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Identification of potential oat phytochrome A mRNA degradation mechanisms

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Identification of potential oat phytochrome A mRNA degradation mechanisms

by

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ABSTRACT

The phytochrome plant photoreceptor is important for perceiving the light environment and initiating plant developmental and environmental responses. After dark-grown oat seedlings are treated with light the phytochrome A (PHYA) mRNA abundance rapidly declines due, in part, to the inherent instability of the PHYA mRNA. Messenger RNA degradation is an important process in regulating eukaryotic gene expression. My primary goal was to investigate both the mechanism of oat PHYA mRNA degradation and factors involved in regulating oat PHYA mRNA degradation. An oat protoplast system was established to investigate its use as a quick and reliable system to study plant mRNA degradation. The reliability of the oat protoplast system to accurately estimate cytoplasmic mRNA degradation rates could not be unequivocally demonstrated, and therefore was not used to study PHYA mRNA degradation. The abundance and decay rates of distinct oat PHYA mRNAs were determined in RNA gel blots with gene-specific oligonucleotide probes. The PHYA3 mRNA makes up ~61% of the total PHYA mRNA pool in etiolated oat seedlings. Two distinct oat PHYA mRNAs (PHYA3 and PHYA4) both had short half-lives, similar to that shown for the average PHYA mRNA half-life. Similar mRNA decay rates suggests a conservation of degradation mechanisms for these unstable PHYA mRNAs. Sequence comparisons identified several highly conserved region within the 5' and 3' untranslated regions, making them candidates for PHYA mRNA instability determinants. Putative PHYA mRNA degradation products were detected with RNA gel blots and were used to investigate the mechanism of PHYA mRNA degradation. Polyadenylated RNA fractions contained a significant amount of PHYA mRNA degradation products while ~25% of the apparently full-length PHYA mRNA was poly(A)-deficient, and endoribonucleases do not appear to be involved in PHYA mRNA degradation. Overall, it appears that PHYA mRNA is degraded by two distinct pathways, one pathway degrades polyadenylated PHYA mRNAs by a 5' to 3' exoribonuclease and the second pathway appears to degrade deadenylated PHYA mRNAs by using both 5' to 3' and 3' to 5' exoribonucleases. Finally, proteins that interact with PHYA mRNA were investigated with ultra-violet light (UV) crosslinking experiments, and two putative RNA-binding proteins, 25-kD and 20-kD in size, were determined from oat total protein and EDTA-treated polysome extracts, respectively. These proteins were concluded to non-specifically interact with PHYA mRNA, reducing the likelihood of them being involved in PHYA mRNA degradation. Currently, a specific PHYA mRNA-binding protein has yet to be identified.
CHAPTER 1. GENERAL INTRODUCTION

Phytochrome

Phytochrome protein and photobiology

The ability of plants to perceive aspects of the light environment is critical to plant development and maturation. Primarily, light is important to the plant for energy production, and, secondarily, light is important for regulated morphological and physiological changes during plant development. At high intensities light can be damaging to the photosynthetic apparatus, increasing the importance of responding accurately to the light conditions (Demmig-Adams and Adams, 1992). Three plant photoreceptors have been identified: the red- and far-red-light absorbing phytochrome photoreceptor, the blue and near-ultra-violet-light (UV-A) absorbing cryptochrome photoreceptor, and the ultra-violet-light (UV-B) absorbing UV-B photoreceptor (Björn, 1994). Of these photoreceptors phytochrome and its mechanism of regulation is the furthest characterized. Phytochrome was first identified in experiments investigating the red-light inhibition of flowering on short-day plants (Parker et al., 1946), and in the well known experiments investigating red-light induction of Lactuca sativa L. (lettuce) seed germination (Borthwick et al., 1952; Borthwick et al., 1954).

Phytochrome is able to perceive both light abundance and light quality, and according to the light environment it initiates cellular signal-transduction pathways that eventually lead to changes in plant growth and physiology. Phytochrome is involved in the regulation of a large number of light-dependent growth and developmental responses throughout the life of a plant. Some key light responses shown to be regulated, in part, by phytochrome are the induction of seed germination, maturation of chloroplasts, synthesis of chlorophyll, regulation of plant height, and the induction of flowering (Smith, 1994). Placement of dark-grown (etiolated) seedlings in a light environment induces many physiological changes, termed de-etiolation. This de-etiolation is controlled, in part, by phytochrome. A de-etiolating light treatment induces phytochrome to alter the expression of genes important for photosynthesis (Furuya, 1993; Vierstra 1993a). Some genes, such as chlorophyll a/b binding protein (CAB), ferredoxin A (FedA), and ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) show an increase in expression following a light treatment (Tobin and Silverthorne, 1985; Kuhlemeier et al., 1987; Mösinger et al., 1988), and other genes, such as protochlorophyllide oxidoreductase (PCR), asparagine synthetase (AS), and the phytochrome gene itself show a decrease in expression following a light treatment due to the action of the phytochrome protein.
The phytochrome protein has a linear-tetrapyrole chromophore covalently attached via a thiol-ether linkage to a unique cysteine residue, producing the chromoprotein (Lagarias and Lagarias, 1989). In vivo the phytochrome holoprotein is a dimer of two chromoproteins, and is currently pictured as a Y-shaped structure with the 55-kD C-terminal domain involved in dimerization (Vierstra, 1993a). Additionally, phytochrome is posttranslationally modified by removal of the N-terminal methionine, N-acetylation of a serine residue, and possibly phosphorylation (Wong et al., 1986; Vierstra, 1993a). The phytochrome protein is soluble and primarily localized in the cytosol (Nagatani et al., 1988; Vierstra, 1993a), although, the possibility can not be ruled out that a small amount of undetectable nuclear-localized phytochrome exists and is biologically significant (Furuya, 1993). Upon light treatment phytochrome is conformationally changed from the Pr (red-light absorbing, non-active) form to the Pfr (far-red-light absorbing, active) form, followed by the induction of light responses (Borthwick et al., 1952; Litts et al., 1983; Vierstra and Quail, 1983). The Pr form in etiolated oat seedlings has absorption maxima at 666 nm (major peak) and 379 nm (minor peak) and the Pfr form has absorption maxima at 730 nm (major peak) and 400 - 402 nm (minor peak) (Litts et al., 1983; Vierstra and Quail, 1983). The two photointerconvertible forms (Pr and Pfr) provide a useful method of experimentally identifying phytochrome-regulated light responses. In the dark the non-active Pr form is synthesized. Red light (R) converts Pr to the active Pfr, inducing various light responses, while, R followed shortly by far-red light (FR) reconverts the Pfr back to the non-active Pr, preventing the induction of a given light response. This R followed shortly by FR (R/FR reversibility) is a key characteristic of phytochrome regulated responses. The exceptions to the R/FR reversibility are phytochrome responses that are induced by very low amounts of the active Pfr form. Such a responses are termed a very low fluence response (VLFR). Two examples of VLFRs are the increase in transcription of the CAB gene and the decrease in transcription of the phytochrome A (PHYA) gene in oats after a FR treatment. The small amount of Pfr produced by FR light is sufficient to induce the transcriptional changes (Lissemore and Quail, 1988).

Two distinguishable types of phytochrome protein exist within plants, a light-labile and light-stable type (Furuya, 1993). The non-active Pr form of the light-labile phytochrome protein is abundant in etiolated seedlings, and is stable with an estimated half-life of 100 hr (Quail et al., 1973b). The Pfr form of the light-labile phytochrome protein is unstable with an estimated half-life of 1 to 2 hr (Quail et al., 1973b; Pratt, 1978). So, in addition to inducing
light responses, R destabilizes the phytochrome protein, causing a rapid decrease in the abundance of the light-labile phytochrome. This posttranslational regulation occurs only with the light-labile phytochrome. The light-stable phytochrome protein does not decrease in abundance after a light treatment, although the initial abundance is low. In etiolated oat and pea seedlings the light-labile phytochrome is 50- to 100- fold higher in abundance than the light-stable phytochrome (Jabben and Deitzer, 1978; Hunt and Pratt, 1979; Konomi et al., 1987). In peas, after a light treatment the light-labile phytochrome decreases in abundance and results in the light-labile and the light-stable phytochromes having similar levels (Konomi et al., 1987). In light-grown oat seedlings the light-stable phytochromes are the dominant types, and the light-labile phytochrome is in exceedingly low abundance (Shimazaki and Pratt, 1985; Wang et al., 1991).

The Pr form is soluble and evenly distributed in the cytosol. After a light treatment and the production of the Pfr form, phytochrome rapidly aggregates and associates with membranes (Quail et al., 1973a). Initially, this aggregation was thought to be part of the signal transduction process, but currently is believed to be part of an intermediate step in the degradation of Pfr (Vierstra, 1993a). It appears that the aggregates contain ubiquitin-conjugated phytochrome, and the phytochrome protein degradation is ubiquitin-dependent (Vierstra, 1993b; Vierstra, 1994).

**Gene families and function**

The light-labile (etiolated-seedling predominant) and light-stable (green-plant predominant) types of phytochromes have been shown to be present in a number of phylogenetic groups including angiosperms (oats, rice, Arabidopsis thaliana, cucumber, tobacco and tomato), gymnosperms, bryophytes, and algae (Furuya, 1993; Vierstra, 1993a). The oat light-labile type is the phytochrome A protein (formerly termed type I or etiolated type). Phytochrome A is highly abundant in etiolated plants (Jabben and Deitzer, 1978; Hunt and Pratt, 1979), and because of this abundance it is the most studied of the phytochromes. Oats have been shown to contain three immunologically distinct phytochrome proteins with different molecular sizes (Wang et al., 1991). The etiolated predominant, light-labile phytochrome A is 124 kD, and at least two light-stable (formerly type II or green type) phytochromes exist and are 123 kD and 125 kD in size. In A. thaliana at least five distinct phytochrome genes exist. Again, phytochrome A protein is the light-labile type and phytochromes B, C, D, and E are the light-stable types present at constitutively low levels (Sharrock and Quail, 1989; Clack et al., 1994).
Among the phytochrome A proteins in monocotyledonous species (corn, oats, and rice) there is about 88% amino acid identity, and in the dicotyledonous species (A. thaliana, pea, and zucchini) there is about 79% amino acid identity (Sharrock and Quail, 1989). Between monocotyledonous and dicotyledonous species phytochrome A has a 63 to 64% amino acid identity. Within A. thaliana phytochrome A protein is 52% identical to phytochrome B, C, and D and 48% identical to phytochrome E, indicating that A. thaliana phytochrome A is more closely related to phytochrome A from other dicotyledonous and monocotyledonous species than it is to A. thaliana phytochrome B, C, D or E (Sharrock and Quail, 1989; Clack et al., 1994). Less is known about the sequence of the light-stable phytochromes. The A. thaliana phytochrome B amino acid sequence is 78% identical to pea PhyII (light-stable) and 72% identical to rice phytochrome B (Dehesh et al., 1991; Furuya, 1993). A. thaliana phytochrome B amino acid sequence is 52% and 56% identical to A. thaliana phytochrome C and E, respectively, but is 80% identical to phytochrome D, suggesting that B and D may have been produced from a recent gene duplication (Clack et al., 1994). Little is known about the phytochrome proteins in gymnosperms, but they do not appear to be highly homologous to any of the angiosperm phytochromes (Furuya, 1993).

In addition to angiosperms and gymnosperms, phytochrome has been cloned and sequenced from the algae Mesotaenium and Mougeotia, the bryophyte Ceratodon purpureus, the lycopod Selaginella, and the fern Adiantum (Hanelt et al., 1992; Thümmler et al., 1992; Winands et al., 1992; Furuya, 1993; Morand et al., 1993). As compared to the angiosperm A. thaliana, the fern Adiantum phytochrome has 54% identity with phytochrome A, 52% with phytochrome B, and 48% with phytochrome C, yet it has 70% identity to the lycopod Selaginella phytochrome. The bryophyte C. purpureus phytochrome gene (phyCer) has three exons. The first two exons have high sequence homology to phytochromes in angiosperms, but the third exon has low sequence homology to any of the reported phytochromes (Thümmler et al., 1992; Clack et al., 1994). The third exon is thought to encode a putative light-regulated protein kinase, and the phyCer is reported to have kinase activity (Algarra et al., 1993). The low sequence homology between the third exon of phyCer, the putative kinase domain, and any angiosperm phytochrome, indicates that such a kinase domain is not represented in the angiosperm phytochromes (Clack et al., 1994). Algal (Mesotaenium and Mougeotia) phytochromes have the highest amino acid identity, 74%, with the lycopod Selaginella phytochrome, 62 to 63% identity, respectively, with A. thaliana phytochrome B, and 50 to 52% identity, respectively, with A. thaliana phytochrome A (Morand et al., 1993).
In hexaploid oats the light-labile phytochrome A is encoded by a small gene family, adding yet another level of complexity to the phytochrome gene-family structure (Hershey et al., 1985a). At least four distinct genes (type 3, 4, 5, and 6) are transcribed to produce phytochrome A mRNAs (PHYA) each encoding the phytochrome A protein (Hershey et al., 1984; Hershey et al., 1985b). Three of these cDNAs (types 3, 4 and 5) have been sequenced, in part (Hershey et al., 1985b). The approximately 140 nt long 5' untranslated region (5' UTR) and the approximately 3.6 kb translated region have about a 98% nucleic acid sequence homology among these oat PHYA mRNA types, but the approximately 250 nt long 3' untranslated region (3' UTR) has only 34% sequence identity. The high identity in the 5' UTR suggest a possible role for the 5' UTR in oat PHYA mRNA regulation, either transcriptionally or post-transcriptionally (Bruce and Quail, 1990). Currently, the relative amount of each distinct type of PHYA mRNA in the total pool of etiolated oat PHYA mRNA is not known.

Little is known about the specific physiological roles of the different types (light-labile and light-stable) of phytochrome proteins. Experiments utilizing either genetic mutants of phytochrome or overexpression of phytochrome proteins in transgenic plants are the primary methods currently being used to assign specific roles to each type of phytochrome protein (Furuya, 1993). The correct interpretation of results from such experiments is not always clear. Overexpression of a phytochrome species from strong constitutive promoters in transgenic plants often produces unnaturally high protein levels and the accumulation of phytochrome proteins in cells or tissues that would not normally contain phytochrome, putting into question the physiological relevance of results from such experiments. The possibility of overlapping functions among the different types of phytochromes can make interpretation of studies involving phytochrome mutants more difficult. Regardless of these possible experimental complications, the assignment of regulatory roles to different phytochromes is beginning to occur.

Overexpression of oat phytochrome A protein in transgenic tomato (Boylan and Quail, 1989), tobacco (McCormac et al., 1991; Cherry et al., 1993; McCormac et al., 1993; Barnes, 1994), and A. thaliana (McCormac et al., 1993) indicates that a monocotyledonous phytochrome can be both expressed and biologically active in a dicotyledonous plant. In general, overexpression of phytochrome A increases seed germination in the dark and it reduces plant height, increases chlorophyll content, and increases anthocyanin content in green plants. From these experiments it also appears that the phytochrome A protein is involved in responding preferentially to FR light. Phytochrome A mutants in A. thaliana have been identified and characterized (Dehesh et al., 1993; Nagatani et al., 1993; Parks and Quail,
Etiolated seedlings of the phytochrome A mutants are not able to respond to continuous FR light, and are taller than wild-type plants after a FR treatment due to a long hypocotyl. Under continuous R or white-light these mutants have a wild-type short height, indicating that phytochrome A is not the key phytochrome involved in responding to R treatments for changes in hypocotyl length.

Overexpression of *A. thaliana* phytochrome B and rice phytochrome B proteins in transgenic *A. thaliana* plants has been used to investigate the role of phytochrome B in seed germination (McCormac et al., 1993) and hypocotyl length (Wagner et al., 1991). Both the monocotyledonous and dicotyledonous phytochrome B proteins increase the percent of seed germination in transgenic *A. thaliana* in the dark, and both phytochrome B proteins are most responsive to R. Dark-grown transgenic plants are equal in height to the wild-type *A. thaliana*, but when grown in white light the transgenic plants have a greatly reduce hypocotyl length as compared to wild type. This indicates that phytochrome B can conformationally photoconvert from the Pr to the Pfr form, as has been shown for phytochrome A (Wagner et al., 1991), and that the Pfr form is the biologically active form as was first shown for phytochrome A (Furuya, 1993). Mutants for the phytochrome B protein in *A. thaliana* (Somers et al., 1991; Reed et al., 1993) and cucumber (López-Juez et al., 1992) have been identified and characterized. In white light the *A. thaliana* phytochrome B mutants are taller than wild-type seedlings, resembling dark-grown seedlings, and are taller than wild-type plants at maturity. In addition, the petioles of phytochrome B mutants are longer than wild-type petioles (Reed et al., 1993). The long hypocotyl (lh) mutant in cucumber is deficient in phytochrome B (type II), and is phenotypically similar to the *A. thaliana* phytochrome B mutant, suggesting that the function of phytochrome B is conserved, at least among dicotyledonous species.

From these studies it is evident that the different types of phytochrome proteins perform different functions in responding to light. In general, the phytochrome A protein seems to be the key photoreceptor involved in de-etiolation responses to continuous FR treatments. This seems logical considering that phytochrome A protein is highly abundant in etiolated seedlings, as would exist while germinating underground, and that the soil acts as a R filter increasing the relative abundance of FR light (Smith, 1994). As an etiolated seedling approaches the soil surface the low amount of light enriched in FR would be detected by the highly abundant phytochrome A protein, and this would induce the de-etiolation response so that the emergence of the seedling into the light would be timed with the development of photosynthetically capable leaves. Phytochrome B is light stable and appears to be a key photoreceptor involved in responding to R induced de-etiolation. In addition, phytochrome B seems to be involved in
regulating plant height (shade avoidance) and other green plant responses as would be logically for a light-stable photoreceptor.

**Phytochrome gene regulation**

Phytochrome A protein decreases in abundance after a light treatment. It is estimated to be 50- to 100-fold more abundant in etiolated oat seedlings as compared to green seedlings (Vierstra, 1993a). The principle reasons for the decrease are the rapid degradation of the Pfr form of the protein and a rapid decline in the abundance of the *PHYA* mRNA. The mechanism of this down regulation of phytochrome gene expression differs among plant species. After a R treatment *PHYA* mRNA abundance in the monocotyledonous species corn (Christensen and Quail, 1989), barley (Colbert *et al*., 1991; Rahim, 1992), oats (Gottman and Schäfer, 1982; Colbert *et al*., 1983; Colbert *et al*., 1985), and rice (Kay *et al*., 1989) decreases rapidly due to a dramatic decrease in transcription and an inherently unstable mRNA (Seeley *et al*., 1992; Vierstra, 1994). After a R treatment transcription of the *PHYA* genes is rapidly down-regulated in oats (Lissemore and Quail, 1988) and rice (Kay *et al*., 1989) seedlings. In oat seedlings a saturating R pulse causes a significant decrease in the amount of *PHYA* transcription within 5 min, transcription is reduced to approximately 15% of dark-grown seedlings by 15 min after the R pulse, and less than 5% of dark-grown seedlings by 1 hr after R (Lissemore and Quail, 1988). Similar results where shown for rice *PHYA* gene transcription (Kay *et al*., 1989), and the rice *PHYA* mRNA also has a dramatic decreases in abundance by 3 hr after a R pulse or in continuous white light (Dehesh *et al*., 1991). Analysis of the oat *PHYA* type 3 promoter (*PHYA3*) has identified two positive cis-acting elements, upstream of the transcription start site, that increase the amount of transcription (Bruce and Quail, 1990) and a negative cis-acting light repressor element that functions to decrease the amount of transcription upon light treatment (Bruce *et al*., 1991). DNA-binding proteins have been identified that specifically interact with these functionally important cis-acting elements in the oat *PHYA3* promoter (Bruce *et al*., 1991), and a trans-acting factor that binds to one of the positive-elements has been cloned (Jorge *et al*., 1994). After an induced transcription termination by R the oat *PHYA* mRNA rapidly decreases with a ~1 hr half-life to 98% reduction in abundance (Colbert *et al*., 1985). Oat seedlings treated with the RNA synthesis inhibitor cordycepin in the dark also have an estimated mRNA half-life of ~1 hr, indicating that the *PHYA* message is inherent unstable with respect to light in etiolated oat seedlings (Seeley *et al*., 1992). In addition to transcriptional regulation and mRNA degradation, oat phytochrome A protein degradation is regulated and this greatly influences the expression. The
Pr form of oat phytochrome A protein has an estimated half-life of 100 hr (Quail et al., 1973b), and the Pfr form has an estimated half-life of 1 to 2 hr (Quail et al., 1973b; Pratt, 1978). The combination of all these levels of regulated expression leads to a very rapid down-regulation of expression for the oat phytochrome A protein after a light treatment.

In some dicotyledonous species the down-regulation of phytochrome A after a R treatment appears to be controlled more at the translational and post-translational levels since the light-labile phytochrome A is greatly reduced upon light treatment yet the PHYA mRNA in these species shows only a slight reduction in mRNA abundance. Zucchini seedling PHYA mRNA decreases to only 60% of the dark control after a R treatment and 40% of the dark control after a continuous white-light treatment (Lissemore et al., 1987). In cucumber cotyledons PHYA mRNA has a transient decrease in abundance, reaching a minimum level that is 20% of the dark control after 3 hr in continuous white light, and returning to approximately 50% of the dark control after 12 hr in continuous white light (Tirimanne and Colbert, 1991).

