* mRNA degradation proceeds via both a polyadenylated pathway and a deadenylated pathway.

METHODS

RNA Isolation and Analysis

Four-day-old dark-grown oat (*Avena sativa* cv Garry) seedlings, excised below the mesocotyl node, were frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated with a small-scale RNA isolation procedure (Seeley et al., 1992). Poly(A)$^+$ and poly(A)$^-$ RNA samples were selected with a poly(U) Sephadex column (Bio-Rad) (Murray et al., 1981; Lissemore et al., 1987) from total RNA and prepared by a large-scale SDS-phenol procedure (Dean et al., 1985). Polysomal RNA was isolated as described by Byrne et al. (1993). RNA gel blots, hybridization with antisense RNA probes, and liquid scintillation spectroscopy were performed as previously reported (Cotton et al., 1990; Seeley et al., 1992).

RNase H Analyses of the 5′ and 3′ Termini

The procedure used in the RNase H experiments was adapted from that previously described by Brewer and Ross (1988). For analysis of the 5′ terminus of phytochrome A (*PHYA*) mRNA, a DNA oligomer of 22 nucleotides was synthesized (5′-dTGATTTCGGAGAGAATACC-TCA-3′) that hybridized 890 nucleotides from the 5′ terminus. In addition to RNA from dark-grown oat seedlings, negative control RNA from 4-day-old dark-grown oat seedlings treated with 10 hr of continuous white light prior to RNA isolation was analyzed. The RNA samples were incubated at 90°C for 8 min in a total volume of 20 μL of 1 mM EDTA, pH 8.0. The oligomer (2 μg) was added and annealed to the RNA for 15 min at 25°C. KCl was added to a final concentration of 50 mM, and annealing was continued for 15 min at 25°C. An equal volume of 56 mM $MgCl_2$, 40 mM Tris-hydrochloride, pH 8.0, was added, followed by 0.8 units of RNase H (Promega), and the reaction was incubated for 30 min at 37°C. RNA samples were phenol-extracted, ethanol-precipitated, and electrophoresed in a 3% NuSieve 3:1 (FMC, Rockland, ME) agarose–3% formaldehyde gel. RNA was blotted to a nylon membrane (GeneScreen; Du Pont) and hybridized with probe 1. The mean size of the cleavage product and the standard deviation for two experiments are presented.

For investigation of the 3′ terminus and poly(A) tail length, RNase H experiments were performed as indicated above, with the following exceptions: (1) 0.5 μg of a 24-nucleotide DNA oligomer (5′-dTCTGA-

CTGCTCCTTGTTCTCCTCC-3′) that hybridized 390 nucleotides from the start of the *PHYA* poly(A) tail was annealed to *PHYA* mRNA; (2) in the indicated samples (Figure 6), 0.3 μg of oligo(dT)$_{12-18}$ (Amersham Corp.) was added simultaneously with the oligomer; and (3) RNA gel blots were hybridized with probe 5. The mean ± SE of the means for the deadenylated sizes (as determined with a shorter exposure than that shown in Figure 6) and the minimum and maximum sizes of the poly(A) tails (as determined with a longer exposure than that shown in Figure 6) were estimated from two independent RNA isolates, and each RNA isolate was analyzed in two different experiments.

Plasmid Constructs and Probe Synthesis

The *PHYA* antisense RNA probes (1 to 5) were in vitro synthesized from partial oat cDNA clones. Fragments were subcloned from various oat *PHYA* parent cDNA clones including pGAP1.7 (Edwards and Colbert, 1990), pAP3.2 (Hershey et al., 1985), and a recently constructed full-length *PHYA* cDNA clone, pFLII (Barnes, 1994). RNA probes were synthesized by linearizing the cDNA template and polymerizing RNA with T7, SP6 (Promega), or T3 (Stratagene) RNA polymerase, according to the instructions of the manufacturer.

The plasmid construct for probe 1 was produced by inserting the 245-bp SacI-KpnI fragment from pFLII into the pBluescript KSII+ (Stratagene) plasmid. Probe 1 was synthesized from an EcoRI-linearized template with SP6 RNA polymerase. The plasmid construct for probe 2 was produced by inserting the 670-bp NcoI-EcoRV fragment into pBluescript KSII+. Probe 2 was synthesized from NcoI-linearized template with T7 RNA polymerase. The plasmid construction and synthesis of the 2.7-kb probe 3 have been previously reported (Seeley et al., 1992). The plasmid construct for probe 4 was produced by inserting the 525-bp XbaI fragment from pAP3.2 into the pGEM3 (Promega) plasmid. Probe 4 was synthesized from the BamHI-linearized template with SP6 RNA polymerase. The plasmid construct for probe 5 was produced by inserting the 164-bp EcoRI-XbaI fragment from pFLII into the pBluescript KSII+ plasmid. Probe 5 was synthesized from the SacI-linearized template with T3 RNA polymerase.

The human H4 histone genomic clone, pHh4A-SP1 (Peltz et al., 1987), was EcoRI linearized, and the antisense RNA probe was synthesized with SP6 RNA polymerase. The 1.4-kb oat β-tubulin partial cDNA clone, pGβ1, was EcoRI linearized, and the antisense RNA probe was synthesized with T7 RNA polymerase (Colbert et al., 1990). An oat actin cDNA clone, pOA24 (Barnes, 1994), was isolated using a soybean actin probe (Shah et al., 1982). The pOA24 plasmid was HindIII linearized, and the antisense RNA probe was synthesized with T3 RNA polymerase.

The pSGUS template containing the β-glucuronidase (*GUS*) transcribed region was EcoRI linearized, and low-specific-activity ^{32}P-labeled, full-length sense mRNA was synthesized with SP6 RNA polymerase (Higgs and Colbert, 1993). This labeled, *GUS* mRNA was added to 5 mg of SDS-phenol–extracted oat total RNA prior to poly(A) selection (Lissemore et al., 1987).

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