Potato PHYA mRNA is 2- to 5-fold less abundant in light-grown shoots verses dark-grown shoots (Heyer and Gatz, 1992). A R treatment produces only a slight decrease in PHYA mRNA abundance in tomato, yet the immunologically detectable phytochrome A protein decreases considerably (Sharrock et al., 1988; Boylan and Quail, 1989). Six-day-old etiolated tobacco seedlings have an approximately 50% reduction in PHYA mRNA abundance after a 4 hr continuous white-light treatment (Barnes, 1994), but the phytochrome A protein declines very rapidly, with an estimated half-life of 5 to 10 min, to less than 10% of dark-grown seedlings (Cherry et al., 1991). The PHYA mRNA in A. thaliana has a small reduction in abundance after R treatment (Sharrock and Quail, 1989; Clack et al., 1994). Tobacco seedling PHYA mRNA is light-down regulated at 1, 2, and 3 days after germination, apparently at the transcriptional level, but by 7 days there is very little light-induced PHYA mRNA reduction, indicating a developmental regulation (Adam et al., 1994). Finally, pea is a dicotyledonous species that shows a significant decline in the abundance of the PHYA mRNA after a R treatment. Overall pea PHYA mRNA decreases to approximately 15% of the dark control after a R treatment (Sato, 1988; Tomizawa et al., 1989). Three distinct pea PHYA mRNAs have been identified and they are all three synthesized from the same gene, most likely through a regulated initiation at alternate transcription start sites, producing mRNAs with heterogenous 5' ends (Sato, 1988). After a R treatment the smallest and most abundant pea PHYA mRNA species shows a rapid decrease in abundance, the second and intermediately sized PHYA mRNA species is constitutively low in abundance, and the third and longest PHYA mRNA species shows a slight increase but is low in abundance (Sato, 1988). Interestingly, the PHYA
mRNAs from *A. thaliana*, pea, and zucchini contain open reading frames in the 5' region upstream of the predicted phytochrome A protein translation start sites, yet *PHYA* mRNA from none of the monocotyledonous species contain upstream open reading frames (Sharrock and Quail, 1989). Similar upstream open reading frames are reported to be involved in translational regulation (Mueller and Hinnebush, 1986), suggesting that similar types of translational regulation might occur for the dicotyledonous phytochrome A proteins (Sharrock and Quail, 1989).

The light-stable phytochromes are constitutively expressed with respect to light (Furuya, 1993). The light-stable oat phytochrome B proteins (123 kD and 125 kD) are constitutively expressed in oat seedlings (Wang et al., 1991), and in rice shoots the *PHYB* mRNA remains at a constant level after a R or FR treatment (Dehesh et al., 1991). Interestingly, the light-grown rice plants contain similar levels of *PHYA* and *PHYB* mRNA due to the drastic decrease in the amount of *PHYA* mRNA and the constitutively low amount of *PHYB* mRNA. *A. thaliana PHY B, C, D,* and *E* mRNAs show no changes in abundance after R or continuous white-light treatments (Sharrock and Quail, 1989; Clack et al., 1994), and the phytochrome B protein level also does not change in abundance after 6 or 24 hr of continuous white light (Somers et al., 1991). Results similar to the constitutive phytochrome B *A. thaliana* expression occur in cucumber seedlings (López-Juez et al., 1992).

Phytochrome A protein and mRNA have been localized in oat and pea seedlings. In oats the protein (Pratt and Coleman, 1974) and the mRNA (Seeley and Colbert, 1992) localization patterns are similar. The oat coleoptile tip and the mesocotyle node contain high amounts of phytochrome A protein and mRNA, and the immature leaf contains relatively low amounts of both. In contrast, barley and rye seedlings have a considerable amount of phytochrome A protein in the basal region of the immature leaf and a low amount in the coleoptile tip (Pratt and Coleman, 1974). Corn seedlings have an even distribution of phytochrome A protein throughout the shoot (Pratt and Coleman, 1974), and etiolated pea seedlings have a high abundance of phytochrome A protein and mRNA in the apical hook region (Tomizawa et al., 1991). Two mechanisms of regulation might produce these varying patterns of expression within seedlings. Transcriptional or post-transcriptional regulation could produce the cell-specific expression of phytochrome. The fact that the *PHYA* mRNA half-life is short might indicate that transcriptional regulation produces the pattern of expression observed in oats (Seeley and Colbert, 1992).
Eukaryotic mRNA Stability

Impact on gene expression

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; Sullivan and Green, 1994). The significance of regulated gene expression in plant growth and development is well documented (Kuhlemeier et al., 1987; Okamura and Goldberg, 1989; Edwards and Coruzzi, 1990). In both soybean embryos and mouse-L-cells, there is a poor correlation between the rates of transcription initiation and mRNA abundance for a number of genes, indicating that post-transcriptional regulation is both a commonly used process for modulating eukaryotic gene expression and a process that has a large impact on gene expression (Cameiro and Schibler, 1984; Walling et al., 1986). In eukaryotic cells cytoplasmic mRNA levels often correlate more closely with the transcript stability than they do with the amount of transcription (Peltz et al., 1991; Binder et al., 1994). In addition to affecting the mRNA abundance mRNA stability also affects the length of time it takes for an mRNA to come to a steady-state level after a change in transcription (Green, 1993). This is advantageous to genes that require rapid changes in expression because a short-lived mRNA will come to a steady-state level much quicker than a long-lived mRNA. Short-lived messages are commonly utilized with genes that respond rapidly to environmental or hormonal signals, such as the mammalian cell-regulated histones (Marzluff and Hanson, 1993), certain mammalian protooncogenes (Brewer and Ross, 1988), the auxin responsive soybean small-auxin-up-RNAs (SAURs) (Franco et al., 1990), and the oat PHYA mRNA (Seeley et al., 1992). The average polyadenylated mRNA half-life in soybean suspension cells is estimated to be 30 hr (Silflow and Key, 1979), and similar half-life values of 10 to 20 hr were estimated for the average mammalian polyadenylated mRNA (Hargrove and Schimdt, 1989; Peltz et al., 1991). The stability of eukaryotic mRNAs varies greatly, some mRNA half-lives range from as short as 10 min to as long as 500 hr, depending upon the gene and growth conditions (Brawerman, 1993; Green, 1993).

On a practical level mRNA stability and understanding the regulation of eukaryotic mRNA stability have been shown to be important in a variety of situations. The Bacillus theringiensis insecticidal crystal protein (Bt-toxin) introduced into transgenic plants has insufficiently low level of expression that is believed to be due, in part, to the instability of the Bt-toxin mRNA (Murray et al., 1991). Large-scale modification of the Bt-toxin mRNA, removing putative destabilizing elements, resulted in the greatly increased expression of the Bt-
toxin (Parlak et al., 1991). The useful technique of down-regulating gene expression by the production of antisense RNA in plant cells can occur through a variety of mechanisms (Smith et al., 1993). One mechanism of antisense-induced, sense-mRNA down regulation is through the destabilization of the sense mRNA (Jiang et al., 1994). Presumably destabilization is due to the formation of the double-stranded mRNA complex in vivo. Finally, the mammalian protooncogene c-myc mRNA is unstable with a half-life of approximately 15 min (Peltz et al., 1991). The naturally occurring rearrangement and expression of the c-myc gene produces a truncated mRNA lacking the 5' UTR. The mutated mRNA is three- to eight-fold more stable than the wild-type c-myc mRNA. This increase in mRNA abundance and subsequently increase in the c-myc protein abundance appears to induce the commonly observed cancer that is manifested as Burkitt's Lymphomas in humans (Peltz et al., 1991).

**mRNA stability determinants**

Efforts have been made to identify cis-acting sequences (cis elements) that are involved in regulating mRNA 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; Shyu et al., 1991; Theodorakis and Cleveland, 1992; Sachs, 1993; Nanbu et al., 1994). The majority of these cis elements have been shown to be destabilizing elements. Several cis elements that destabilize plant mRNAs have been identified (Newman et al., 1993; Ohme-Takagi et al., 1993).

The sequence AUUUA (A+U rich element or ARE) was first identified in the mammalian granulocyte-macrophage colony stimulating factor (GM-CSF) 3' UTR as a cis-acting instability determinant (Peltz et al., 1991). An ARE was added to the 3' end of the stable β-globin mRNA, and the chimeric Globin-ARE message was unstable. Commonly AREs are found in multiple repeats in the 3 UTR of unstable mammalian protooncogene mRNAs such as c-fos and c-myc. The AUUUA sequence and not just the high A+U content is necessary for mRNA destabilization (Peltz et al., 1991; Ohme-Takagi et al., 1993). In tobacco plants 11 repeats of the mammalian ARE inserted into the 3' UTR function to significantly destabilize both globin and β-glucuronidase mRNAs, indicating that some mRNA degradation mechanisms are conserved among eukaryotes (Ohme-Takagi et al., 1993). The presence of A+U rich regions have been noted in a few endogenous plant mRNAs, but the half-lives nor the effect of the A+U rich regions on the half-lives have not been determined (Green, 1993; Ohme-Takagi et al., 1993). The Bt-toxin mRNA is very A+U rich and contains putative AREs
that are thought to destabilize the mRNA in plants (Murray et al., 1991; Parlak et al., 1991). Cytoplasmic proteins that specifically interact with the ARE have been identified in mammals (Bohjanen et al., 1991; Gillis and Malter, 1991; Vakalopoulou et al., 1991). The function of these binding proteins in mRNA degradation is not known. It has been postulated that in mammalian cells the ARE in the 3' UTR might compete with the poly(A) tail for binding of the poly(A)-binding-protein (PABP), a protein that in mammals binds to and protects the poly(A) tail from degradation (Brewer and Ross, 1988). Removal of PABP from the poly(A) tail could increase the rate of poly(A) tail degradation, leading to the destabilization of the ARE-containing mRNA.

The small-auxin-up-RNAs (SAURs) are a conserved set of plant mRNAs that are rapidly induced by the hormone auxin and have among the shortest reported half-lives for any plant mRNA, 10 to 50 min (McClure and Guilfoyle, 1989, Franco et al., 1990). The 3' UTR of SAURs contain a unique and conserved region, termed DST due to its down-stream location. This DST sequence appears to be a 3' UTR instability determinant because the 3' UTR insertions of two tandem repeats of a synthetic DST destabilizes both the β-glucuronidase and the globin messages (Newman et al., 1993). Recent evidence suggests that the coding region from the soybean SAUR mRNA contains a cis-element that is involved in translation-dependent destabilization, but estimates of mRNA decay rates have not been done to confirm this observation (Li et al., 1994).

Stem-loops in the 3' UTR have been shown to be important for the degradation of some cytoplasmic mRNAs, primarily the human non-polyadenylated histone mRNAs and transferrin receptor mRNA (Harford, 1993; Marzluff and Hanson, 1993). In histone mRNAs the 3' terminus has a stem-loops instead of the standard eukaryotic mRNA poly(A) tail. The 3' stem-loop appears to act similar to the poly(A) tail in stabilizing the mRNA, but it has also been shown to act as a stability-regulating element when DNA synthesis is inhibited, as would normally occur after S-phase has ended in the cell cycle (Levine et al., 1987). In the G1 and S phases of the cell cycle, histone mRNA is stable, but in G2 phase histone mRNA is destabilized. This post-transcriptional regulation greatly influences the rapid decrease in histone mRNA upon entering the G2 phase (Harris et al., 1991). The stability of a chimeric mRNA containing the globin 5' UTR and coding region and a histone 3' UTR is regulated similar to histone mRNAs, indicating that the 3' stem-loop acts to regulate the mRNA degradation and not just to stabilize the message (Levine et al., 1987).

The human transferrin receptor mRNA contains a series of stem-loops in the 3' UTR, collectively termed the iron regulatory element (IRE) (Klausner and Harford, 1989; Koeller et
The transferrin receptor protein functions in the uptake of iron. The IREs regulate the transferrin receptor mRNA stability according to the cellular iron concentration so as to maintain the necessary level of internal iron (Harford, 1993). Under low iron the mRNA is stable, increasing the uptake of iron, and under high iron the mRNA is unstable, decreasing the uptake of iron. The IREs have been shown to be binding sites for mRNA stability-regulating proteins (IRE-BP). Under low iron conditions IRE-BP binds to the 3' stem-loops. The bound IRE-BP is thought to protect the transferrin receptor mRNA from a site-specific endoribonuclease that cleaves within the IRE (Harford, 1993).

The unstable c-fos and c-myc human protooncogene mRNAs contain multiple instability determinants (Shyu et al., 1991; Wisdom and Lee, 1991). Both of these mRNAs contain the destabilizing ARE in the 3' UTR, and a second destabilizing element within the coding regions. The c-fos and the c-myc coding-region instability determinants are unique and function independently of the ARE element, implying two possible pathways of degradation for each of these mRNAs. A 75-kD protein binds to c-myc coding-region instability determinant and regulates the rate of mRNA degradation. It is thought that this protein blocks an endoribonucleolytic cleavage site and thereby stabilizes the mRNA when the 75-kD protein is bound (Bernstein et al., 1994). In addition, the removal of the c-myc 5' UTR leads to an increase in the stability of this mRNA, suggesting that yet a third instability determinant resides in the 5' UTR (Rabbitts et al., 1985). The yeast STE3 mRNA also contains both coding-region and 3' UTR instability determinants, indicating that organisms other than mammals have mRNAs with multiple cis-elements (Heaton et al., 1992).

Recently, the porcine urokinase-type plasminogen activator (uPA) mRNA was reported to contain multiple instability determinants within the large 3' UTR (Nanbu et al., 1994). The uPA mRNA half-life is estimated to be 70 min (Altus et al., 1987). A 3' UTR ARE element was identified and shown to be responsible, in part, for the uPA mRNA instability (Nanbu et al., 1994). In addition a stem-loop in the uPA 3' UTR was also shown to destabilize the uPA mRNA. Finally, a third independent cis-element in the 3' UTR was postulated to also destabilize the uPA mRNA (Nanbu et al., 1994). It is not known if additional destabilizing cis-elements exist in the uPA mRNA coding region or 5' UTR.

The β-tubulin instability determinant appears to function through a unique mechanism of destabilization. The abundance of free β-tubulin subunits influences the expression of mammalian β-tubulin (Theodorakis and Cleveland, 1992). This regulated expression is controlled post-transcriptionally by changes in the mRNA stability, and the β-tubulin mRNA is destabilized by a small coding-region instability determinant (Yen et al., 1988).
thirteen translated nucleotides are necessary and sufficient for mRNA destabilization, and alterations within the first 13 nucleotides that maintain the wild-type amino acid sequence also maintain the wild-type destabilizing activity of this cis-element. In addition the mRNA destabilization also requires translation elongation. From these data it is postulated that the first four amino acids of the nascent peptide are key to the mRNA destabilization and not the mRNA sequence (Theodorakis and Cleveland, 1992).

The redundancy of instability determinants within the c-myc, c-fos, SAURs, and uPA mRNAs shows the complexity of eukaryotic mRNA degradation, since, presumably, each cis-element functions through a distinct pathway of degradation. Currently, the process through which a cis-acting sequence destabilizes an mRNA has been postulated for only a few transcripts, and the complete mechanism of action has not been determined for any instability determinant. In addition to instability determinants, conditions that reduce the rate of translation elongation also appear to destabilize some mRNAs. Messages from both yeast and plants are reported to be destabilized because of a rare codon usage, causing a decrease in the rate of translation and an increase in the rate of mRNA degradation (Hoekema et al., 1987; Murray et al., 1991). The expression of antisense RNAs has been shown to destabilize sense mRNAs (Jiang et al., 1994), and the presence of a naturally occurring antisense RNA has been reported in barley (Rogers, 1988) and maize (Dolfini et al., 1993), suggesting that antisense-induced sense-mRNA destabilization might be an endogenous regulatory mechanism. From all the above mentioned instability determinants it is clear that the regulation of eukaryotic mRNA stability is complex, yet there appears to be some conservation in mechanisms of eukaryotic mRNA degradation.

The majority of eukaryotic mRNAs contain both a 5' cap structure and a 3' poly(A) tail. The roles of these two structures are still under investigation. The 5' cap is important for both mRNA stabilization (Furuichi et al., 1977; Shimotohno et al., 1977; Sachs, 1993) and translation initiation (Callis et al., 1987, Gallie et al., 1989). The poly(A) tail also affects mRNA stability (Peltz et al., 1991; Sachs, 1993) and translation efficiency (Gallie, 1993). Poly(A)-binding proteins (PABP) have been identified in a number of organisms (Baker, 1993, Sachs, 1993) including plants (Belostotsky and Meagher, 1993; Hilson et al., 1993). In mammalian cells the PABP is thought to bind and protect the poly(A) tail from ribonucleases (Baker, 1993). In yeast cells the PABP appears to have a different role, and it is necessary to work cooperatively with poly(A)-nuclease (PAN) to remove the poly(A) tail (Sachs and Deardorff, 1992).
Aside from the 5' cap and the 3' poly(A) tail, little is known about cytoplasmic mRNA stabilizing elements. Unlike nuclear encoded eukaryotic mRNAs, prokaryotic and organellar (chloroplastic and mitochondrial) mRNAs commonly have mRNA stabilizing elements (Gruissem and Schuster, 1993; Higgins et al., 1993). These stabilizing elements are frequently inverted repeats that form stem-loops. The mRNA stabilizing stem-loops prevent processive exoribonucleases from degrading the message. Comparison of in vitro and in vivo analyses indicate that prokaryotic and organellar proteins bind to the stem-loop structures, increasing the stability of the stem-loop and enhancing the effectiveness of these elements. At this point it is difficult to know if prokaryotic-like stabilizing elements are present or even functional in cytoplasmic mRNAs, but conceptually it seems possible that cytoplasmic mRNAs could contain general cis-elements that interact with trans-acting factors to stabilize messages.

*Cytoplasmic mRNA degradation pathways*

To date, the majority of the reported mRNA degradation pathways are initiated by the removal of the poly(A) tail, either by a 3' to 5' exoribonuclease (poly(A) nuclease-PAN) or an endoribonuclease cleavage near the 3' terminus, followed by the degradation of the body of the message (Peltz et al., 1991; Sachs, 1993; Sullivan and Green, 1994). The mammalian mRNAs, β-globin (Albrecht et al., 1984), c-myc (Brewer and Ross, 1988), and apolipoprotein II (Binder et al., 1989), that have been investigated for a mRNA degradation pathway are initiated by removal of the poly(A) tail then hydrolysis of the body of the message by a 3' to 5' exoribonuclease. The non-polyadenylated human H4 histone is initiated by endoribonucleolytic removal of the 3' stem-loop followed by 3' to 5' exoribonucleolytic digestion of the body of the message (Peltz et al., 1987). Poly(A) tail removal is reported to be a first step in the degradation of a variety of yeast mRNAs followed by the hydrolysis of the 5' cap (Vreken and Raué, 1992; Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994). After deadenylation and 5' cap hydrolysis, these yeast mRNAs are then degraded by a 5' to 3' exoribonuclease. Frequently, an endoribonuclease cleavage in the 3' UTR or less frequently in the 5' UTR are early events in the degradation of mRNAs (Binder et al., 1989; Stoeckle and Hanafusa, 1989; Brown and Harland, 1990; Stoeckle, 1992; Brown et al., 1993; Pastori and Schoenberg, 1993; Binder et al., 1994). Very recently the yeast phosphoglycerate kinase mRNA (PGKI) was shown to be degraded by two different pathways (Muhlrad and Parker, 1994). In the first pathway the polyadenylated PGKI mRNA is decapped then degraded by a 5' to 3' exoribonuclease prior to the shortening of the poly(A) tail, and in the second degradation pathway the PGKI mRNA is deadenylated by PAN and then degraded by...
a 3' to 5' exoribonuclease. Endoribonucleolytic cleavages do not appear to be involved in either of the degradation pathways for the PGK1 mRNA. The soybean rbcS mRNA is reported to be degraded by a stochastic endoribonuclease early in the pathway followed, most likely, by the exoribonucleolytic degradation of the RNA fragments (Thompson et al., 1992; Tanzer and Meagher, 1994). Many discrete bands of rbcS RNA degradation products are detected in RNA gel blots, indicating that the putative stochastic endoribonuclease would have some sequence or structure specificity and would not produce truly random cleavages (Tanzer and Meagher, 1994). The rbcS RNA degradation products might also have been produced by simultaneous 3' to 5' and 5' to 3' exoribonucleases that stalled at specific sequences, generating distinct RNA fragments. The degradation products of rbcS mRNA lack a poly(A) tail, suggesting that deadenylation occurs prior to the degradation of the body of the rbcS message (Thompson et al., 1992). RNA-oligonucleotides are reported to exist in the vacuoles of cultured tomato cells (Abel et al., 1990). This suggests that the transport of RNA into the vacuole might occur at some point in a pathway of mRNA degradation. The vacuole contains 70 - 80% of all the plant ribonucleases (Green, 1994), and vacuolar targeting could be a step that occurs in the degradation of all plant mRNAs or for a subset of plant mRNAs. The direct involvement of RNA transport into the vacuole and vacuolar ribonuclease in the mechanism of cytoplasmic mRNA degradation has yet to be shown.

Some eukaryotic mRNAs have been shown to be degraded while associated with polysomes (Pei and Calame, 1988; Brewer and Ross, 1990; Byrne et al., 1993; Tanzer and Meagher, 1994). Many mRNAs require ongoing translation for mRNA instability, and commonly short-lived transcripts are stabilized when translation is inhibited (Franco et al., 1990; Altus and Nagamine, 1991; Koeller et al., 1991; Gil et al., 1994). This mRNA stabilization could either be due to a translation elongation-associated degradation mechanism or due to a necessary but labile degradation factor that requires constant synthesis to maintain the mRNA degradation activity. For the transferrin receptor mRNA a labile factor is the cause for mRNA stabilization by protein inhibition (Koeller et al., 1991). Translation inhibition can affect mRNA degradation for reasons other than the decrease in a labile degradation factor. For example, message-specific translation of the mammalian β-tubulin and yeast PGK1 mRNAs is necessary for the degradation of these specific messages (Theodorakis and Cleveland, 1992; Peltz et al., 1993). Both plant and animal in vitro polysome-based degradation systems have been shown to accurately reflect both degradation rates and mechanisms (Brewer and Ross, 1990; Byrne et al., 1993; Tanzer and Meagher, 1994). In addition, ribonucleases have been found to associate with polysomes. Exoribonucleolytic removal of the c-myc poly(A) tail
occurs on cell-free polysomes, implying that a PAN-like enzyme is polysome associated (Brewer and Ross, 1988). A nuclease that selectively degrades Xenopus albumin mRNA was found to associate with the assembled ribosomes but not the free subunits (Pastori and Schoenberg, 1993).

**Plant mRNA degradation**

The regulation of mRNA stability is as important to the control of plant gene expression as it is to other eukaryotic organisms (Green, 1993; Sullivan and Green, 1994). It has been suggested that plants might have a greater need for rapid regulation of gene expression than animals since they are unable to relocate during extreme environmental conditions, forcing plants to respond to environmental changes (Green, 1993). As previously mentioned, post-transcriptional regulation is commonly used to alter gene expression in plants (Walling et al., 1986), and the average soybean suspension cell mRNA half-life is estimated to be 30 hr (Silflow and Key, 1979). A number of plant mRNAs have been identified as being post-transcriptionally regulated. Frequently, this is determined by observing discrepancies between transcriptional run-off and mRNA abundance data. In some cases mRNA half-lives have been experimentally determined to confirm that mRNA stability has a regulatory effect.

The nuclear encoded \( rbcS \) mRNA is regulated at the transcriptional, mRNA stability, translational, and post-translational levels in response to light and development (Wanner and Gruissem, 1991). In potato \( rbcS \) mRNA is stabilized by light which leads to an increase in message abundance (Fritz et al., 1991). In immature soybeans \( rbcS \) mRNA abundance decreases in light-treated plants while transcription increases, a result that might be due to a decrease in the \( rbcS \) mRNA stability in light (Thompson and Meagher, 1990). In the same report, mature-soybean \( rbcS \) mRNA abundance increased considerably in response to light while transcription only marginally increased, suggesting that mature-soybean \( rbcS \) mRNA might be stabilized by light. In *A. thaliana* different \( rbcS \) genes are regulated by different stimuli. Some \( rbcS \) mRNAs are induced by R, via phytochrome, while other \( rbcS \) mRNAs are induced by blue light (Dedonder et al., 1993). In light-grown tobacco the \( rbcS \) mRNA half-life is estimated to be 5 hr (Jiang et al., 1994). In addition to light-regulated post-transcriptional control, duckweed (*Lemna gibba* G3) \( rbcS \) mRNAs show organ specific post-transcriptional regulation (Silverthorne and Tobin, 1990). In the photosynthetic fronds and the roots of duckweed certain \( rbcS \) genes have roughly equal rates of transcription initiation, as determined by transcription run-off experiments, but the mRNAs accumulate to high levels in the fronds while the roots have very low levels of these \( rbcS \) mRNAs. Differences in the stability of \( rbcS \)
mRNA in the frond versus the root might explain this post-transcriptional regulation, but rates of RNA processing or transport into the cytosol could also explain this organ-specific regulation.

Another light-regulated gene that has been studied with regard to mRNA stability is the \textit{CAB} message. \textit{CAB} gene transcription is induced by light via phytochrome (Lissemore and Quail, 1988). In potato the \textit{CAB} mRNA appears to be slightly less stable in the dark, as measured with the RNA synthesis inhibitor cordycepin (Romero and Lam, 1993). The \textit{CAB} mRNA half-life in light-grown tobacco plants is estimated to be 45 min (Jiang \textit{et al}., 1994). Oat seedling β-tubulin mRNA also has a light-regulated mRNA decay, with an estimated half-life of 125 min in the dark and 90 min after a R treatment (Byrne \textit{et al}., 1993). This is a slight but significant β-tubulin mRNA destabilization by the R treatment. A similar R destabilization of β-tubulin mRNA was also observed with a polosome-based oat \textit{in vitro} degradation system (Byrne \textit{et al}., 1993). In the same report the more stable actin mRNA was determined to have a half-life of 200 min in oat seedlings.

The nuclear encoded pea ferredoxin I (\textit{Fed-1}) mRNA is positively light regulated (Dickey \textit{et al}., 1992). In seedlings this \textit{Fed-1} mRNA light induction is mostly transcriptionally regulated (Gallo-Meagher \textit{et al}., 1992). In light-grown pea leaves the 4- to 5-fold light induction is due to mRNA cis-elements (Dickey \textit{et al}., 1992). This internal light regulatory element (iLRE) resides in the 5' portion of the \textit{Fed-1} message, and requires translation of the \textit{Fed-1} mRNA to cause light induction (Dickey \textit{et al}., 1994). Light-regulated mRNA stability is a likely explanation for the \textit{Fed-1} mRNA light induction, but alternative post-transcriptional processes have not been ruled out. Attempts to estimate \textit{Fed-1} mRNA half-life by the use of the transcriptional inhibitor actinomycin-D have lead to the artifactual stabilization of this mRNA (Dickey \textit{et al}., 1994), and alternative methods for estimating the mRNA turnover have not been reported. Interestingly, the \textit{Fed-1} mRNA from the cyanobacteria \textit{Synechococcus} has a post-transcriptional regulation pattern very similar to the pea \textit{Fed-1} mRNA except in response to iron (Bovy \textit{et al}., 1993). The 5' most 207 nucleotides are sufficient to confer iron-induced mRNA stabilization on a chimeric mRNA, and translation is required for this stabilization (Bovy \textit{et al}., 1993).

In suspension cultured cells of bean (\textit{Phaseolus vulgaris}) the proline-rich protein 1 (\textit{PvPRPl}) mRNA is down-regulated at the mRNA stability level by a fungal elicitor (Zhang \textit{et al}., 1993). Elicitor treated beans have a \textit{PvPRPl} mRNA half-life that is approximately three times less stable than in the non-treated control cells, as measured with actinomycin-D inhibitor experiments. The actinomycin-D treatment greatly reduced the rate of mRNA degradation.
either with or without elicitor treatment. Cells treated with fungal elicitor alone indicate that the destabilized \( \text{PvPRPI} \) mRNA has a half-life that is 45 min at the most, but this could be an overestimation since transcription might still be occurring (Zhang et al., 1993). The binding of a 50-kD protein to the 3' end of the \( \text{PvPRPI} \) mRNA is reported to be regulated by the cellular reduction/oxidation potentials because the addition of the reducing agent dithiothreitol (DTT) greatly influences binding of the 50-kD protein. The functional role of this 50-kD RNA-binding protein in the degradation of \( \text{PvPRPI} \) mRNA has not been determined (Zhang and Mehdy, 1994).

Finally, as previously mentioned the soybean SAURs are among the least stable known plant mRNAs (McClure and Guilfoyle, 1989; Franco et al., 1990). These mRNAs are rapidly induced by auxin, and have an estimated mRNA half-lives between 10 and 50 min. The lower time is estimated from non-transcription-inhibited experiments and the upper time is estimated from actinomycin-D inhibited soybeans, resulting in an artifically increased mRNA half-life (Franco et al., 1990). Translational inhibition causes a dramatic increase the abundance of the SAURs, termed super-induction. This super-induction has also been shown to occur with the \( \text{A. thaliana} \) SAUR-AC1 mRNA (Gil et al., 1994). It is not certain if auxin alters the stability of the SAURs, but, as previously mentioned, the DST instability determinant identified in the 3' UTR of SAURs destabilizes a GUS chimeric mRNA, but does not confer auxin regulation (Newman et al., 1993). This would suggest that the SAURs are inherently unstable. The DST element located in the 3' UTR was first singled-out as a candidate for an instability determinant because of its high sequence conservation among SAURs yet it is unique to these transcripts (McClure et al., 1989). Another message that is hormonally-regulated is the tomato fruit E17 message. This mRNA encodes a protein with an unknown function and is thought to be regulated at the mRNA stability level by ethylene (Lincoln and Fischer, 1988).

**Oat phytochrome A mRNA degradation**

The oat \( \text{PHYA} \) mRNA is unstable with an estimated half-life of approximately 1 hr (Seeley et al., 1992). Etiolated oat seedlings exposed to R or treated with cordycepin, an RNA synthesis inhibitor, in the dark and in the light have very similar initial rates of mRNA degradation, indicating that the mRNA is inherently unstable (Seeley et al., 1992). In an \textit{in vitro} polysome-based oat degradation system \( \text{PHYA} \) mRNA is degraded with a half-life of 45 min, very near the \textit{in vivo} estimated half-life (Byrne et al., 1993). RNA gel blot analysis of etiolated oat total RNA and polysome RNA reveals the full-length 4.2-kb \( \text{PHYA} \) message, plus a large amount of smaller than full-length \( \text{PHYA} \) RNA fragments (Seeley et al., 1992; Byrne et
These PHYA RNA fragments were shown to be in vivo produced, and are most likely degradation products, due to the high abundance of the message and rapid degradation (Seeley et al., 1992). The putative degradation products are associated with polysome fractions (Byrne et al., 1993), suggesting that the PHYA RNA fragments are originating in the cytoplasm and are not a result of premature transcription termination.

In etiolated oat seedlings the PHYA mRNA is primarily localized in the coleoptile and not in the immature leaf (Seeley and Colbert, 1992). The stability of PHYA mRNA has yet to be determined in organs or in species other than the coleoptiles of etiolated oat seedlings. It is formally possible that in different organs or cell-types of oats the PHYA mRNA has a longer or shorter half-life from that determined in the etiolated coleoptiles. Because the PHYA promoter is down-regulated by light, expression of PHYA mRNA from a constitutive promoter in transgenic plants would be required to estimate the mRNA half-life in organs and cell-types present in light-grown plants. Also, PHYA is a multi-gene family and at least four different types of PHYA mRNAs accumulate in etiolated oats as determined by DNA gel blot analysis and cDNA cloning (Hershey et al., 1985a; Hershey et al., 1985b). As mentioned previously, the different types of PHYA mRNAs have 98% nucleic acid homology except for the 3' UTR which has approximately 34% homology (Hershey et al., 1985b). The estimated 1 hr half-life is an average of all types of PHYA mRNAs since the probes used in RNA gel blots originated from the coding region of cDNA clones and would detect all PHYA mRNAs. Each type of PHYA mRNA could have a different half-life.

To date a cis-acting instability determinant has not been identified for PHYA mRNA. Due to the size of the message, approximately 4.2 kb, large 3' to 5' nested deletions were used to investigate PHYA mRNA instability determinants in transgenic tobacco plants (Barnes, 1994). Unfortunately, the full-length PHYA message in the heterologous tobacco plants had an mRNA half-life four times longer than that estimated for etiolated oats, putting into question whether tobacco cells can properly recognize an oat PHYA mRNA destabilizing cis-element. Likely candidates for putative PHYA mRNA destabilizing cis-elements have yet to be determined. Interestingly, the 5' UTR is highly conserved among the three sequenced types of PHYA mRNA, implying a possible post-transcriptional regulatory role for the 5' UTR.

Methods of analysis

Analyzing mRNA decay rates can be technically difficult in multicellular eukaryotic organisms (Atwater et al., 1990; Belasco and Brawerman, 1993). A variety of techniques have been successfully used to estimate mRNA half-lives in eukaryotic organisms, and the best
method of analysis used to estimate mRNA decay rates depends on the biological system and mRNA under investigation. Ideally, data from several different methods are combined to provide the best estimate of the rate of mRNA degradation (Harrold et al., 1991). The determination of cis-acting stability determinants commonly uses deletion and chimeric mRNA constructs that are reintroduced into an mRNA decay system for analysis and identification of critical sequences.

Single-celled organisms or tissue cultured cells lend themselves more easily to techniques utilizing in vivo labeling than do complex multicellular eukaryotic organisms such as higher plants. The approach to steady-state method involves quantification of an incorporated radiolabeled tracer into cellular mRNA, commonly an excess amount of 32P-orthophosphate. The length of time it takes for the labeled mRNA to reach a steady-state level indicates the overall mRNA degradation rate. Unstable mRNAs reach a steady-state level more rapidly than stable mRNAs (Belasco and Brawerman, 1993). A major drawback to this technique is that the half-life for an individual mRNA species can not easily be determined. Alternatively, a tightly controlled, rapidly induced promoter can be used in a gene-specific approach to steady-state method of analysis, utilizing an RNA gel blot or ribonuclease protection assay to quantify the increasing abundance of the individual mRNA. Unfortunately, identification of useful, inducible promoters plus the time required to produce transgenic organisms makes this method difficult.

Pulse-chase experiments employing radiolabeled 32P-orthophosphate that is incorporated into mRNAs have been used to estimate mRNA half-lives (Silflow and Key, 1979; Harrold et al., 1991). The radiolabeling pulse is followed by a chase of non-radiolabeled orthophosphate and the determination of the decay rate for the in vivo radiolabeled mRNAs. A quick and efficient chase that completely replaces the radiolabeled nucleotide pool is necessary for accurate mRNA half-life estimates. Again, these pulse-chase experiments will determine the overall mRNA decay rates, but not an individual mRNA decay rate.

The most commonly used method of mRNA half-life estimation is the administration of a general RNA synthesis inhibitor followed by RNA gel blot analysis to measure the rate of mRNA decay (Atwater et al., 1990; Belasco and Brawerman, 1993). A variety of eukaryotic RNA synthesis inhibitors have been used including decay and 1,10-phenanthroline in yeasts (Belasco and Brawerman, 1993), actinomycin-D in animals and plants (Harrold et al., 1991; Newman et al., 1993), 5,6-dichloro-18-D-ribofuranosylbenzimidazole (DRB) in animals (Nanbu et al., 1994), and cordycepin in plants (Edelman and Schopfer, 1989; Seeley et al., 1992). As general transcription inhibitors these have a severe impact on cell physiology that
can lead to inaccurate estimates of mRNA decay rates (Belasco and Brewerman, 1993). Short time courses with a minimal concentration of an inhibitor that efficiently terminates transcription can result in accurate estimates of mRNA decay rates (Harrold et al., 1991).

Several endogenous down-regulated promoters have been used to estimate mRNA half-lives. In animals the c-myc promoter can be induced by growth factors or neurotransmitters and 30 - 40 min after induction transcription from this promoter is rapidly terminated, allowing mRNA half-life measurements (Belasco and Brawerman, 1993). In plants the oat PHYA promoter is rapidly down-regulated by light, allowing mRNA half-life measurements (Lissemore and Quail, 1988; Seeley et al., 1992). Currently, only the endogenous oat PHYA mRNA half-life has been measured with the PHYA promoter, but it is possible that chimeric genes with the PHYA promoter could be used in transgenic oats to determine other mRNA decay rates. The use of endogenous down-regulated promoters is advantageous because it results in gene-specific transcription termination, preventing the mRNA stability artifacts caused by general transcription inhibitors.

The use of a chemically controlled, rapidly down-regulated promoter, providing a transcriptional chase has recently been used in both plant and animal systems (Gossen and Bujard, 1992; Weinmann et al., 1994). The addition of tetracycline to transgenic tobacco plants expressing the GUS mRNA from the Top-10, tetracycline down-regulated, promoter leads to a rapid gene-specific transcription termination (Weinmann et al., 1994). The Top-10 promoter contains DNA sequence from the Escherichia coli tetracycline operator that is a binding site for the tetracycline repressor protein (Gossen and Bujard, 1992). A second gene is included that constitutively expresses a chimeric transcription activator. This transcriptional activator is able to bind and activate the Top-10 promoter in the absence of tetracycline, but is unable to bind and activate transcription in the presence of tetracycline (Gossen and Bujard, 1992). After the addition of tetracycline and the subsequent transcription termination the mRNA decay rate is estimated. Although this method is complex and a transgenic organism expressing both the chimeric tetracycline down-regulated transcription factor and the mRNA of interest, under the control of the Top-10 promoter, is required, the gene-specific transcription termination and avoidance of general transcription inhibitors is a significant advantage (Weinmann et al., 1994).

The introduction of in vitro synthesized, radiolabeled mRNAs into animal cells or plant protoplasts has been used to measure mRNA half-lives (Gallie et al., 1989; Gallie, 1991). After the introduction of labeled transcripts total RNA is isolated at various time points and analyzed for the abundance of the introduced, labeled mRNA, providing an estimate of the
mRNA decay rate. The advantage of this method is that time-consuming transformation is not required and the use of general transcription inhibitors is avoided. An alternative method that estimates the functional mRNA half-life, the rate of decay of translatable mRNAs, has also been used with plant protoplasts (Gallie, 1991). In vitro synthesized GUS mRNA and luciferase mRNA were electroporated into protoplasts, and the approach to steady-state level of enzyme activity was determined. When the enzyme activity stops increasing it is assumed that all of the introduced translatable mRNA has been degraded. The functional mRNA half-life is estimated by determining the length of time it takes from electroporation to when the enzyme activity reaches half of the steady-state activity (Gallie, 1991). Two important assumptions must be meet for this method to accurately reflect the cytoplasmic mRNA half-life. First, the reporter protein must be very stable with respect to the mRNA stability and secondly, transcription and translation must be constant in the protoplasts for the duration of the analysis.

Finally, the use of in vitro polysome-based degradation systems have been used to determine mRNA half-lives. In mammalian cells the endogenous c-myc and histone mRNA half-lives were determined in an in vitro system (Peltz et al., 1987; Pei and Calame, 1988). In an oat in vitro system half-lives for the endogenous PHYA, β-tubulin, actin mRNAs were estimated (Byrne et al., 1993). After isolation the polysomes are frozen then thawed to start the mRNA degradation time course followed by RNA isolation and RNA gel blot analysis. Currently, only endogenous mRNA decay rates have been determined and exogenous addition of in vitro synthesized mRNAs to the polysome systems has met with only limited success with regard to studying mRNA degradation (Tanzer and Meagher, 1994).

Questions To Be Addressed

Primarily I have been involved in investigating aspects about oat PHYA mRNA stability. My general research goal was to learn more about why oat PHYA mRNA is degraded more rapidly than other mRNAs in the same cells and more rapidly than the average plant mRNA. To address this general goal I established and performed experiments that were concerned with four main research questions. First, can an oat protoplast system be used as a quick and reliable method to measure the rates of mRNA degradation. In a successful oat protoplast system either mutant PHYA mRNAs or chimeric mRNAs containing PHYA mRNA sequences could be analyzed in an attempt to determine PHYA cis-acting regulatory elements. Secondly, what is the percentage of etiolated oat PHYA mRNA that is the type 3 PHYA mRNA and what is the mRNA half-life of two distinct PHYA mRNAs, type 3 and type 4, as
compared to the average oat PHYA mRNA half-life. This would provide a better understanding of oat PHYA mRNA regulation and mRNA stability making it possible to use results from sequence comparisons between distinct PHYA mRNAs as a means to identify possible cis-elements. Thirdly, what is the mechanism by which PHYA mRNA is degraded in etiolated oat cells. Determining early events in PHYA mRNA degradation would be important to the understanding of plant mRNA degradation pathways and their regulation. Fourthly, can RNA-binding proteins that specifically interact with the oat PHYA mRNA be identified. PHYA mRNA-binding proteins might be involved in the mRNA degradation process, and determining the location of binding could help in identifying cis-acting regulatory elements.

**Dissertation Organization**

This dissertation is composed of three papers prepared for publication. Chapters 2 and 4 are papers that have been published in the journals Plant Cell Reports and Plant Cell, respectively, and authorizations for copyright transfer have been given by each of the publishing companies. Chapter 3 is a manuscript that is intended for submission to the journal of Plant Molecular Biology. Chapter 5 investigated a method used in preliminary studies. The references cited in Chapters 2, 3, 4, and 5 are given at the end of each chapter. The references cited in Chapter 1, the General Introduction and Chapter 6, the General Summary, are given at the end in the Literature Cited Section. The research presented in these chapters was performed by myself under the supervision of Dr. James T. Colbert in the Department of Botany at Iowa State University, between the Spring of 1989 and the Fall of 1994.
CHAPTER 2. β-GLUCURONIDASE GENE EXPRESSION AND mRNA STABILITY IN OAT PROTOPLASTS


David C. Higgs and James T. Colbert

Summary

Protoplasts derived from oat (Avena sativa L.) suspension culture cells (7 days after subculturing) were electroporated with plasmid DNA containing the Escherichia coli uidA gene encoding the β-glucuronidase reporter enzyme. Consistently high enzyme activity was observed with electroporation conditions of 500 μF and 1125 volts/cm. Enzyme activity and mRNA accumulation time courses were determined. The maximum enzyme activity was detected at 24 hours after electroporation, while the maximum mRNA level was detected at 12 hours after electroporation. β-glucuronidase mRNA was in vitro synthesized with and without a 5’ methylated cap and then electroporated into protoplasts. Only capped mRNA produced significant enzyme activity. By electroporating radiolabeled, in vitro synthesized mRNA, the β-glucuronidase mRNA half-life was estimated to be ~35 minutes in oat protoplasts.

Key words
mRNA stability - GUS - transient expression - oat protoplasts

Abbreviations
GUS: β-glucuronidase; mRNA: messenger RNA; ICP: insecticidal crystal proteins; OCS: octopine synthase; CAT: chloramphenicol acetyltransferase; nt: nucleotide; kb: kilobase; MS0D3%: Murashige and Skoog media with zero 2,4-dichlorophenoxy acetic acid and 3% sucrose; MU: 4-methyl umbelliferone; ATA: aurintricarboxylic acid
Introduction

Introduction of DNA into protoplasts by electroporation or polyethylene glycol treatment is routinely done (Krens et al., 1982; Paszkowski et al., 1984; Fromm et al., 1985; Potrykus et al., 1985; Hauptmann et al., 1987; Davey et al., 1989). Transient transformation of protoplasts with DNA is a common method of estimating the level of promoter activity in plant cells (Maas et al., 1990). The Escherichia coli uidA gene that encodes β-glucuronidase (GUS) is often used as a reporter gene to determine the expression from a specific promoter in these transient assays, and also in stable transformation experiments (Jefferson et al., 1987a). However, little is known about the stability of GUS mRNA. Variability of GUS mRNA stability among plant organs or plant species could influence the expression of this reporter gene. There is some evidence suggesting that GUS mRNA stability in plants is regulated by light (Dickey et al., 1992).

Electroporation of DNA into protoplasts to study transiently produced mRNA is less frequently reported. Carrot protoplasts were electroporated with an insecticidal crystal protein (ICP) gene or octopine synthase (OCS) gene, and transiently expressed mRNA from electroporated DNA was analyzed to determine why the ICP activity was low in transgenic tobacco plants (Murray et al., 1991). The ICP mRNA was concluded to be less stable than the OCS mRNA. In addition to mRNA stability, RNA processing has been studied during transient expression in protoplasts. To determine minimum intron length, artificial genes were transiently expressed in Nicotiana plumbaginifolia and maize protoplasts, and the mRNAs were analyzed with RNase protection assays (Goodall and Filipowicz, 1990). Finally, splicing efficiency of transiently expressed GUS and firefly luciferase mRNAs were measured in maize protoplasts (Luehrsen and Walbot, 1991).

The importance of posttranscriptional regulation, specifically mRNA stability, in gene expression is becoming more apparent (Atwater et al., 1990). Presently, little is known about the regulation or the variation of mRNA stability in plant cells (Okamuro and Goldberg, 1989). The use of transient gene expression in protoplasts to study mRNA stability and RNA processing has been reported (Callis et al., 1987a; Gallie et al., 1989; Goodall and Filipowicz, 1990; Peterhans et al., 1990; Luehrsen and Walbot, 1991; Gallie, 1991).

In vitro synthesized mRNA has been electroporated into protoplasts (Callis et al., 1987b; Fromm et al., 1987) to study translation efficiency and mRNA stability. Tobacco protoplasts were electroporated with GUS mRNA (Gallie et al., 1989). The 5' cap and
poly(A) tail of the electroporated mRNA were shown to affect translation efficiency, and the GUS mRNA half-life was estimated to be 87 min. Firefly luciferase mRNA was electroporated into tobacco protoplasts, and the mRNA half-life was estimated to be 100 min (Gallie, 1991).

In this report, we describe electroporation and culturing conditions that yielded high GUS activity from DNA electroporated into oat protoplasts. The time courses of transient GUS mRNA accumulation and enzyme activity after electroporation with DNA were determined to identify the points of maximum mRNA abundance and enzyme activity. GUS mRNA was in vitro synthesized, electroporated, and protoplasts were assayed for GUS activity to confirm that the mRNA was delivered to and translated in the oat cytoplasm. Finally, radiolabeled in vitro synthesized GUS mRNA was electroporated, and the half-life was estimated. To our knowledge this is the first report of transient expression in oat protoplasts from electroporated DNA or RNA, and the first estimation of GUS mRNA stability in protoplasts from a monocotyledenous species.

**Materials and Methods**

**Protoplast isolation**

Protoplasts were prepared from oat (*Avena sativa* L.) suspension cultured cells. The cells were subcultured weekly in fresh media by adding 10 ml suspended cells to 40 ml MS0D3%S media consisting of MS salts (Murashige and Skoog, 1962) with 30 g/L sucrose, 0.5 mg/L pyridoxin-HCl, 0.5 mg/L thiamine-HCl, 0.5 mg/L nicotinic acid, and 100 mg/L myo-inositol, pH 5.8. Protoplasts were prepared by a procedure similar to that used for *Triticum monococcum* (Young *et al.*, 1989). Three or seven days after subculturing protoplasts were prepared by adding 10 ml digestion solution per 1 gram fresh weight of suspension cells, and incubated 16 to 20 h at room temperature in the dark on a 40 RPM shaker. The digestion solution consisted of 0.5% Cellulysin (Calbiochem), 0.75% hemicellulase (Sigma), 0.2% Driselase (Sigma) in 50% artificial sea water (ASW), 0.3 M mannitol. The 100% ASW consisted of 18.2 g/L NaCl, 2.26 g/L MgSO₄, 1.0 g/L CaCl₂(2H₂O), 2.14 g/L MES (2-[N-morpholino]ethanesulfonic acid), 0.52 g/L KCl, 3.40 g/L MgCl₂(6H₂O), 0.148 g/L NaHCO₃, pH 6.0. Protoplasts were passed through a 100 µm screen, and collected by centrifugation at 100xg for 5 min. Protoplasts were washed twice by resuspending in 50% ASW, 0.3 M mannitol and centrifuging at 100xg for 5 min, and once in
electroporation buffer before resuspending in an appropriate volume of electroporation buffer to give 2x10^6 protoplasts per ml. Electroporation buffer contained 20 mg/L KH2PO4, 115 mg/L Na2HPO4(7H2O), 7.5 g/L NaCl, 0.6 g/L CaCl2(2H2O), 0.2 M mannitol, (Fromm et al., 1987) with the addition of 0.2 mM spermidine (Sigma; Brault and Miller, 1992), pH 7.2.

**Transient expression**

In 0.4 cm wide electroporation cuvettes, 500 µl of protoplasts (2x10^6 per ml), 20 µg pMON8678 plasmid DNA (6.5 kb), 40 µg of carrier DNA (salmon sperm, Sigma # D-1626), and enough electroporation buffer to make a total volume of 1 ml were combined and set on ice for 5 min. In the cuvettes, the protoplasts were gently mixed then electroporated (Gene pulser, Bio-Rad). Protoplasts were set on ice for 10 min then added to 5 ml of the MS0D3%S, 0.4 M mannitol media. Protoplasts were incubated in the dark at room temperature for 24 h. They were collected by centrifugation, 100xg for 5 min, and resuspended in GUS extraction buffer. The GUS fluorometric assay was performed as reported (Jefferson, 1987b), except that the GUS extraction buffer lacked sodium lauryl sarcosine. The fluorescence spectrophotometer (F-2000, Hitachi) was standardized with 4-methyl umbelliferone (MU) and sample fluorescence was converted to moles of MU in the GUS assay reaction tube. Bradford's total protein assays were performed, and each sample was standardized to mg of protein in the GUS assay (Bradford, 1976).

**Plasmid DNA and in vitro RNA synthesis**

The pMON8678 plasmid contains the cauliflower mosaic virus 35S promoter, the first intron of the maize alcohol dehydrogenase gene, the β-glucuronidase gene, and the nopaline synthase 3' termination sequence. Large scale plasmid DNA isolation was done with Qiagen's Maxi isolation column (Qiagen). Plasmid DNA was quantified by measuring the absorbance at 260 nm (Sambrook et al., 1989).

The 1.9 Kb BamHI-SacI fragment from pBI221 (Clontech), containing the GUS gene, was cloned into the BamHI-SacI sites of the *in vitro* transcription vector pSP64(polyA) (Promega), producing pSGUS. This plasmid was linearized with EcoRI, and GUS mRNA was synthesized with SP6 RNA polymerase (Promega). GUS mRNA with a poly(A)30 tail was *in vitro* transcribed with or without a 5' methylated cap. The large scale *in vitro* transcription reactions were done as previously reported (Yisraeli and Melton, 1989). This mRNA was quantified using absorbance at 260 nm, and the integrity of the mRNA was
determined by electrophoresis in a 1% agarose/3% formaldehyde gel (Sambrook *et al.*, 1989). Capping of the mRNA was done by adding methylated cap substrate (m7G(5')ppp(5')G, Pharmacia) to a final concentration of 1.5 mM and decreasing the GTP concentration to 150 μM in the transcription reaction. These conditions are reported to cap virtually all the transcripts synthesized in the *in vitro* reaction (Yisraeli and Melton, 1989). Labeling of GUS mRNA was done by adding 50 μCi of [α-32P]UTP (Amersham) to the transcription reaction.

**Electroporation of RNA**

GUS *in vitro* synthesized mRNA was electroporated into oat protoplasts essentially as described for electroporation of DNA (above). Instead of carrier DNA, 15 μg of carrier RNA (yeast tRNA, Sigma) was added to protoplasts (prepared three days after subculturing suspension cells) and incubated on ice for 5 min. Capped or non-capped GUS mRNA (4 μg) was added to cuvettes and electroporated (500 μF, 1125 volts/cm) within 10 sec of adding mRNA. Protoplasts were assayed for GUS activity 24 h after electroporation.

**Analysis of mRNA**

Transiently expressed GUS mRNA from electroporated pMON8678 DNA was analyzed with both an RNase protection assay and an RNA gel-blot (northern). Protoplasts for RNA isolations were centrifuged at 100xg for 5 min, frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated with a small-scale procedure that uses aurintricarboxylic acid (ATA) as an RNase inhibitor (Wadsworth *et al.*, 1988; Seeley *et al.*, 1992). Instead of grinding with mortar and pestle, protoplasts were broken while thawing in the ATA buffer with phenol/chloroform and vigorous vortexing. RNA was quantified by measuring absorbance at 260 nm (Sambrook *et al.*, 1989). Total RNA, to be used in the RNase protection assay or the RNA gel-blot assay, was treated with 2 units of RQ1 DNase (Promega) to remove any plasmid DNA from the electroporation that could co-isolate with the total RNA.

The reagents and procedure used for the RNase protection assay were from the Ribonuclease Protection Assay Kit II (RPA Kit II™, Ambion). The probe used to estimate the amount of GUS mRNA in the RNase protection assay was derived from pBGEV. The pBGEV plasmid was made by inserting the 230 bp EcoRV internal GUS fragment from pBI221 into the *in vitro* transcription vector pBluescript KSII+ (Stratagene). A 310 nt labeled, anti-sense probe was made by linearizing pBGEV with HindIII and synthesizing RNA with T7 RNA polymerase (Promega). Template DNA was removed with 2 units RQ1 DNase.
(Promega) and the probe was gel purified in a 5% polyacrylamide/7 M urea gel. This gel was exposed to x-ray film to allow detection of the full-length transcript. The full-length band was excised and then eluted at 37°C in 300 μl of elution buffer (20 mM tris, 1 mM Na-EDTA, 0.5 % SDS [pH 8.0]). The 310 nt pBGEV probe (4.5x10^4 CPM) was added to total oat protoplast RNA (3.2 μg) and hybridized for 14 h at 45°C in 20 μl of 80% deionized formamide, 40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)], 400 mM sodium acetate, 1 mM EDTA, pH 6.4. Single stranded RNA was digested by adding 200 μl of 0.5 units/ml RNase A and 100 units/ml RNase T1, and incubating for 30 min at 37°C. The RNA was analyzed in a 5% polyacrylamide/7 M urea gel, vacuum dried for two hours at 80°C, and exposed to x-ray film at -80°C with an intensifying screen. GUS mRNA signal intensity was quantified by excising the 230 nt band from the gel and using liquid scintillation spectroscopy.

RNA gel-blots, RNA probe hybridization, and band quantification were done as previously reported (Cotton et al., 1990; Seeley et al., 1992). A high specific activity 1.9 kb, anti-sense GUS RNA probe was used to detect GUS mRNA in total oat protoplast RNA (5 μg). This full-length GUS probe was made by inserting the BamHI-SacI fragment, containing the GUS gene from pBI221, into pBluescript KSII+. This plasmid, pBSGUS, was linearized with BamHI, and the RNA probe was synthesized with T7 RNA polymerase. GUS mRNA hybridization was quantified by excising the 1.9 kb band from the membrane and using liquid scintillation spectroscopy.

Estimation of mRNA half-life

The GUS mRNA half-life was estimated by electroporating labeled, in vitro synthesized, capped GUS mRNA into oat protoplasts. GUS mRNA synthesis and electroporation were performed as described in previous sections with some modification. After labeled mRNA was synthesized from linearized pSGUS, it was precipitated and resuspended in 36 μl H2O then quantified by liquid scintillation spectroscopy (Sambrook et al., 1989). Four samples of protoplasts were electroporated with 8 μl (6.8x10^5 cpm/μl) mRNA and incubated on ice for 10 min. Two washes in 5 ml of MS0D3%S, 0.4 M mannitol media, followed by centrifugation (100xg for 5 min), were done to remove labeled mRNA not introduced into protoplasts. Each sample was resuspended in 5.5 ml MS0D3%S, 0.4 M mannitol and this was considered to be time zero (about 45 min after electroporation). One ml from each sample was pooled for each time point. In these RNA isolations 110 μg carrier RNA (total oat seedling RNA) was added to the frozen protoplasts to improve the recovery of
labeled GUS mRNA. Total RNA was isolated (described above). RNA samples were quantified by liquid scintillation spectroscopy. Labeled RNA samples (500 CPM per lane) were electrophoresed in a 1% agarose/3% formaldehyde gel and transferred, by using a 25 mM sodium phosphate buffer, to a nylon membrane (GeneScreen, Du Pont). The RNA was ultraviolet light cross-linked to the membrane (UV Stratalinker 1800, Stratagene), and exposed to x-ray film at -80°C with an intensifying screen. The 1.9 kb GUS full-length band was quantified using scanning densitometry (GS 300 scanning densitometer, Hoefer Scientific Instruments). Densitometric data were analyzed (GS 365 Data System, Hoefer Scientific Instruments) to determine the area of the peak that corresponded with the GUS full-length RNA, and plotted as percent of time zero.

Results

DNA electroporation

Prior to analyzing GUS mRNA levels, GUS enzyme activity was used to determine the electroporation and culturing parameters that efficiently introduced nucleic acids into oat protoplasts. An array of capacitance (μF) and Voltages (volts/cm) were used to electroporate 10 μg of pMON8678 into oat protoplasts derived from suspension cells 3 days after subculturing (Figure 1). Four conditions gave reproducible high GUS activity (500 μF, 875 volts/cm; 500 μF, 1125 volts/cm; 960 μF, 625 volts/cm; and 960 μF, 875 volts/cm).

To test the effect of suspension culture age on expression, the four conditions that resulted in high GUS activity were used to electroporate DNA into protoplasts derived from suspension cells either 3 or 7 days after subculturing (Figure 2). Significantly higher GUS activity was observed in protoplasts derived from suspension cells 7 days after subculturing. With protoplasts derived from suspension cells 3 days after subculturing, the four conditions tested gave equivalent GUS activity. With protoplasts derived from suspension cells 7 days after subculturing the 500 μF, 1125 volts/cm and the 960 μF, 875 volts/cm conditions produced the highest GUS activity.

The amount of plasmid DNA electroporated into the oat protoplasts was altered to measure the impact on GUS activity (Figure 3). Protoplasts for these experiments were derived from suspension cells 3 days after subculturing. Five different amounts of pMON8678 plasmid DNA were electroporated (0, 10, 20, 40, and 80 μg). The total mass of DNA (pMON8678 plus carrier) was kept at 80 μg per sample by adding an appropriate amount
Figure 1. The effect of electroporation conditions, capacitances (μF) and Voltages (volts/cm), on transient GUS activity. Protoplasts, derived from suspension cells 3 days after subculturing, were electroporated at various conditions with 10 μg of pMON8678 DNA and 40 μg of carrier DNA. Protoplasts were incubated 24 h then assayed for GUS activity. Error bars indicate standard error of the mean of two experiments, except that 875 and 1125 volts/cm at 500 μF and 625 and 875 volts/cm at 960 μF are the mean of three experiments.
Figure 2. The effect of suspension cell age on transient GUS activity. Protoplasts were isolated from suspension cells 3 days and 7 days after subculturing, and electroporated at the four best conditions (Figure 1) with 10 µg of pMON8678 DNA and 40 µg of carrier DNA. Protoplasts were incubated 24 h, then assayed for GUS activity. Error bars indicate standard error of the mean of two experiments.
Figure 3. The effect of plasmid DNA concentration on transient GUS activity. Between 0 and 80 μg of pMON8678 plasmid DNA was electroporated (960 μF, 625 volts/cm) into protoplasts isolated from suspension cells 3 days after subculturing. An appropriate amount of carrier DNA was added to make a total of 80 μg per sample. Protoplasts were incubated 24 h, then assayed for GUS activity. Error bars indicate standard error of the mean of two experiments with two replicates per experiment.
of carrier DNA. As previously reported (Fromm et al., 1985) and observed here, increasing he plasmid DNA resulted in a linear increase in GUS activity. Overall, the maximum GUS activity was obtained by electroporating protoplasts, derived from suspension cells 7 days after subculturing, with 80 µg pMON8678 plasmid DNA (no carrier DNA) at 500 µF, 1125 volts/cm.

Analysis of GUS enzyme activity and mRNA levels

Time courses of GUS activity and mRNA levels after DNA electroporation were estimated. The GUS activity was monitored for 48 h after electroporation with 20 µg of pMON8678 DNA (Figure 4). By 4 h significant GUS activity was detected. GUS activity increased linearly until 24 h, and by 48 h activity had decreased to 48% of the maximum. At 24 h a carrier-DNA-only control had 0.1% of the activity of the 24 h sample electroporated with 20 µg of pMON8678 (data not shown). A similar enzyme activity time course was reported for carrot protoplasts expressing chloramphenicol acetyltransferase (CAT) (Hauptmann et al., 1987).

By adding cordycepin (an inhibitor of RNA synthesis) to tobacco protoplasts, it was determined that the majority of CAT mRNA synthesis occurred prior to 1 h after electroporation with DNA (Pröls et al., 1988). Carrot protoplasts had maximum mRNA levels at 4 h and 8 h after electroporation with ICP DNA and OCS DNA, respectively, as analyzed with RNA gel-blots (Murray et al., 1991). From these reports and the enzyme activity time course (Figure 4), it was expected that the highest GUS mRNA levels would occur prior to 24 h. Initially, GUS mRNA accumulation was analyzed in an RNase protection assay. Subsequent mRNA analyses were done with RNA gel-blots. For the RNase protection assay a 310 nt RNA probe (pBGEV) that protects 230 nt internal to the GUS mRNA was used to estimate the mRNA abundance (Figure 5A). The GUS mRNA level was maximum at 12 h after electroporation and had decreased considerably by 24 h (Figure 5B). In addition to the expected 230 nt protected fragment, a smaller fragment estimated to be ~189 nt was detected in the 4, 8, and 12 h time points in the RNase protection assay (Figure 5A). This band could have been an RNA degradation product, or an RNase protection assay artifact. It was assumed to be the latter since a corresponding band was not seen in RNA gel-blots (data not shown).
Figure 4. Time course of GUS activity after electroporation. Protoplasts derived from suspension cells 3 days (first experiment) and 7 days (second experiment) after subculturing were electroporated (500 μF, 1125 volts/cm) with 20 μg of pMON8678 DNA and 40 μg of carrier DNA. Seven samples were electroporated per experiment. At various times after electroporation (1, 2, 4, 8, 18, 24, and 48 h) 0.7 ml from each sample was pooled for each time point, and assayed for GUS activity. The seven samples were pooled at each time point to reduce potential experimental error caused by variability in electroporation efficiency from sample-to-sample. Error bars indicate standard error of the mean of the two experiments.
Figure 5. Time course of GUS mRNA accumulation after electroporation with DNA. Protoplasts derived from suspension cells 7 days after subculturing were electroporated (500 μF, 1125 volts/cm) with 80 μg of pMON8678 DNA (no carrier DNA). Four samples were electroporated for each time point, and total RNA was isolated from the four pooled samples. The abundance of GUS mRNA was estimated at each time point. (A) An RNase protection assay was performed with a 310 nt GUS RNA probe that protects a 230 nt internal GUS fragment from RNase digestion. The Probe lane contained the probe (310 nt) and non-electroporated protoplast RNA (3.2 μg). The negative control lane (C) contained the probe and non-electroporated protoplast RNA (3.2 μg). The six time points (0, 4, 8, 12, 18, and 24 h) contained total RNA from electroporated protoplasts (3.2 μg) and probe. All samples except the probe lane were digested with RNase. Low molecular weight RNA markers (Bethesda Research Laboratories) were end-labeled and electrophoresed simultaneously with the RNA samples to confirm the sizes of the bands. The gel was exposed to x-ray film for 20 h. (B) Quantification of GUS mRNA detected in the RNase protection assay (A) and an RNA gel-blot experiment (not shown). The time points were 0, 1, 2, 4, 8, 12, 18, and 24 h after electroporation. Error bars indicate the standard error of the mean of the two experiments, except those for the 1 and 2 h points which are from one RNA gel-blot experiment.
**Estimating GUS mRNA half-life**

*In vitro* synthesized GUS mRNA was electroporated into the oat protoplasts to estimate its half-life. The GUS mRNA included 30 adenylate residues, enough to function as a poly(A) tail (Gallie et al., 1989). First, it was necessary to confirm that the mRNA was delivered to and translated in the cytoplasm. To do this GUS enzyme activity was measured in protoplasts that were electroporated with 4 µg of capped or non-capped GUS mRNA. The optimal DNA electroporation conditions (Figure 1) were used to electroporate GUS mRNA into oat protoplasts derived from suspension cells 3 days after subculturing. Only the protoplasts electroporated with capped mRNA showed significant GUS activity (Figure 6). This result indicated that mRNA capping had occurred, that some of the capped, full-length mRNA reached the cytoplasm, and that cytoplasmic proteins required for translation associated with the *in vitro* synthesized GUS mRNA. Also, this confirms the requirement for a 5' cap in the translation of mRNA as reported (Drummond *et al*., 1985; Moldave, 1985; Callis *et al*., 1987b; Gallie *et al*., 1989).

Under the same conditions radiolabeled, *in vitro* synthesized GUS mRNA was electroporated into oat protoplasts. The protoplasts were washed to remove labeled mRNA remaining outside of the protoplasts. RNA gel-blotts were used to determine the amount of full-length GUS mRNA at varying times after electroporation (Figure 7). These data lead to an estimate of ~35 min for the half-life of GUS mRNA in oat protoplasts. GUS mRNA degradation during RNA isolation was controlled for by adding labeled GUS mRNA to frozen non-electroporated protoplasts, and immediately followed by the isolation of total RNA. This isolated, labeled GUS mRNA was predominantly full-length (GUS lane, Figure 7A). Degradation of the GUS mRNA in protoplasts produced a continuous smear of fragments from the 1.9 kb full-length band to less than 200 nt (Figure 7A). This suggests that the GUS mRNA was not degraded by specific endonucleolytic cleavages as are some mRNAs (Stoeckle and Hanafusa, 1989; Vreken and Raué, 1992). Unidirectional or bidirectional exonuclease activity, or a random endonuclease, activity could explain the continuous smear of fragments seen in GUS mRNA degradation.
Figure 6. Electroporation of in vitro synthesized GUS mRNA. Protoplasts were electroporated with either 4 µg of capped or non-capped GUS mRNA and 15 µg of carrier RNA. The controls were electroporated with carrier RNA alone. Protoplasts were incubated for 24 h, then assayed for GUS activity. Error bars indicate standard error of the mean of four replicates from two experiments.
Figure 7. Degradation of GUS mRNA in protoplasts. Protoplasts were electroporated with labeled, in vitro synthesized, capped GUS mRNA and 15 μg of carrier RNA. Protoplasts were washed to remove excess labeled mRNA. At various times total RNA was isolated from the protoplasts. (A) Visualization of GUS mRNA after electroporation. For the GUS lane, labeled GUS mRNA was added to frozen non-electroporated protoplasts immediately prior to RNA isolation. C0 and C5 are non-electroporated controls in which labeled GUS mRNA was added to protoplasts, without electroporation, and subjected to washes prior to RNA isolation at 0 (C0) and 5 (C5) h. RNA was isolated from protoplasts at 0, 0.5, 1, 2, 4, and 5 h after electroporation with labeled GUS mRNA. All samples were electrophoresed in a 1% agarose/3% formaldehyde gel and transferred to a nylon membrane. This membrane was exposed to x-ray film for 3 days at -80°C with intensifying screen. (B) Full-length GUS mRNA was quantified at the various times after electroporation. Error bars indicate the standard error of the mean of three experiments including the experiment in panel (A), except the 2, 2.3, and 4 h time points are the mean of two experiments.
Discussion

We have determined DNA electroporation conditions that yield high GUS enzyme activity in oat protoplasts. The age of the suspension cells, since the previous subculturing, had a dramatic effect on GUS expression. Protoplasts derived from 7-day-old suspension cells were either more competent for transformation or more competent for expression of the DNA once it had entered the protoplasts. The GUS mRNA level was maximum at 12 h after DNA electroporation. This indicates that transcription is occurring for at least 12 h, if not longer, after electroporation. This length of time for transcription is longer than was reported for the CAT reporter gene in tobacco protoplasts (Pröls et al., 1988). GUS enzyme activity was maximum at 24 h after DNA electroporation. Again, this length of time to maximum enzyme activity was longer than that reported for the CAT reporter gene in tobacco protoplasts, which had a maximum activity at 7 h after electroporation (Pröls et al., 1988). The GUS enzyme activity time course reported here is similar to that reported for CAT activity in carrot protoplasts (Hauptmann et al., 1987). The length of time over which transcription occurs would influence the time course of GUS protein accumulation. The 12 h lag between the maximum GUS mRNA abundance and maximum enzyme activity is not unreasonable when the complex relationship between mRNA accumulation and protein accumulation is considered (Price, 1992).

The GUS mRNA half-life in oat protoplasts, as estimated with electroporated labeled mRNA, was ~35 min. This value is about 40% of the reported value of 87 min for GUS mRNA in tobacco protoplasts (Gallie et al., 1989). The GUS mRNA measured in tobacco protoplasts had a poly(A) tail with 50 adenylate residues (Gallie et al., 1989). The GUS mRNA electroporated into oat protoplasts had a poly(A) tail with 30 adenylate residues. The difference between these two estimates of GUS mRNA half-life might be due to the difference in plant species or the difference in the length of the poly(A) tails (Atwater et al., 1990).

The observation of the continuous smear of GUS RNA fragments is not novel. Other plant mRNAs have been reported to produce a smear of RNA fragments, that may be in vivo degradation products (Seeley et al., 1992; Dickey et al., 1992). The GUS RNA fragments observed here are most likely in vivo degradation products because non-electroporated GUS mRNA that was subjected to the RNA isolation procedure resulted in predominantly full-length GUS mRNA.
Some questions can be raised about using electroporated labeled mRNA to estimate cytosolic mRNA half-lives. The primary concern is the cellular location of the mRNA after electroporation. We know that some of the electroporated mRNA is localized in the cytosol because translation occurs and GUS enzyme activity is detected in the oat protoplasts. If the majority of the electroporated GUS mRNA is localized in the cytosol, then the apparent half-life of ~35 min seems reliable. On the other hand, if a significant percentage of mRNA is electroporated into cellular compartments other than the cytosol, e.g., the vacuole, then the ~35 min half-life might not accurately reflect the cytosolic mRNA half-life. The percentage of electroporated mRNA that is localized in the cytoplasm is unknown. Also, the poly(A) tail length and capping efficiency, during in vitro mRNA synthesis, could affect mRNA stability. Sequential nuclear synthesis, processing, and transport to the cytosol could influence mRNA stability. Proteins that regulate stability could potentially associate with an mRNA during these stages of RNA maturation prior to entering the cytosol. The same mRNA synthesized in vitro and electroporated might not be complexed with such proteins after delivery into the cytosol, and therefore might have a different half-life. These possibilities need to be considered when using electroporated labeled mRNA to estimate in vivo mRNA stability.

Alternative approaches to estimating mRNA half-lives in protoplasts include pulse-chase and RNA synthesis inhibitor studies. Pulse-chase experiments have been performed in soybean suspension-cultured cells (Silflow and Keys, 1979), however, it is difficult to equilibrate intracellular nucleotide pools (Atwater et al., 1990). Protoplast mortality during the pulse-chase would further complicate such experiments. The addition of an RNA synthesis inhibitor to protoplasts that were transiently transcribing mRNA from electroporated DNA is another possible method to estimate mRNA stability. Although, such an experiment would estimate the half-life of an in vivo-produced and localized mRNA it would have other problems. Principally, does the inhibitor completely stop transcription of the electroporated DNA, and will the inhibitor cause artifacts, altering the rate of RNA degradation? Ideally, data from several approaches would be combined when trying to estimate the half-life of mRNAs in protoplasts (Harrold et al., 1991).

Acknowledgments

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References


CHAPTER 3. THE ABUNDANCE AND DEGRADATION OF THE DISTINCT OAT PHYTOCHROME A3 AND A4 mRNAs

A paper to be submitted to the journal of Plant Molecular Biology

David C. Higgs, Linda J. Barnes, and James T. Colbert

All of the experiments presented in this chapter and the primary authorship were performed by myself under the supervision of Dr. James Colbert. Dr. Linda Barnes' primary contributions were in the cloning and characterization of a key construct used in these experiments and in manuscript review.

Abstract

Gene-specific oligonucleotide probes were used to determine the relative abundance and half-lives of distinct oat phytochrome A (PHYA) mRNAs. Oat PHYA mRNAs are highly conserved in the 5' untranslated region and the coding region, but the 3' untranslated region has an overall lower sequence conservation and was the source of gene-specific probes. PHYA3 mRNA was estimated to be ~61% of the oat PHYA mRNA pool present in poly(A)+ RNA from dark-grown seedlings, and PHYA4 mRNA appeared to make up a much lower percentage. The half-lives for PHYA3 and PHYA4 mRNAs were both estimated to be ~30 min, and a similar similar short half-life was estimated for the average PHYA mRNA. Sequence comparisons of PHYA mRNAs identified highly conserved sequences within the 5' and 3' untranslated regions that might be important for PHYA mRNA degradation.

Key words

Avena sativa, gene expression, PHYA, gene-specific probes, light regulation, RNA stability.
Introduction

The phytochrome photoreceptor is involved in many light-regulated plant responses including seed germination, de-etiolation, and flowering (Furuya, 1993). The phytochrome protein functions, in part, by altering the transcription of light-regulated genes, including the phytochrome A (PHYA) genes (Vierstra, 1993). Oat PHYA mRNA abundance is rapidly down regulated in response to light through the activity of the phytochrome protein (Colbert et al., 1983; Colbert et al., 1985). A red-light (R) pulse leads to a significant inhibition of oat PHYA transcription within 5 min, and by 30 min PHYA transcription is inhibited by 90% (Lissemore and Quail, 1988). By 5 hr after a transcription-terminating R pulse the PHYA mRNA abundance is reduced by greater than 90% (Colbert et al., 1985). This rapid decrease in mRNA abundance is due to the unstable message (Seeley et al., 1992). The PHYA mRNA has an estimated half-life of ~1 hr in etiolated oat seedlings when either treated with light or when maintained in the dark and treated with the RNA synthesis inhibitor cordycepin (Seeley et al., 1992). These results support the conclusion that the oat PHYA mRNA is inherently unstable. In a polysome-based in vitro degradation system the oat PHYA mRNA half-life is estimated to be 45 min, similar to the in vivo estimate, indicating that the PHYA mRNA is degraded while associated with ribosomes (Byrne et al., 1993). In vivo the oat PHYA mRNA appears to be degraded by two distinct pathways (Higgs and Colbert, 1994) with mechanisms similar to those reported for the yeast PGK1 mRNA (Muhlrad and Parker, 1994).

The oat phytochrome A protein is encoded by a multi-gene family (Hershey et al., 1985a) and at least four distinct PHYA mRNAs accumulate in etiolated seedlings (Hershey et al., 1985b). A PHYA multi-gene family is not surprising because oats are hexaploid. The three sequenced PHYA partial cDNA clones from etiolated oats (types PHYA3, PHYA4, and PHYA5) show ~98% nucleic acid identity in both the coding region and known portions of the 145 nt long 5' untranslated region (5' UTR) (Hershey et al., 1985b; Hershey et al., 1987). In contrast, the approximately 250 nt long 3' UTR shows a 34% identity between the PHYA3 and PHYA4 mRNAs when the sequences are aligned at the translation stop site (Hershey et al., 1985b). An estimated 72% identity exists in the 3' UTR between PHYA3 and PHYA4 mRNAs if realignment with gaps is permitted to account for deletions and insertions (Hershey et al., 1985b). The partial PHYA5 cDNA clone does not contain the 3' UTR (Hershey et al., 1985b). Previous studies of oat PHYA mRNA expression and degradation have used RNA probes derived from the highly conserved coding-region and, therefore, would have detected all known oat PHYA mRNAs.
An oat PHYA3 genomic clone containing the PHYA3 promoter has been used to determine functionally important cis-sequences involved in transcriptional regulation (Bruce and Quail, 1990; Bruce et al., 1991). When a +10 to +1377 fragment (+1 indicates the first transcribed nucleotide) was inserted 3' of the cauliflower mosaic virus 35S promoter expression of the chloramphenicol acetyltransferase reporter enzyme increased fivefold (Bruce and Quail, 1990). This +10 to +1337 PHYA3 fragment contains the 85-nt long first exon that makes up part of the 5' UTR, the 1.2-kb long first intron, and 75 nt of the second exon, including the remaining 60 nt of the 5' UTR and the first 15 nt of the coding region. This five-fold increase in expression could be caused by transcriptional, post-transcriptional, or translational events. It has been suggested that the intron may be primarily responsible for this effect, but this remains to be determined (Bruce and Quail, 1990).

Post-transcriptional regulation of gene expression is an important level of control in plants and other eukaryotes (Brawerman, 1993; Sachs, 1993; Sullivan and Green, 1993). Cytoplasmic mRNA levels often correlate more closely with transcript stability than with the level of transcription (Carneiro and Schibler, 1984; Walling et al., 1986; Peltz et al., 1991). The average soybean suspension-cultured cell mRNA half-life is estimated to be 30 hr (Silflow and Key, 1979). Unstable messages are commonly transcribed from genes that are rapidly regulated in response to environmental or hormonal stimuli, and unstable messages have been shown to be destabilized by cis-acting instability determinants (Sullivan and Green, 1993). To date the cytoplasmic plant mRNAs with the shortest estimated half-lives (10 to 50 min) are the small-auxin-up-RNAs (SAURs) present in soybeans (McClure and Guilfoyle, 1989; Franco et al., 1990), mung beans (Yamamoto et al., 1992) and Arabidopsis thaliana (Gil et al., 1994). A unique sequence termed DST was identified in the soybean SAUR 3' UTR by the high sequence conservation (McClure et al., 1989), and a synthetic DST has been shown to function as an mRNA instability determinant in tobacco (Newman et al., 1993).

In this report we have used gene-specific oligonucleotide probes for PHYA3 and PHYA4 mRNAs to estimate the contribution of each of these distinct messages to the etiolated-oat PHYA mRNA pool. The PHYA mRNA pool was determined with an oligonucleotide probe that detected all known types of oat PHYA mRNAs. After a PHYA transcription-inhibiting light treatment the mRNA half-lives for the PHYA3 and PHYA4 mRNAs were determined and compared to the average half-life of all oat PHYA mRNAs. Sequence comparisons of different unstable PHYA mRNAs from several grass species identified highly conserved sequences in the 5' and 3' untranslated regions.
Materials and Methods

*Estimating mRNA abundance and half-lives*

Total RNA was isolated from 4-day-old etiolated oat seedlings (*Avena sativa* cv Garry) as previously described (Dean *et al.*, 1985), and poly(A)+ mRNA was purified from this total RNA with a poly(U) Sephadex column (Bio-Rad) as previously described (Lissemore *et al.*, 1987). Poly(A)+ RNA was electrophoresed in 1% NuSieve 3:1 (FMC, Rockland, ME) agarose-3% formaldehyde gels and transferred to a nylon filter (GeneScreen, Du Pont) as indicated (Cotton *et al.*, 1990). RNA gel blots were prehybridized in 0.2 ml/cm² hybridization buffer for 30 min at 52°C (Church and Gilbert, 1984). The hybridization buffer contained 1% (w/v) acetylated bovine serum albumin (BSA) (Promega), 7% SDS (sodium dodecyl sulfate), 0.25 M Na₂HPO₄/NaH₂PO₄ buffer, pH 6.8, and 1 mM EDTA (ethylenediaminetetraacetic acid, disodium). Radiolabeled oligonucleotide probe was added to a final concentration of 5x10⁵ cpm/ml of hybridization buffer, and incubated overnight (12 to 20 hr) at 52°C (4°C below the calculated melting temperature, see below). Blots were washed two times for 5 min in 5% SDS, 40 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 6.8, at room temperature (22°C), followed by two washes for 15 min in 1% SDS, 40 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 6.8, at 52°C. Blots were exposed to x-ray film at -80°C with an intensifying screen (Du Pont). RNA bands were quantified by liquid scintillation spectroscopy as previously described (Cotton *et al.*, 1990).

To test the specificity of the oligonucleotide probes, a full-length *PHYA3*-like RNA (pFL) was *in vitro* synthesized. The pFLII plasmid (Barnes, 1994) was digested with *XhoI* to linearize the plasmid, and the pFL RNA was synthesized with T7 RNA polymerase as indicated by the manufacturer (Promega). This pFL RNA was 3.8-kb in size and lacked a poly(A) tail. The *in vitro* synthesized pFL RNA was 100% complementary to oligonucleotide probe A and probe 3, but at the same region to which probe 3 hybridizes to pFL RNA the probe 4 was only 20% complementarity to pFL RNA. At a different region of pFL RNA a maximum complementarity between probe 4 and pFL RNA was estimated as 58% with the Bestfit Program [Genetic Computer Group (UWGCG) software package (Version 7, April, 1991, Madison, WI)]. The region of maximum complementarity between probe 4 and pFL RNA (a *PHYA3*-like RNA) was a single site in the 3' UTR. The pFL RNA (4 ng) was added to yeast total RNA (10 μg) and RNA gel blotted. To investigate the cross-hybridization of the gene-specific probes the three oligonucleotide probes (A, 3, and 4) were hybridized to the pFL RNA blots, and the radioactivity at the pFL band was quantified for each probe by liquid
scintillation spectroscopy. The percentage of radioactivity for each probe as compared to oligonucleotide probe A was calculated.

To control for differences in hybridization efficiencies between the oligonucleotide probe A (detects all known PHYA mRNAs) and probe 3 (detects PHYA3 mRNA), smaller in vitro synthesized PHYA RNAs were added to poly(A)+ samples as reference RNAs. The pAPSX2.7 plasmid, containing an internal fragment of the PHYA5 mRNA (Seeley et al., 1992), was linearized with XbaI and T7 RNA polymerase was used to synthesize a 2.7-kb PHYA5 mRNA termed RA (reference RNA for probe A). RA contained a region that was 100% complementary to probe A. The pPhyl 1A plasmid contained the 3' half of a PHYA3-like mRNA (Barnes, 1994) and was linearized with XhoI and T7 RNA polymerase was used to synthesize a 2.3-kb sense PHYA3-like mRNA termed R3 (reference RNA for oligonucleotide 3). Like PHYA3 mRNA, the R3 RNA contained a region that was 100% complementary to probe 3. The terminal 206 nt of the 3' UTR of pPhyl 1A plasmid was sequenced twice from both the 5'-end and 3'-end at the Iowa State University Nucleic Acid Facility to confirm 100% complementarity to probe 3. Before RNA gel blotting the reference RNAs were quantified by ultra-violet light spectroscopy, assuming 1 A$_{260}$ = 40 µg RNA/ml (Sambrook et al., 1989), and equal molar amounts of the reference RA and R3 RNAs were added to the poly(A)+ RNA samples as the molar equivalent of 4 ng of endogenous full-length PHYA mRNA, as estimated to be 4.0 kb in poly(A)+ RNA (Higgs and Colbert, 1994). When equal molar amounts of reference RNAs were added to poly(A)+ RNA samples differences in signal intensity between the probes were observed, assumed to be due to differences in hybridization efficiencies. These differences were corrected for by determining the ratio of endogenous full-length RNA/reference RNA. The percent of total PHYA mRNA that was the distinct PHYA3 mRNA was determined by comparing the ratio of radioactivity at the endogenous PHYA3 mRNA/reference RNA (R3) to the ratio of radioactivity at the endogenous total PHYA mRNA/reference RNA (RA). The typical ratio for the total PHYA mRNA (PHYA/RA) as determined with oligonucleotide probe A was 0.171 and for the PHYA3 mRNA (PHYA3/R3) as determined with oligonucleotide probe 3 was 0.111.

The half-lives of distinct PHYA mRNAs were determined by RNA gel blot analysis of poly(A)+ RNA isolated from etiolated oat seedlings at time points after a Red-light (R) pulse. RNA from these R-pulse time courses were hybridized with either oligonucleotide probe A (detects all known PHYA mRNA types), probe 3 (detects PHYA3 mRNA) or probe 4 (detects PHYA4 mRNA). The full-length PHYA mRNA bands were quantified by liquid scintillation spectroscopy and made relative to the time-zero point for each probe. Reference RNAs were
not included in these experiments because differences in hybridization efficiencies between the probes would not influence the mRNA half-life estimation.

**Oligonucleotide probes**

Three single-stranded DNA-oligonucleotides, 40-nt in length, were synthesized (Keystone Co., Menlo Park, CA) to be complementary to oat PHYA mRNAs such that all the oligonucleotides had the same G+C content in an attempt to minimize differences in hybridization efficiencies between the oligonucleotide probes. Probe A (5'-dGGGCTCTCATGGTGCAAAACAAGGAGGCCCCAGACTTTC-3') was 100% complementary to +1008 to +1127 nt of all the known oat PHYA mRNAs, probe 3 (5'-dTACACTACAGGCTGCAGCATTCCATCTCCTGAGCACCAGG-3') was 100% complementary to +3497 to +3536 nt of the oat PHYA3 mRNA and had a maximum complementarity of 70% to oat PHYA4 mRNA, and probe 4 (5'-dACTTTTCACCTGACGCGCAACAGTGTTGAGCAGCGACATTC-3') was 100% complementary to +3497 to +3536 nt of the oat PHYA4 mRNA and had a maximum complementarity of 58% to oat PHYA3 mRNA (Hershey et al., 1985b). The melting temperatures for the specific RNA/DNA complexes were calculated to be 56°C under the hybridization conditions used here (Ausubel et al., 1987). The melting temperatures for the PHYA3/probe 4 complex, 58% complementarity, and the PHYA4/probe 3 complex, 70% complementarity, were calculated to be 43°C and 47°C, respectively, under the hybridization conditions used here. The oligonucleotides were 32P end-labeled by T4 polynucleotide kinase with the forward kinase reaction by incubating 0.250 µg (18 pmoles) of a single-stranded 40-mer-oligonucleotide with 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM dithiothreitol (DTT), 0.250 µg acetylated bovine serum albumin (BSA)(Promega), 1 µM [γ32P]-ATP (>3000 Ci/mmol), and 8 units of T4 polynucleotide kinase (Gibco-BRL, Grand Island, NY) for 1 hr at 37°C according to Ausubel et al. (1987), followed by stopping the reaction with the addition of EDTA, final concentration of 15 mM, and then separating the probe from the unincorporated label with a Bio-Gel P-60 (100-200 mesh) exclusion column (Bio-Rad). The calculated specific activities were typically ~2x10⁸ cpm/µg DNA for all three oligonucleotide probes.

**Results**

**Abundance of distinct oat PHYA mRNAs**

To estimate the relative abundance of distinct types of PHYA mRNA, gene-specific mRNA probes must be of the same length, the same specific activity (cpm/µg), the same G+C
content, and hybridize to only one location per mRNA. Gene-specific probes needed to fit these criteria so that hybridization efficiencies might be equal. This would make it possible to measure the amount of radioactivity at a specific band in an RNA gel blot as a direct method of estimating the relative abundance of a distinct PHYA mRNA. Three 40-nt long oligonucleotides were synthesized, each having the similar G+C content (0.58 for oligonucleotides A and 3 and 0.55 for oligonucleotide 4), generating similar melting temperatures for the different RNA/DNA complexes. Melting temperatures were calculated to be ~56°C with all three probes under the hybridization conditions used here (Ausubel et al., 1987). In addition the two gene-specific oligonucleotide probes (3 and 4) were made to be complementary to a region with sufficient sequence divergence. The 3' UTR is the only portion of oat PHYA mRNA with a low enough sequence identity between the known message types to generate gene-specific mRNA probes. The sequence of the 3' UTR is reported only for PHYA3 and PHYA4 mRNAs (Hershey et al., 1985b). When aligned at the translation stop site the PHYA3 and PHYA4 mRNAs have a 34% identity in the 3' UTR, and within the 3' UTRs a 40-nt region has only 20% identity between these mRNAs. Oligonucleotides 3 and 4 were synthesized from this region of 20% identity and used as gene-specific probes to specifically detect the PHYA3 and PHYA4 mRNAs, respectively.

Figure 1 indicates the region of hybridization for each of the oligonucleotide probes and the percent complementarity between the probes and the different PHYA mRNAs. A region of maximum complementarity was identified between the probes and each PHYA mRNA (Figure 1C). The 58% and 70% maximum complementarities for the PHYA3/probe 4 and PHYA4/probe 3 complexes, respectively, were significantly less than 100% complementarity. Although PHYA3 and PHYA4 mRNAs have a lower sequence conservation in the 3' UTR, it is possible that the gene-specific probes 3 and 4 might hybridize to unknown PHYA mRNAs for which the sequence is very similar to either PHYA3 or PHYA4 mRNA. Sequence comparisons showed that each probe was highly complementary to only one region of the specific mRNA, as indicated (Figure 1A). Oligonucleotide A was synthesized to hybridize to the PHYA mRNA coding region with 100% sequence complementarity for all three sequenced PHYA mRNAs. Signal intensity obtained from RNA gel blots with oligonucleotide A determined the total PHYA mRNA pool.

RNA gel blot experiments with 4 ng of in vitro synthesized pFL (PHYA3-like RNA) added to 10 μg of yeast total RNA resulted in high amounts of radioactivity at the size expected for pFL RNA with both probe A (100% complementarity to all known PHYA mRNAs, including PHYA3 mRNA) and probe 3 (100% complementarity to PHYA3 mRNA). Low
amounts of radioactivity (4.5 ± 1.9% of probe A) was detected with probe 4 (58% maximum complementarity to PHYA3 mRNA) (Figure 2A). This indicates that there was sufficiently low cross-hybridization between gene-specific probes and PHYA mRNAs. A PHYA4 cDNA plasmid was not available to synthesize a known amount of PHYA4 mRNA that might have been used in a reciprocal cross-hybridization control experiment with probe 3. The discrepancy between the signal intensity with probe A and probe 3 was unexpected (Figure 2A). The in vitro synthesized pFL RNA was 100% complementary to both probes A and 3, yet much more radioactivity was observed with probe 3. This indicates that some variable in addition to the oligonucleotide length, G+C content, and specific activity had influenced the hybridization efficiency. Differences in predicted intramolecular secondary structure of the oligonucleotide probes was a possible explanation for the discrepancy between probe A and 3 signal intensity (data not shown).

In order to control for the discrepancy in hybridization efficiencies, reference RNAs were in vitro synthesized and added to poly(A)+ RNA samples, a technique previously shown to control for variability in hybridization when analyzing an mRNA (Pape et al., 1990). A 2.7-kb PHYA5 RNA (RA) (Hershey et al., 1985b) was used as the reference RNA for probe A, and a 2.3-kb PHYA3 RNA (R3) was used as the reference RNA for probe 3. The PHYA3 reference RNA was synthesized from a partial cDNA clone (pPhy11A) recently isolated from an etiolated oat-seedling cDNA library (Barnes, 1994). The pPhy11A clone contains a 2.1-kb partial PHYA cDNA insert (Barnes, 1994). Restriction mapping and preliminary sequencing data indicated that pPhy11A cDNA was very similar to the PHYA3 cDNA (Hershey et al., 1985b). Confirmation of 100% complementarity between the pPhy11A cDNA clone and probe 3 was critical if pPhy11A was to be used as template to synthesize the PHYA3 reference RNA (R3). The nucleic acid sequence of the 3' most 206 nucleotides of the 3' UTR from pPhy11A was determined and compared to the PHYA3 3' UTR sequence (Hershey et al., 1985b). Overall there was 97.1% identity in the 3' UTR between these two independently isolated clones, and a 100% identity in the 40-nucleotide region complementary to probe 3 (Figure 1B). The 2.9% difference in sequence was due to 6 additional bases in the pPhy11A clone. These data might indicate that the pPhy11A cDNA was derived from a distinct oat PHYA mRNA that was very closely related to PHYA3 mRNA. Alternatively, it is possible that the pPhy11A and PHYA3 cDNAs represent the same mRNA and the difference between these sequences was due to small modifications or errors that occurred during the cloning and sequencing of one or both of the cDNAs.
Figure 1. A diagrammatic representation of the oat PHYA mRNA and the three oligonucleotide probes. A. The endogenous full-length PHYA mRNA is shown along with the percent nucleic acid sequence identity among the known PHYA mRNA types (Hershey et al., 1985b). The 5' UTR and the 3' UTR are indicated with a solid line and the coding region is indicated with an open box, the 5' cap structure (7mG) and the 3' poly(A) tail (AA(A)n) are indicated. The in vitro synthesized full-length PHYA3 mRNA (pFL) and the reference RNAs used with oligonucleotide probe A (RA) and probe 3 (R3) are aligned to their respective locations. The molecular size in kilobases (kb) is indicated to the right of each RNA. The three probes (A, 3, and 4) are aligned to their respective region of complementation. The length corresponding to 500 nucleotides (nt) is indicated. B. Sequences complementary to each of the oligonucleotide probes (A, 3, and 4) are shown for the PHYA3, PHYA4, PHYA5 mRNAs (Hershey et al., 1985b), and the in vitro synthesized pFL, RA and R3 reference RNAs. A period in the sequence indicates a nucleotide identical to that of top most PHYA mRNA sequence. The percent of complementarity to the indicated probe is given for each of the PHYA mRNAs aligned by the translation stop site. The sequence of PHYA5 mRNA 3' UTR is not available and not shown under oligonucleotide 3 and 4. C. The Bestfit program from the Genetics Computer Group (UWGCG) was used to identify the percent of maximum complementarity between the probes and the indicated mRNAs.
A

**in vitro RNAs**

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</tr>
<tr>
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<td>-</td>
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</tr>
<tr>
<td>R3:</td>
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**B**

Region complementary to probe A

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<td>pFL &amp; RA:</td>
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<tr>
<td>PHYA4:</td>
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<td>PHYA4:</td>
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Region complementary to probe 4

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<th>RNA</th>
<th>Complementarity to probe 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHYA4:</td>
<td>GGAATGTGCTGCAACCTGTGGCGGC TCAGGGTGAAAGT 100%</td>
</tr>
<tr>
<td>PHYA3:</td>
<td>CCTGG.GA.GAGG.TGGAA.T.T.CT.CAGCCTGT.GTGA 20%</td>
</tr>
<tr>
<td>pFL &amp; R3:</td>
<td>CCGTG.GA.GAGG.TGGAA.T.T.CT.CAGCCTGT.GTGA 20%</td>
</tr>
</tbody>
</table>

**C**

Maximum Complementarity
Figure 2. Estimation of the abundance of PHYA3 mRNA in the oat poly(A)+ PHYA mRNA pool. A. Oligonucleotide probes A, 3, and 4 were hybridized to in vitro synthesized pFL RNA (PHYA3-like RNA) to determine probe specificity. The full-length band was quantified for each probe and indicated as the percent of probe A. For each probe the average from two independent experiments and the standard deviation, in parenthesis, is indicated at the bottom. The blot shown was exposed to film for 12 hr. B. Poly(A)+ RNA (2 μg) from etiolated oats was mixed with equal molar amounts of either RA (2.7 ng) or R3 (2.3 ng) reference RNAs. Each RNA mixture was RNA gel blotted, hybridized with either probe A or probe 3, and exposed to film for 2 days or 12 hr, respectively. Both the endogenous full-length PHYA band and reference RNA band were quantified, and the endogenous PHYA mRNA signal was standardized to the reference RNA signal as indicated in Materials and Methods. The standardized PHYA3 signal was compared to the standardized PHYA signal to estimate the percent of PHYA3 in the PHYA mRNA Pool, as indicated at the bottom. The value indicated is the average of two independent experiments and the standard deviations are given within the parenthesis.
Although there are slight differences in the pPhyl 1A sequences as compared to the PHYA3 sequence the pPhyl 1A clone was useful in the production of a PHYA3-like reference RNA (R3) due to the 100% complementarity to probe 3. Equal molar amounts of RA and R3 were added to different samples of poly(A)+ RNA from etiolated oat seedlings, RNA gel blotted, and probed with the appropriate labeled oligonucleotide (Figure 2B). The endogenous full-length PHYA mRNAs were quantified and compared to the respective reference RNA. The PHYA3-like mRNA was estimated to be 61±3% of the total PHYA mRNA in etiolated oat seedlings by comparing the ratios of endogenous PHYA mRNA/reference RNA from probe 3 to probe A. Due to the lack of a PHYA4-like reference RNA for probe 4 the relative abundance of PHYA4 mRNA was not able to be accurately determined.

**Half-lives of distinct oat PHYA mRNAs**

Etiolated oat seedlings were treated with a R pulse to inhibit PHYA transcription followed by the isolation of poly(A)+ RNA at various time points. RNA gel blot analyses with probes A, 3, and 4 were used to estimate the full-length mRNA half-lives for the average PHYA mRNA, the PHYA3 mRNA, and the PHYA4 mRNA, respectively (Figure 3A). PHYA mRNA half-lives of ~30 min were estimated with all three probes (Figure 3C). The distinct PHYA3 and PHYA4 mRNA types had similar half-lives, indicating a conservation in the rate of mRNA degradation. In addition to the full-length PHYA mRNAs, smaller than 4-kb RNAs were detected with all three probes (Figure 3, A and B). These smaller RNAs did not show a rapid decrease in abundance after a light treatment as did the full-length PHYA mRNA, and were present in poly(A)+ RNA from 10 hr white-light treated seedlings (Figure 3B). The signal detected with each probe resulted from the abundance of the distinct PHYA mRNA and the hybridization efficiency (Figure 3, A and B).

**Sequence comparisons of PHYA mRNAs**

Sequences from PHYA mRNAs with apparently short half-lives, similar to the oat PHYA mRNAs were compared, and small conserved sequences were identified in the 5' UTR (Figure 4) and the 3' UTR (Figure 5). 5' UTR sequences from unstable PHYA mRNA were compared. Sequences for unstable PHYA mRNAs were obtained from the oat partial PHYA3, PHYA4, and PHYA5 cDNA clones (Hershey et al., 1985b), the corn PHYA genomic clone (Christensen and Quail, 1989), and the rice PHYA genomic clone (Kay et al., 1989a). After a light treatment the PHYA mRNAs in corn and rice decrease rapidly with time courses similar to the oat PHYA mRNA and indicative of unstable messages (Christensen and Quail, 1989; Kay
Figure 3. The mRNA half-lives were determined for the average of all PHYA mRNAs, and for the distinct PHYA3 and PHYA4 mRNAs in oat poly(A)+ RNA. A. Poly(A)+ RNA (3 µg) from 4-day-old etiolated oat seedlings at 0, 30, 60, and 120 min after a R pulse RNA gel blotted and hybridized with oligonucleotide probes A, 3, and 4. The molecular size markers (kb) are indicated on the left. B. Poly(A)+ RNA from either dark-grown seedlings (D) or dark-grown seedlings placed in continuous white light for 10 hr (L), to deplete the RNA samples of PHYA mRNA, were RNA gel blotted and hybridized with oligonucleotide probes A, 3, and 4. These data are representative of two independent experiments. C. The full-length PHYA mRNA bands in the R time courses, panel A, were quantified and presented as the percent of the 0 min time point on a semi-log plot as determined with each probe. These data are averages from two independent experiments and the error bars indicate the standard deviation.
Figure 4. Sequence comparison between 5' untranslated regions of unstable PHYA mRNAs from three grass species. Comparisons were made with the Pileup program in the Genetics Computer Group (UWGCG) software package (Version 7, April 1991, Madison, WI). The sequences include the ATG translation start site. The oat PHYA3 (OatPHYA3) and oat PHYA4 (OatPHYA4) mRNAs are not complete for the 5' untranslated regions. The oat sequences are from cDNA clones (Hershey et al., 1985b), the corn (Christensen and Quail, 1989) and rice (Kay et al., 1989a) sequences were from genomic clones. Dots within the sequences represent introduced gaps, and nucleotides shown with upper-case letters in the Consensus RNA are perfectly conserved among the mRNAs. Nucleotides shown in lowercase in the Consensus RNA are perfectly conserved but in a region where sequence from only two mRNAs was available.
Figure 5. Sequence comparison between 3' untranslated regions of unstable PHYA mRNAs from two grass species. Comparisons were made with the Pileup program in the Genetics Computer Group (UWGCG) software package (Version 7, April 1991, Madison, WI). The sequences include the TGA translation stop site, except for the oat PHY11A partial sequence that was missing the portion adjacent to the stop site (Barnes, 1994). The oat PHYA3 and PHYA4 sequences were from cDNA clones (Hershey et al., 1985b), and the barley PHYA (BarPHYA) sequence was also from a cDNA clone (Rahim, 1992). Dots within the sequences represent introduced gaps, and nucleotides shown with upper-case letters in the Consensus RNA are perfectly conserved among the mRNAs. Nucleotides shown in lower-case in the Consensus RNA are perfectly conserved but in a region where sequence from only two mRNAs was available.
et al., 1989b). Overall, a low identity (18%) exist among these five \textit{PHYA} mRNA 5' UTRs from three different grass species. A conserved region with 10 out of 13 identical nucleotides (77\% identical), termed PA5 (for \textit{PHYA} mRNA 5' UTR, conserved region) was identified about 46 nucleotides 5' of the ATG translation start sites.

The sequences of unstable \textit{PHYA} mRNAs with available 3' UTR sequences were compared from partial oat pPhyl1A (Barnes, 1994), \textit{PHYA3}, and \textit{PHYA4} cDNA clones (Hershey et al., 1985b), and the partial barley \textit{PHYA} cDNA clone (Rahim, 1992) (Figure 5). The barley \textit{PHYA} mRNA appears to be unstable because it decreases rapidly after a R treatment with a time course similar to the oat \textit{PHYA} mRNAs (Rahim, 1992). The length of the 3' UTRs varied among these four \textit{PHYA} mRNAs from two grass species, and the overall identity was estimated to be 27\%. Two regions with highly conserved sequence were identified, termed PA3-1 and PA3-2 (for \textit{PHYA} mRNA 3' UTR, conserved regions 1 and 2). The second conserved region, PA3-2, was the longer of the two, and had 20 identical nucleotides out of 22 (91\% identity). The 5' most four nucleotides (CTAC), of the PA3-2 region, were separated by introduced gaps in the oat \textit{PHYA} mRNAs. The barley \textit{PHYA} mRNA contained 13 additional nucleotides that separated the 5' four nucleotides (CTAC) from the 3' most 16 nucleotides, but this additional sequence (TATGTAATATGTA) appeared to be a partial duplication of the downstream 16 conserved nucleotides (TGTGTAAGGTTCA--TTT).

\textbf{Discussion}

The 40-nt long DNA-oligonucleotide probes provided sufficient hybridization specificity to be useful as gene-specific mRNA probes, and the addition of \textit{in vitro} synthesized reference RNAs allowed for the accurate determination of the abundance of the \textit{PHYA3} mRNA. The reference RNAs made it possible to correct for discrepancies in hybridization efficiencies between oligonucleotide probes A and 3. \textit{PHYA3}-like mRNA was estimated to be \textasciitilde 61\% of the oat \textit{PHYA} mRNA (Figure 2). This value seems reasonable considering that of all the oat \textit{PHYA} cDNA clones isolated the \textit{PHYA3}-like mRNAs are the most commonly represented (Hershey et al., 1985b; Barnes, 1994). The discrepancy in hybridization efficiencies, between what appeared to be very similar oligonucleotide probes, and the lack of an available \textit{PHYA4} reference RNA prevented the accurate estimation of the \textit{PHYA4} message abundance. At best, the \textit{PHYA4} mRNA would seem to make up a low percentage of the \textit{PHYA} mRNA pool in etiolated-oat seedlings.
Unlike PHYA mRNA (Colbert et al., 1985) some of the smaller than 4-kb RNAs detected in poly(A)+ RNA samples with the DNA-oligonucleotide probes did not decline after a R treatment. In addition, these smaller RNAs were present in 10 hr white-light treated poly(A)+ RNA (Figure 3B). RNA gel blots with antisense RNA probes, more sensitive than DNA-oligonucleotide probes used here, detect putative PHYA mRNA degradation products in poly(A)+ and total RNA samples, and these putative degradation products decrease in abundance after a light treatment with a similar time course as the full-length PHYA mRNA (Seeley et al., 1992; Byrne et al., 1993; Higgs and Colbert, 1994). From these observations it would appear that in addition to PHYA mRNA the DNA-oligonucleotide probes detected non PHYA RNAs.

The oat PHYA3 promoter has been shown to be transcriptionally down-regulated by light (Bruce et al., 1989; Bruce and Quail, 1990); this has also been shown for the overall transcription of the oat PHYA genes (Lissemore and Quail, 1988). We estimated that the PHYA3-like mRNA, the most abundant PHYA mRNA in etiolated seedlings, had a half-life of ~30 min. Similar values were estimated for the average PHYA mRNA half-life and the distinct PHYA4 mRNA half-life (Figure 3). The in vivo oat PHYA mRNA half-life has previously been estimated as ~1 hr (Seeley et al., 1992). The difference in the mRNA half-life estimates may be due to the greatly lowered sensitivity of RNA gel blots with oligonucleotide probes used here as compared to the previous report that used more sensitive RNA probes. The large decrease in signal strength with the oligonucleotide probes resulted in a reduced signal to background ratio, reducing the accuracy of mRNA half-life estimates as compared to estimates with RNA probes.

The uniformity in transcriptional down-regulation and mRNA instability between the average PHYA messages and the distinct PHYA3 message might suggest that all PHYA mRNAs are coordinately down-regulated by light. The high abundance of the PHYA3 mRNA would support the use of this oat PHYA gene or mRNA in experiments investigating either transcriptional and post-transcriptional regulation, as has already been done with PHYA3 promoter studies (Bruce and Quail, 1990; Bruce et al., 1991). The existence of either oat PHYA genes that are not transcriptionally down-regulated or PHYA mRNAs that are stable can not be ruled out. However, it seems certain that if present such PHYA genes or messages, not down-regulated by light, would make up a very small percentage of the total PHYA mRNA pool because 5 hr after a light treatment the PHYA mRNA abundance is reduced by greater than 90% (Colbert et al., 1985).
Conserved sequences in the 3' UTR of a number of mRNAs have been shown to function as eukaryotic mRNA instability determinants, including the DST element in plant SAUR transcripts (Newman et al., 1993) and most notably the A+U rich elements first identified in mammalian protooncogene mRNAs (Peltz et al., 1991). This A+U rich element also functions as an instability determinant in the 3' UTR of plant mRNAs (Ohme-Takagi et al., 1993). The involvement of coding region and 5' UTR instability determinants has been reported for eukaryotic mRNAs (Peltz et al., 1991). The similarity in the mRNA half-lives between the distinct oat PHYA3 and PHYA4 mRNAs, and the average oat PHYA mRNA, suggests a conservation of mRNA degradation mechanisms. Putative, instability determinants might be conserved and located anywhere within the PHYA message. Sequence comparisons of 5' UTRs and 3' UTRs from known unstable PHYA mRNAs identified several regions with highly conserved sequences. A single region, PA5, in the 5' UTR and two regions, PA3-1 and PA3-2, in the 3' UTR had high sequence identity. Of these, the PA3-2 sequence in the 3' UTR was the most conserved (91% identity) over a stretch of 22 nucleotides. The functional importance of these sequences remains to be determined, but it is possible that they might be involved in PHYA mRNA degradation. These sequences might also be involved in any number of processes that regulate PHYA expression, such as transcription, RNA processing, or translation initiation.

Acknowledgments

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References


CHAPTER 4. OAT PHYTOCHROME A mRNA DEGRADATION APPEARS TO OCCUR VIA TWO DISTINCT PATHWAYS


David C. Higgs and James T. Colbert

Abstract

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 contains a pattern of putative degradation products that was 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 ranges from ~14 to 220 nucleotides. Early PHYA mRNA degradation events did not appear to involve site-specific endoribonucleases. About 25 percent 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 were polysome associated.
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 (Cameiro 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 Vreken and Raué, 1992). 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 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 codes for 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 about 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 due to 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 and both remain 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, which were 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
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 - 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 are representative of two independent experiments.
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 ribosomal RNAs, 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 approximately 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 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 contain 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)^− 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.
Figure 2. Poly(A)+ selection does not degrade in vitro-synthesized, labeled GUS mRNA nor 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 minute (1200) of GUS mRNA was 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 are representative of 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 are representative of two independent experiments.
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 endoribonucleolytic cleavage occurs 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 a 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 of detecting 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 - 6). In total, poly(A)^+, poly(A)^-, and polysomal RNA a band of 888±11 nucleotides was detected (Figure 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
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 - 6 contain 10 μg total RNA; 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 are representative of two independent experiments.
isolation. No RNase H cleavage product was detected in this RNA sample (Figure 3A, lanes 1 and 2).

**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 due to 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.

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
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 (1 pass), and poly(A) reselected. Again, the poly(A)\(^{-}\) faction was collected (2 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 (1 - 4 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 spectrosopy. The amount of radioactivity is indicated as a percent of total [2323 counts per minute for the blot in (A)]. The mean of two independent experiments was determined and error bars indicate the standard deviation.
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 percent of the respective total RNA. From the blot in (A), the counts per minute 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 means from two independent experiments and error bars indicate the standard deviation.
(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-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 - 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.
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)12-18 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 3-7, 10 µg of total RNA; and lanes 8-10, 1 µg of poly(A)+ RNA. 

(B) Lanes 1-3 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 are representative of two independent experiments.
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 determined in samples that included oligo(dT) for each type of RNA, from the size of the adenylated cleavage products, as 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, while 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.

In all 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 the 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, due to 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 (XRNI and HKEI/RATI) 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 (Hsu and Stevens, 1993; Stevens and Maupin, 1987). 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; Stoeckle and Hanafusa, 1989; Binder et al., 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 polyosome-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 \textit{PHYA} mRNA in the 3' untranslated region, it seems more likely that an enzyme similar to poly(A) nuclease might exoribonucleolytically deadenylate \textit{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 \textit{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 \textit{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 \textit{PHYA} mRNA. Both models include two pathways of \textit{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 the first model (a), the polyadenylated \textit{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 \textit{PHYA} message by the hydrolysis of mononucleotides. The abundance of the \textit{PHYA} RNA fragments in poly(A)+ RNA was high, suggesting that a high percentage of \textit{PHYA} messages are degraded by this pathway. This pathway appears capable of degrading the entire \textit{PHYA} mRNA molecule to mononucleotides.

The second pathway of model (a) is involved in degrading the poly(A)-deficient \textit{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 \textit{PHYA} mRNA appears to be completely degraded by a combination of a 5' -> 3' exoribonuclease and a 3' -> 5' exoribonuclease. From the \textit{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 \textit{PHYA} mRNA.

Currently, there is only indirect evidence for a 3' -> 5' exoribonuclease degradation of poly(A)-deficient \textit{PHYA} mRNA. Frequently, deadenylation of an mRNA precedes
Figure 7. Two tentative models for the degradation of \textit{PHYA} mRNA in oat seedlings.

Ribosomal subunits are indicated by filled ovals. 7mG indicates the position of the 7-methyl guanosine 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.
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 the second 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 endoribonucleases, with very little or no specificity to internal PHYA mRNA sequence or structure; or 2) a stochastic endoribonuclease with some specificity followed, very quickly, by rapid exoribonucleases, such that the immediate products of a stochastic endoribonucleases 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, prepared by a large-scale SDS-phenol procedure (Dean et al., 1985). Polysomal RNA was isolated as described (Byrne et al., 1993). RNA gel blots, hybridization with anti-sense 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 (Brewer and Ross, 1988). For analysis of the 5' terminus of PHYA mRNA a DNA oligomer of 22 nucleotides was synthesized (5'-dTGATTTCGGAGAGAATACCTCA-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 RNA isolation, was analyzed. The RNA samples were incubated at 90°C for 8 min in a total volume of 20 μl (in 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 MgCl₂, 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'-dTCTGACTGCTCCTTGTTCTCCTCC-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 anti-sense 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, (L.J. 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 Sacl-KpnI fragment from pFLII into the pBluescript KSII+ (Stratagene) plasmid. Probe 1 was synthesized from 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 has 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 Sacl-linearized template with T3 RNA polymerase.

The human H4 histone genomic clone, pHh4A-SP1 (Peltz et al., 1987), was EcoRI-linearized and anti-sense RNA probe was synthesized with SP6 RNA polymerase. The 1.4-kb oat β-tubulin partial cDNA clone, pGB1, was EcoRI-linearized and anti-sense RNA probe was synthesized with T7 RNA polymerase (Colbert et al., 1990). An oat actin cDNA clone, pOA24 (L.J. Barnes, 1994), was isolated using a soybean actin probe (Shah et al., 1982). The pOA24 plasmid was HindIII-linearized and anti-sense RNA probe was synthesized with T3 RNA polymerase.

The pSGUS template containing the β-glucuronidase (GUS) transcribed region was EcoRI linearizing, and low-specific-activity 32P-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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CHAPTER 5. PHYTOCHROME A mRNA-PROTEIN INTERACTIONS

Introduction

RNA-protein interactions are continuously occurring throughout the life of a cytoplasmic mRNA, from transcription through degradation. The precise control of these interactions is critical to the processes of RNA capping and polyadenylation, splicing, nuclear-transport, and translation, and involves many different nuclear and cytoplasmic proteins (Burd and Dreyfuss, 1994). RNA-protein interactions have also been shown to be important for the regulation of mRNA stability (Peltz et al., 1991; Sachs, 1993).

Messenger RNA instability determinants commonly act as binding sites for trans-acting protein factors. The human transferrin receptor mRNA has a series of stem-loops in the 3' untranslated region (UTR) that are involved in regulating the message stability according to the cellular iron concentration (Koeller et al., 1991). These stem-loops are collectively termed the iron regulatory element (IRE), and are the binding site for the iron regulatory element-binding protein (IRE-BP) that stabilizes the mRNA when bound (Harford, 1993). The binding of this IRE-BP protein is controlled by the iron concentration. In the presence of high amounts of iron the IRE-BP does not bind, causing destabilization of the transferrin receptor mRNA. A cis-acting A+U-rich element (ARE) has been shown to destabilize mRNAs (Peltz et al., 1991), including plant mRNAs (Newman et al., 1993). Different proteins, termed AU-BPs, have been identified that bind to the A+U-rich element (Malter, 1989; Brewer, 1991; Vakalopoulou et al., 1991; Bohjanen et al., 1992; You et al., 1992) and appear to be involved in regulating mRNA stability (Brewer, 1991; Vakalopoulou et al., 1991; You et al., 1992). It is not known how these AU-BP affect the stability of mRNA molecules (Grenberg and Belasco, 1993). The mammalian cell-cycle regulated histone mRNAs are nonpolyadenylated and instead have a stem-loop at the 3' terminus (Marzluff and Hanson, 1993). This stem-loop regulates the mRNA stability according to the cell-cycle, and an RNA-binding protein has been shown to specifically bind to this stem-loop (Pandey et al., 1991).

In prokaryotic organisms and eukaryotic organelles RNA-binding proteins are important for controlling message stability. The majority of prokaryotic mRNAs are thought to have 3' stem-loops (Higgins et al., 1993), and the removal of this stabilizing 3' stem-loop would produce an mRNA that would be degraded within seconds by 3' to 5' exoribonucleases (Belasco, 1993). The highly conserved repetitive extragenic palindromic (REP) sequences...
from *Escherichia coli* are well-characterized mRNA stabilizing stem-loops. The REP from the *malE-malF* intergenic region is a 90-nt sequence that can potentially fold into two stable forms. One form is a large single stem-loop that has a $\Delta G^0$ value of -56 kcal/mol (Higgins et al., 1993). The second form has two stem-loops with $\Delta G^0$ values of -21.7 and -22.8 kcal/mol, respectively. The REP stabilizing structure is believed to block 3' to 5' exoribonucleases (PNPase or RNase II), and the 3' terminus of these prokaryotic mRNAs map to the 3' base of the stem-loop, as would be expected. *In vivo* the REP stem-loops can impede mRNA degradation eight times longer than it can *in vitro* (Higgins et al., 1993). Presumably this increase in stability *in vivo* is due to cellular RNA-binding proteins that enhance the stabilizing effect of this element.

Chloroplast mRNAs are similar to prokaryotic mRNAs because they lack poly(A) tails and have stabilizing 3' stem-loops. The *petD* mRNA that encodes the cytochrome b6f subunit IV from spinach chloroplasts contains a 3' stem-loop that stabilizes that message (Gruissem and Schuster, 1993). A 63-nt region contains the *petD* 3' stem-loop with a theoretical $\Delta G^0$ value of -28.1 kcal/mol. This sequence was also shown to be a binding site for proteins that might be involved in stabilizing the stem-loop (Stern et al., 1989). The spinach *petD* 3' stem-loop can stabilize the chloroplast *aptB* mRNA in the green alga *Chlamydomonas reinhardtii* (Stern et al., 1991). This indicates that the spinach *petD* 3' stem-loop functions autonomously in chloroplasts from an evolutionarily distant organism.

In animals, plants, and yeast, poly(A)-binding proteins (PABP) have been identified (Sachs et al., 1987; Bernstein and Ross, 1989; Yang and Hunt, 1992). The function of the PABP is not completely understood (Matunis et al., 1993). In mammals the PABP appears to protect the poly(A) tail from ribonuclease degradation, but, in contrast, the yeast PABP is required for poly(A)-tail degradation (Sachs, 1993). Also, the PABP is involved in the poly(A) tail-dependent translation, suggesting that the PABP interacts with the 5' cap structure and ribosome initiation (Gallie, 1991). RNA-binding proteins have also been identified that interact with specific mRNA sequences for which the function is not known. Aside from the PABP, only one cytoplasmic RNA-binding protein has been reported in plants (Zhang and Mehdy, 1994). This RNA-binding protein has been shown to specifically associate with the 1-kb full-length *Phaseolus vulgarus* proline-rich protein 1 (PvPRP-1) mRNA (Zhang and Mehdy, 1994). This PvPRP-1 mRNA is down-regulated by the addition of a fungal elicitor, and the binding of the PvPRP-binding protein (PvPRP-BP) is induced by the fungal elicitor, suggesting that the PvPRP-BP might be involved in destabilizing that message (Zhang and Mehdy, 1994). Truncated RNAs were used to determine that a 27-nt sequence region in the 3'
UTR is part of the binding site for the PvPRP-BP, but this 27-nt region has not been established as a functional mRNA instability determinant. It is interesting to note that this 27-nt binding site is uridine-rich and contains a single copy of the consensus AUUUA sequence, present in the mammalian protooncogene ARE instability determinant (Zhang and Mehdy, 1994).

From studies involving many types of RNA-protein interactions a number of RNA-binding motifs have been identified. The most widely found and best characterized RNA-binding motif is the ribonucleoprotein (RNP) motif, also termed the RNA recognition motif (RRM), the RNP consensus sequence (RNP-CS), or the consensus sequence RNA-binding protein (CS-RNP) (Kenan et al., 1991; Burd and Dreyfuss, 1994). This RNP motif is ~90 to 100 amino acids in size with four predicted β-sheets and two predicted α-helixes, and the RNP motif is present in one or more copies. Two short amino acid sequences, RNP-1 and RNP-2, are the identifying features of the RNP motif, but not all RNP motifs have the canonical RNP-1 or RNP-2 sequences (Burd and Dreyfuss, 1994). The consensus RNP-1 sequence is (Lys/Arg)-Gly-(Phe/Tyr)-(Gly/Ala)-Phe-Val-X-Phe and found in one of the predicted β-sheets (Burd and Dreyfuss, 1994). The RNP-2 sequence is also found in one of the predicted β-sheets with a variable region of about 26 to 38 amino acids between it and the RNP-1 sequence. The two common consensus sequences for RNP-2 are Leu-Phe-Val-Gly-Asn-Leu and Ile-Tyr-Ile-Lys-Gly-Met (Burd and Dreyfuss, 1994). A number of variable regions exist within the RNP, and it is these variable regions that are important for RNA-binding specificity. PABP, some heteronuclear RNPs (hnRNPs), U1A and U2B small nuclear RNPs (snRNPs), the eukaryotic translation initiation factor 4B (eIF4B), and two 33-kD tobacco chloroplast proteins with unknown functions are some of the proteins that have the RNP motif (Kenan et al., 1991; Burd and Dreyfuss, 1994).

Other RNA-binding motifs have been identified. The arginine-rich motif (ARM) is a 10 to 20 amino acid sequence rich in arginine, and is present in viral, bacteriophage, and ribosomal proteins that bind RNAs (Burd and Dreyfuss, 1994). A 20 to 25 amino acid long motif with closely spaced Arg-Gly-Gly (RGG) is termed the RGG motif that has been found in the hnRNP U protein (Burd and Dreyfuss, 1994). A number of RNA-binding proteins have high homology with the human hnRNP K protein over a ~60 amino acid sequence and contain the hnRNP K homology (KH) motif. An interesting note is that the common human genetic mutation of the fragile X mental retardation-1 protein has a change in one of the highly conserved amino acid in the KH motif, suggesting functional importance (Burd and Dreyfuss, 1994). Finally, a double-stranded RNA binding motif (DSRM) is conserved among at least
eleven proteins that bind double-stranded RNA. The DSRM is ~70 amino acids in size and is present in one or more copies (Burd and Dreyfuss, 1994). Conserved positions include the two basic amino acids, Arginine and Lysine, and some hydrophobic amino acids.

The identification and specificity of RNA-binding proteins are determined primarily by two techniques, RNA-gel shift assays and ultra-violet light (UV) crosslinking assays. The RNA-gel shift assay involves incubating small radiolabeled RNAs with protein extracts to form RNA-protein complexes. This mixture is electrophoresed in non-denaturing polyacrylamide gels, and the labeled RNA-protein complexes migrate more slowly than the free labeled RNA, producing detectable bands of high molecular mass. To determine the specificity of the RNA-protein complex non-labeled specific and non-specific RNAs are added to compete with the labeled RNA for the binding of proteins. UV crosslinking assays involve incubating internally radiolabeled RNAs with protein extracts to allow the formation of RNA-protein complexes. These complexes are then exposed to ultra-violet light to form covalent bonds between the labeled RNA and the associated proteins, followed by ribonuclease digestion of the RNA. The bound protein protects regions of the RNA from digestion, leaving small labeled fragments of RNA covalently bound to the protein. These proteins are then electrophoresed in denaturing SDS-polyacrylamide gels to determine the intensity and molecular mass of the labeled RNA-binding proteins. To determine binding specificity non-labeled specific and non-specific RNAs can be added prior to UV treatment to compete with the labeled RNA for the binding of the proteins.

RNA gel-shift assays require RNA-protein complexes that are not denatured during electrophoresis, and because of this RNA gel-shift assays place an additional demand upon the RNA-protein complex as compared to UV crosslinking assays. In general fewer non-specific RNA-binding proteins are detected with RNA gel-shift assay, but UV crosslinking assays provide an estimate of the RNA-binding protein molecular weight. Finally, large labeled RNAs can be used in UV crosslinking experiments; these RNAs are eventually degraded, except for the portion of the RNA bound to and protected by the RNA-binding protein. For these reasons the UV crosslinking assay seemed to be the better method for initial investigations of PHYA RNA-binding proteins.

Full-length, radiolabeled PHYA mRNA was in vitro synthesized and used in UV crosslinking experiments with oat coleoptile total protein extracts and EDTA-treated polysome extracts. The RNA-protein binding conditions and specificity were investigated. Two apparent RNA-binding proteins were consistently identified but the binding specificity of these proteins could not be satisfactorily determined.
Materials and Methods

Protein isolation

Total soluble protein was isolated from 4-day-old dark-grown oat seedlings (Avena sativa cv Garry) by a method that yields photoreversible phytochrome from oats (Vierstra and Quail, 1982; Vierstra et al., 1984) and active DNA binding proteins from maize (Tirimanne and Colbert, unpublished data). To 10 g of oat coleoptiles 3 ml of cooled extraction buffer was added [100 mM MOPS, 5 mM EDTA, 28 mM β-mercaptoethanol, 4 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor, prepared just prior to addition)] and the coleoptiles were ground on ice. The extract was filtered through three layers of cheese cloth and centrifuged at 48,000 x g for 20 min at 4°C. The supernatant was removed and 3 M CaCl2, pH 7.4 was cooled to 4°C and added to the supernatant to a final concentration of 15 mM; this was stirred at 4°C for 10 min to precipitate cellular membranes. The membranes were pelleted by centrifuging at 48,000 x g for 20 min at 4°C, and the supernatant was aliquoted into small tubes to be stored at -80°C. The Bradford assay was used to quantitate total protein (Bradford, 1976). Typical yields were 5 μg/μl of protein, as compared to an acetylated bovine serum albumin (BSA) standard (Promega).

Polysome extracts were isolated as indicated (Byrne et al., 1993) and stored at -20°C. Protein abundance was determined with the Bradford protein assay (Bradford, 1976). Polysomes were treated with EDTA by adding 0.5 M EDTA, pH 8.0 to a final concentration of 50 mM, and incubating 1 hr on ice (Pastori and Schoenberg, 1993).

In vitro RNA synthesis

Both radiolabeled and non-radiolabeled RNAs were synthesized in vitro. High specific activity, sense PHYA RNA (3.8 kb) was uniformly radiolabeled by first linearizing the pFLII plasmid (Barnes, 1994) with XhoI, and synthesizing RNA with T7 RNA polymerase (Promega) in a low concentration of non-labeled rUTP plus [α32P]-rUTP (>3000 Ci/mmol), as indicated by manufacturer (Promega). The in vitro synthesized RNA was separated from unincorporated nucleotides by passing through a Bio-Gel P-60 (100 - 200 mesh) exclusion column (1 g/ml of elution buffer) (Bio-Rad) with an elution buffer (20 mM Tris-HCl, 1 mM EDTA, and 0.5% SDS), and then ethanol precipitated and dissolved in ribonuclease free H2O (Sambrook et al., 1989). RNAs were quantified by scintillation spectroscopy to determine radioactive counts per minute (cpm) and by spectrophotometric analysis, assuming 1 A260 = 40
μg RNA/ml, to determine RNA mass. Non-radiolabeled full-length, sense PHYA RNA was synthesized by the same method except that the amount of non-labeled rUTP equaled the other three nucleotides and labeled rUTP was not added. Both labeled and non-labeled β-glucuronidase (GUS) sense RNA (1.9 kb) was synthesized from the plasmid pSGUS (Higgs and Colbert, 1992) by linearizing with EcoRI and polymerized with SP6 RNA polymerase (Gibco-BRL) as indicated above for PHYA RNA. Two partial PHYA RNAs were synthesized as non-labeled molecules. A 1.7-kb sense PHYA 5' half RNA was produced from the PvuII 1.7-kb fragment of the pFLII plasmid (Barnes, 1994) digested with T7 RNA polymerase. A 2.2-kb sense PHYA 3’ half PHYA RNA was produced from the Xhol linearized pPhyl 1A plasmid with T3 RNA polymerase (Barnes, 1994). Finally, the pOA24 oat actin cDNA clone (Barnes, 1994) was used to synthesize a 1.2-kb antisense actin RNA by HindIII digested and T3 RNA polymerase. The concentration in fmoles of the different in vitro synthesized RNAs was calculated by assuming that the average RNA nucleotide = 320 g/mole and by knowing the molecular weight of each RNA. For example, the 3.8-kb in vitro synthesized PHYA RNA is equal to 0.82 fmol/ng.

Ultra-violet light crosslinking

The standard ultra-violet light (UV) crosslinking experiment is presented, and specific alterations are indicated in the figure legends. Total protein extracts (20 μg) and labeled, in vitro synthesized, sense PHYA RNA (10 fmoles) were added to 40 mM KCl, 10 mM HEPES buffer, pH 7.6, 3 mM MgCl₂, 1 mM dithiothreitol (DTT), and 5% glycerol to give a final volume of ~12 μl and incubated for 30 min at room temperature (22°C) to allow RNA-protein complexes to form (Bohjanen et al., 1992). Each sample was spotted onto a baked Pyrex petri dish and UV crosslinked (254 nm light) for 10 min in a Stratalinker 1800 (Stratagene) on ice. Samples were transferred back to microcentrifuge tubes and unprotected RNA was degraded by adding RNase T1 (20 units) and RNase A (0.5 units) (Ambion) followed by an incubation for 30 min at 37°C. Sample buffer (6X) (Ausubel et al., 1987) for SDS-polyacrylamide gel was added and proteins were denatured by heating for 3 min at 90°C. Samples were electrophoresed in a 4% stacking/10% separating SDS-polyacrylamide gel (Ausubel et al., 1987). Gels were stained for 2 hr in 0.25% (w/v) Coomassie blue (Sigma, R250), 45% methanol, 10% glacial acetic acid, and destained overnight in the same solution without the dye with three changes of the destain solution. Gels were placed on 3MM Chromatography Paper (Whatman) and vacuum dried for 1 hr at 65°C, followed by exposure to x-ray film at -80°C with an intensifying screen (Du Pont).
Results

It was first necessary to establish conditions that resulted in the crosslinking of full-length, *in vitro* synthesized, labeled *PHYA* RNA to oat proteins. The synthesized *PHYA* RNA lacked a 3' poly(A) tail and 5' cap so that proteins that associate with either of these two structures should not complex with the labeled *PHYA* RNA. The addition of increasing amounts of an oat total-protein extract to the incubation buffer containing labeled *PHYA* RNA produced increasing intensities of several distinct bands (55 kD, 40 kD, and 25 kD) and the strongest signals occurred when 20 μg of protein were added, suggesting that the detected signals result from RNA-protein complexes (Figure 1, lanes 2 to 4). The overall background signal in the control lane without protein was high. The high background might have been due to either ribonucleases in the added protein extract that helped degrade non-complexed labeled *PHYA* RNA or that in the absence of oat proteins the *PHYA* RNA forms secondary structures that are crosslinked and resistant to RNase T1 digestion alone, for RNase A was not included in this experiment. A 10 min UV (254 nm) crosslinking treatment resulted in the highest signal to noise ratio as compared to 5 min and 20 min treatments (data not shown). Magnesium (Stolow and Berget, 1990) and dithiothreitol (DTT) (Zhang and Mehdy, 1994) concentrations have been shown to affect RNA-protein interactions. Preliminary experiments indicated that 1 mM magnesium concentration resulted in a slightly higher 25-kD signal than with the standard 3 mM magnesium and different DTT concentrations (0 to 25 mM) had no apparent effect (data not shown). The addition of 25X molar excess of either *PHYA* or GUS non-labeled competitor RNAs resulted in a decrease in both the 55-kD and the 40-kD bands (Figure 1, lanes 5 and 6), but not the 25-kD band.

Further investigation into the source of the high background observed in the no-protein control treatment was necessary. The addition of RNase T1 and RNase A decreased the background signal considerably, indicating that the high background observed in Figure 1 was caused, in part, by insufficient amounts of added ribonuclease (data not shown). More controls were tested to determine if the detected bands were dependent upon active protein. The lack of the distinct bands (55 kD, 40 kD, and 25 kD) in the samples that either had no protein extract added or had boiled protein extract added prior to mixing with *PHYA* RNA is evidence to support the conclusion that these detected bands resulted from RNA-binding protein interactions (Figure 2, lanes 1 to 3). Surprisingly when *PHYA* RNA-protein complexes were allowed to form but not UV crosslinked the diffuse signals around the 55-kD
Figure 1. A UV crosslinking experiment between oat total protein extracts and full-length in vitro synthesized *PHYA* RNA to identify RNA-binding proteins. No protein extract was added to the sample in lane 1. To the labeled RNA, 2 μg (lane 2), 10 μg (lane 3), and 20 μg (lanes 4 to 6) were added. To determine binding specificity 25X molar excess of a non-labeled specific (*PHYA*)RNA (lane 5) or non-specific (GUS) (lane 6) RNAs were added. Specific RNA-binding proteins are indicated on the left, and molecular weight markers (Gibco-BRL) are indicated on the right. The gel was exposed to x-ray film for 4 days.
Figure 2. A UV crosslinking experiment with labeled PHYA RNA to determine RNA-binding proteins. The presence (+) or absence (-) of total protein extract (20 μg) and a crosslinking treatment (XL) is indicated for each lane. In lane 3 the added protein extract was boiled (b) for 10 min then placed on ice for 3 min, prior to mixing with PHYA RNA. Specific RNA-binding proteins are indicated on the left, and molecular weight markers (Gibco-BRL) are indicated on the right. The gel was exposed to x-ray film for 12 hr.
and 40-kD regions were still detected (Figure 2, lane 5). The small 25-kD band was detected only in the presence of active proteins and after UV crosslinking, indicating that it is likely to be a UV-crosslink-labeled protein (Figure 2, lane 6), although the signal at this band is not competed away with 25X molar excess of specific or non-specific competitor RNAs (Figure 1, lanes 5 and 6). The 55-kD and 40-kD bands could have resulted from either the protection of the PHYA RNA from ribonucleases by RNA-binding proteins and the migration of free PHYA RNA fragments that corresponded to the 55-kD and 40-kD bands, or from the less likely event of non-covalent RNA-protein interactions that were maintained even after denaturing by a 90°C treatment in SDS and electrophoresis in the SDS-polyacrylamide gel. This observation puts into question whether the 55-kD and 40-kD bands are labeled proteins and may instead be detection of free RNA fragments, as has been reported to occur in other UV crosslinking experiments (Furuya and Lai, 1993). Full-length, labeled PHYA RNA remained primarily in the well of a 10% SDS-polyacrylamide gel (data not shown). Results from the non-crosslinked-plus-protein controls, as described here, are less frequently reported, so it is difficult to know how wide spread this possible protein-protection in the absence of covalent bonds effect is in UV crosslinking experiments.

The addition of excess specific (PHYA) and non-specific (GUS) non-labeled competitor RNAs greatly reduced the signal at the 55-kD and 40-kD bands (Figure 1, lanes 5 and 6), indicating that this putative protection of PHYA RNA from ribonucleases by oat RNA-binding proteins can be competed away and is non-specific. To analyze the quality of possible RNA fragments after ribonuclease digestion PHYA RNAs were incubated in the presence or absence of the protein extract then ribonuclease digested without UV crosslinking. Instead of SDS-polyacrylamide gel analysis these mixtures were treated with phenol/chloroform to remove proteins and the resultant RNA was ethanol precipitated and analyzed in a 3% NuSieve 3:1 agarose/3% formaldehyde gel. The RNA from the treatment without oat protein was degraded while the PHYA RNA incubated with oat protein contained some full-length RNA (data not shown). This preliminary experiment would suggest non-crosslinked oat RNA-binding proteins are remaining bound and protecting the PHYA RNA from RNase A and T1 degradation.

Heparin, a poly-anion, mimics RNA and acts as a general non-specific competitor of RNA-protein interactions (Bohjanen et al., 1992; You et al., 1992). The effect of heparin on PHYA RNA-oat protein interactions was tested (Figure 3). In addition to the 55-kD, 40-kD, and 25-kD bands, larger faint bands (~85 kD, ~95 kD, and ~110 kD) were also detected in these experiments (Figure 3, lane 2). The addition of heparin (1 µg/µl) decreased the intensity
Figure 3. A UV crosslinking experiment with labeled PHYA RNA and increasing amounts of competitor RNAs. The presence (+) or absence (-) of heparin (1 μg/μl), total protein extract (20 μg), and competitor RNA is indicated for each lane. The amount of competitor RNA in molar excess (10, 25, and 50X) of the labeled PHYA RNA (10 fmoles) is indicated for the non-labeled specific (PHYA) and non-specific (GUS) RNAs, lanes 4 to 9. In lane 10, 3X more labeled GUS (G) RNA (30 fmoles) was added in place of labeled PHYA RNA (10 fmoles). Specific RNA-binding proteins are indicated on the left, and molecular weight markers (Gibco-BRL) are indicated on the right. The gel was exposed to x-ray film for 4 days.
of the 85-kD and 110-kD bands, but it increased the intensity of the 25-kD band (Figure 3, lane 3). In the presence of heparin, non-labeled specific (PHYA) and non-specific (GUS) RNAs were added to be in 10, 25 and 50X molar excess of the labeled PHYA RNA (Figure 3, lanes 4 to 9). The 95-kD and 40-kD bands were affected little by either the specific or the non-specific competitors. The 25-kD signal appeared to be reduced more with 10X and 25X excess of the specific competitor than with the same level of the non-specific competitor (Figure 3, lanes 4 and 5 compared to lanes 7 and 8). These data would indicate a slight specificity of the 25-kD protein for the PHYA RNA. To determine the binding activity of a protein to a labeled, non-specific RNA is a second method of investigating the RNA-binding specificity. This was done by adding 3X excess amounts (30 fmoles) of labeled, in vitro synthesized GUS RNA (Figure 3, lane 10). The addition of labeled GUS RNA resulted in the detection of the 95-kD, 55-kD, 40-kD and 25-kD bands. These putative GUS RNA-protein interactions might appear to be slightly less specific than the PHYA RNA-protein interactions because similar signal intensities were observed when 3X more GUS RNA was added. It is also possible that the GUS RNA sequences that were bound to and protected by RNA-binding proteins contain a low percent of labeled-UTP nucleotides as compared to the binding site of the PHYA RNA. A lower percentage of labeled-UTP in the binding site would effectively reduce the specific activity (cpm/µg of RNA), resulting in a lower signal intensity after ribonuclease treatment even if the GUS RNA-protein interaction was as strong as the PHYA RNA-protein interaction. All the putative protein bands detected with both labeled PHYA RNA and GUS RNA appeared to result from non-specific RNA-protein interactions.

A second type of non-specific competitors commonly added to UV crosslinking experiments include yeast total RNA (You et al., 1992), yeast tRNA (Furuya and Lai, 1993), and Escherichia coli tRNA (Stolow and Berget, 1990). The effect of yeast tRNA (0.25 µg/µl) on PHYA RNA-protein interactions was tested (Figure 4, lanes 2 and 3). The tRNA non-specific competitor appeared to reduce the signal only at the 25-kD band and had little effect on other bands. When heparin was added in combination with tRNA the 25-kD signal increased but not to the same intensity as when tRNA was not added (Figure 4, lanes 3 and 4). The mechanism by which heparin would increase the 25-kD signal is unknown.

An experiment to test the effects of specific and non-specific competitor RNAs was repeated (Figure 4, lanes 5 to 10). Addition of 30 and 60X molar excess of the specific (PHYA) and the non-specific (GUS) RNAs lead to an equivalent decrease in the 25-kD signal. The addition of 30 and 60X molar excess of a second non-specific competitor RNA (antisense actin) did not result in an equivalent decrease in the 25-kD signal (Figure 4, lanes 9 and 10) as
Figure 4. A UV crosslinking experiment with labeled PHYA RNA to test the effects of heparin, yeast tRNA, and competitor RNAs on the RNA-binding proteins. The presence (+) or absence (-) of total protein extract (20 μg), heparin (1 μg/μl), yeast tRNA (0.25 μg/μl), and non-labeled competitor RNAs is indicated for each lane. Both 30 and 60X molar excess amounts of the non-labeled specific (PHYA) and non-specific (GUS and antisense actin) competitor RNAs were added. Specific RNA-binding proteins are indicated on the left, and molecular weight markers (Gibco-BRL) are indicated on the right. The gel was exposed to x-ray film for 2 days.
compared to the PHYA specific competitor (Figure 4, lanes 5 and 6). These data would indicate that the putative 25-kD protein has some affinity for the PHYA RNA and the GUS RNA, but a poor affinity for the antisense actin RNA.

Non-labeled in vitro synthesized PHYA 5' half and PHYA 3' half RNAs were added in molar excess to investigate the binding location of the putative PHYA RNA-binding proteins. These experiments were analyzed in 15% versus 10% SDS-polyacrylamide separating gels. Increasing amounts of full-length PHYA RNA (25 and 250X molar excess) resulted in the decrease in signal at the 25-kD band whereas the same molar excess of the non-specific antisense actin RNA appeared to cause less of a decrease in signal at the 25-kD band (Figure 5, lanes 1 to 3 compared to lanes 4 to 6). The addition of 250 and 500X molar excess of the PHYA 5' half RNA did not appear to compete with the labeled full-length PHYA RNA for binding to the putative 25-kD protein as well as the non-labeled full-length PHYA RNA (Figure 5, compare lanes 3 and 7). On the other hand the addition of 250 and 500X molar excess of the PHYA 3' half RNA appeared to compete away the 25-kD signal similarly to the full-length PHYA RNA and slightly better than the PHYA 5' half RNA. These data would suggest that the putative 25-kD RNA-binding protein binds to the 3' half of the PHYA RNA with higher affinity.

PHYA mRNA is degraded in association with polysomes (Byrne et al., 1993; Higgs and Colbert, 1994). Therefore, polysome fractions might appear to be a good source of PHYA mRNA-binding proteins that might be involved in mRNA degradation. Oat polysome extracts were incubated with labeled PHYA RNA in place of total oat protein, UV crosslinked, and analyzed. In addition to proteins the polysome extracts contained RNA in the forms of ribosomal RNAs and messenger RNAs. The affect of these RNAs from the polysome extract on the UV crosslinking assay is not known. Initial experiments with polysome extracts resulted in high background and poorly resolved bands (data not shown). The chelating action of EDTA can cause dissociation of ribosomal subunits and proteins loosely associated with polysomes (Pastori and Schoenberg, 1993). A similar EDTA treatment of oat polysomes was conducted prior to using the polysomes in UV crosslinking experiments (Figure 6). The effect of increasing heparin concentration was also tested with the EDTA-treated polysomes (Figure 6). A number of distinct bands were observed, two bands of medium signal intensity at ~50 kD and ~30 kD, a faint ~35-kD signal, and a band with strong signal intensity at ~20 kD (Figure 6, lanes 3 to 5 and 7 to 9). Two control treatments, a crosslinked RNA without polysomes and a non-crosslinked RNA with polysomes, both resulted in some background signal. Background in the 35-kD to 65-kD size range might have been caused by the protection
Figure 5. A UV crosslinking experiment with labeled PHYA RNA to test the effects 5' and 3' PHYA RNA halves as competitor RNAs. Total protein extract (20 μg), heparin (1 μg/μl), and yeast tRNA (0.25 μg/μl) were added to each sample. Samples in lanes 1 and 4 did not include non-labeled competitor RNAs. Non-labeled specific (PHYA) and non-specific (antisense actin) RNAs were added as 25 and 250X molar excess amounts of the labeled PHYA RNA. Both 5' and 3' halves of non-labeled PHYA RNAs were added as 250 and 500X molar excess of the labeled PHYA RNA. Specific RNA-binding proteins are indicated on the left, and molecular weight markers (Gibco-BRL) are indicated on the right. The gel was exposed to x-ray film for 6 days.
of the labeled RNA from ribonucleases by polysome extracts (Figure 6, lane 2). It would appear that the smaller ~30-kD and ~20-kD bands were RNA-labeled proteins, but it is difficult to be certain about the sources of the faint ~35-kD signal and the stronger ~50-kD signal. Increasing heparin in the presence or absence of yeast tRNA had little effect at the 0.01 and 0.1 μg/μl concentrations but 1.0 μg/μl of heparin resulted in a dramatic decrease in signal at all bands (Figure 6, lanes 6 and 10). The putative RNA-binding proteins from polysomes were sensitive to heparin at a lower concentration than oat total proteins (Figure 3, lanes 2 and 3). The addition of tRNA had no apparent effect on the signal strength for any of the observed bands. A direct comparison of putative PHYA RNA-labeled proteins from polysomes and total protein extracts confirmed that some of the detected bands (35 and 20 kD) from polysome-crosslinking experiments differed in size from bands detected in total protein-crosslinking experiments (data not shown).

Competitor RNAs were added to EDTA-treated-polysome UV crosslinking experiments to investigate binding specificity. Both 100 and 250X molar excess amounts of either non-labeled PHYA or antisense actin competitor RNAs resulted in the decreased signal at all of the detected bands (~50, 35, 30, and 20 kD), although the 20-kD signal appeared to have less affinity for the non-specific competitor RNA (antisense actin) than the specific (PHYA) competitor RNA (Figure 7, lanes 3 and 4 compared to 6 and 7). This suggests that most of these bands corresponded to non-specific RNA-protein interactions, and at best the putative 20-kD protein shows twice the affinity to PHYA RNA versus antisense actin RNA.

Discussion

Several oat seedling proteins have been identified that consistently interact with PHYA mRNA. An apparent 25-kD protein was detected only in presence of active total protein extract treated polysomal extracts but not in total protein extracts. Presumably, this was due to the higher specific activity of this 25-kD putative RNA-binding protein in the polysomal extracts as compared to total protein extracts. It would also seem that the putative 25-kD RNA-binding protein was not polysome associated, or at least was not active if it was polysome associated. Due to the addition of the covalently-bound labeled RNA fragment to the protein, the apparent molecular weight of the detected protein would be a maximum estimate, and the actual protein would be of a lower molecular weight.

Besides the putative 25-kD RNA-binding protein, additional distinct bands (40-kD, 55-kD, 85-kD, 95-kD, and 110-kD in weight) have been detected with total protein extracts. The
Figure 6. A UV crosslinking experiment with labeled PHYA RNA and EDTA-treated polysomal extracts to test the effects of heparin and yeast tRNA on the RNA-binding proteins from polysomes. The presence (+) or absence (-) of EDTA-treated polysomal protein extracts (10 µg), heparin (1 µg/µl), yeast tRNA (0.25 µg/µl), and a crosslinking treatment (XL) is indicated for each lane. Increasing amounts of heparin (0.01, 0.1, or 1.0 µg/µl) was added to some samples, lanes 4 to 5 and 7 to 10. Specific RNA-binding proteins are indicated on the left, and molecular weight markers are indicated on the right. The gel was exposed to x-ray film for 1 day.
Figure 7. A UV crosslinking experiment with labeled PHYA RNA and EDTA-treated polysomal extracts to test the effects of heparin, and yeast tRNA on the RNA-binding proteins from polysomes. To each sample heparin (0.1 μg/μl) and yeast tRNA (0.25 μg/μl) were added. The presence (+) or absence (-) of EDTA-treated polysomal protein extracts (10 μg) and non-labeled competitor RNAs is indicated for each lane. Non-labeled specific (PHYA) and non-specific (antisense actin) competitor RNAs were added as 100 and 250X molar excess of the labeled PHYA RNA. Specific RNA-binding proteins are indicated on the left, and molecular weight markers are indicated on the right. The gel was exposed to x-ray film for 8 days.
40-kD and 55-kD bands appeared to result from the protection of portions of a labeled PHYA RNA from ribonucleases by some factor of the total protein extract in the absence of UV crosslinking. This would suggest that these bands were free labeled-RNA fragments instead of small, labeled pieces of PHYA RNA bound to proteins. The addition of either non-labeled specific (PHYA) or non-specific (GUS) competitor RNAs decreased the signal at the 40-kD and 55-kD bands, indicating that these resulted from non-specific interactions. The 85-kD, 95-kD, and 110-kD proteins were not consistently detected, and the addition of the non-specific competitor heparin prevented detection of the 85-kD and 110-kD proteins, but the 95-kD protein was affected little by the heparin.

When non-specific labeled GUS RNA was added in place of PHYA RNA at a 3X higher concentration, to determine non-specific RNA-protein interactions, the 95-kD, 55-kD, 40-kD, and 25-kD were detected in total protein extracts. At best, this would indicate only a 3X higher binding of these proteins to PHYA RNA as compared to GUS RNA. It is likely that differences in signal intensities were due to differences in the percent of labeled-UTP within the binding sites of the GUS and PHYA RNAs instead of binding affinity. Excess amounts of non-labeled specific (PHYA) and non-specific (GUS and antisense actin) RNAs were added as competitors. The 25-kD protein showed a slight binding specificity for PHYA RNA verses antisense actin RNA, but showed equivalent binding specificity for either PHYA or GUS RNAs. These observation support the conclusion that the putative 25-kD RNA-binding protein is not specific to the PHYA message. The binding site within the RNA is unknown, but presumably it would not be a cap structure or poly(A) tail because these are absent from the in vitro synthesized PHYA RNA.

The putative 20-kD RNA-binding protein detected in EDTA-treated polysome extracts also appeared not to be specific for PHYA RNA, as determined by non-labeled RNA competition assays and by adding labeled GUS RNA in place of PHYA RNA. From previous reports (Pastori and Schoenberg, 1993), it is likely that the EDTA treatment caused dissociation of the oat ribosomal subunits and proteins loosely associated with the ribosomes. It is interesting to note that no distinct bands were detected with polysome extracts not treated with EDTA. Presumably the EDTA treatment decreased the divalent cation concentrations and it is possible that such a change in concentration increased the activity of RNA-binding proteins, as has been shown to be true for magnesium (Stolow and Berget, 1990).

The inability to detect RNA-binding proteins that specifically interacted with PHYA RNA could have resulted from two possible explanations. Either PHYA mRNA specific RNA-binding proteins exist in oat cells but the current method of UV crosslinking is unable to
detect them, or PHYA mRNA specific RNA-binding proteins do not exist and the PHYA mRNA is destabilized through a method independent of a specific RNA-binding protein. The inability to detect a PHYA mRNA-binding protein for technical reasons would seem the more likely explanation because of reports that show the binding of trans-acting protein factors to cis-acting destabilizing sequences, and these proteins factors are involved in regulating the mRNA stability (Brewer, 1991; Koeller et al., 1991; Vakalopoulou et al., 1991; You et al., 1992).

Two major technical changes might make it possible to detect a PHYA mRNA specific RNA-binding protein. First, it is possible that the method of protein isolation used might have selected against a PHYA mRNA-binding protein activity. To test this, alternative methods of protein isolation could be investigated. Second, UV crosslinking and RNA gel-shift assays are commonly performed with small labeled RNAs containing sequences with functional importance, such as an mRNA instability determinant. Using a small portion of the PHYA mRNA would likely eliminate much of the non-specific binding, and increase the relative signal intensity for a possible specific PHYA mRNA-binding protein. In addition, a smaller RNA would likely reduce the abundance of background bands that are a result of large non-degraded free RNA fragments. Currently, an instability determinant has not been identified in PHYA mRNA.

References


CHAPTER 6. GENERAL SUMMARY

My general research goal was to determine why the oat PHYA mRNA is unstable as compared to other plant mRNAs. To date, only a few plant mRNAs have been investigated regarding mRNA stability and degradation mechanisms, so any gain in the understanding of PHYA mRNA degradation would be progress toward the better understanding of how plant and eukaryotic mRNA degradation is controlled. In the course of the research presented here I addressed four main questions that were selected to investigate a method of measuring plant mRNA degradation rates, the expression and half-lives of distinct oat PHYA mRNAs, and the mechanisms of oat PHYA mRNA degradation.

The first question I addressed was whether an oat protoplast system could be used to quickly and reliably determine mRNA half-lives. Protoplasts derived from suspension cultured cells were electroporated to introduce either plasmid DNA or in vitro synthesized mRNA; both DNA and RNA were successfully expressed in protoplasts, as determined by enzymatic assays. This indicated that cytoplasmic factors that were involved in gene expression interacted normally with the introduced nucleic acids, a step that would be critical for correct mRNA degradation. In vitro synthesized, labeled GUS mRNA was electroporated into the protoplasts, and the mRNA half-life was estimated to be ~35 min. This estimate is somewhat lower than the published values for the GUS mRNA half-life in tobacco cells. The accuracy of this protoplast system in estimating endogenous mRNA half-lives can be questioned because of the uncertainty of the cellular location of the electroporated mRNA. For this reason the protoplast system was not used in further experiments to study mRNA half-lives. The DNA and RNA electroporation methods developed here have been used in experiments investigating different translational regulation mechanisms with the barley yellow dwarf virus (BYDV) (Dinesh-Kumar and Miller, 1993).

The second group of experiments was designed to determine the abundance of distinct PHYA mRNAs in etiolated oat seedlings and to estimate the mRNA half-life for two distinct PHYA mRNAs as compared to the average of all the PHYA mRNAs. Gene-specific DNA-oligonucleotide probes were used with RNA gel blots to detect distinct PHYA mRNAs. The addition of in vitro synthesized reference RNAs to etiolated oat poly(A)+ RNA samples to correct for differences in hybridization efficiencies between probes was critical to the accurate determination of abundance for a distinct PHYA mRNA. The PHYA3 mRNA was estimated to be the most abundant, ~61% of the total PHYA mRNA pool in etiolated oat seedlings. In addition, PHYA3 is the most studied of the distinct PHYA mRNAs with regard to the promoter
activity and up-stream transcriptional elements. With DNA-oligonucleotide probes the *PHYA3* and *PHYA4* mRNA half-lives were both estimated to be ~30 min, and a similar value was estimated for the average *PHYA* mRNA half-life. These data plus the reported transcriptional down-regulation data imply that all *PHYA* mRNAs are coordinately down-regulated after a light treatment via the phytochrome protein. Since the distinct *PHYA* mRNAs and average *PHYA* mRNA were all degraded with similar apparent half-lives, it is likely that all oat *PHYA* mRNAs are degraded by similar mechanisms. The 5' UTR and the coding sequences of the known oat *PHYA* partial cDNAs have 98% nucleic acid identity, and the 3' UTRs have 72% identity if realignment with gaps is allowed. Because of the lower sequence conservation the 3' UTR was the portion of the *PHYA* mRNA to which the gene-specific oligonucleotide probes were hybridized, providing sufficiently low cross-hybridization to detect distinct *PHYA* mRNAs.

Sequence comparisons of the unstable *PHYA* mRNAs from different grass species identified one highly conserved sequence of 13 nucleotides in length (85% identical) in the 5' UTR, termed PA3, and two highly conserved sequences in the 3' UTR, one of 18 nucleotides in length (89% identical), termed PA3-1, and the other of 22 nucleotides in length (91% identical), termed PA3-2. Of these conserved regions the PA3-2 sequence appears to be the most strongly conserved as analyzed in three oat *PHYA* mRNAs and one barley *PHYA* mRNA. The PA3-2 sequence is currently the best candidate for a putative *PHYA* mRNA instability determinant. Aside from sequence conservation, there are no additional data that confirm the functional importance for any of the three conserved sequences.

The third question was to determine the *in vivo* degradation mechanism of oat *PHYA* mRNA. Antisense RNA probes that detected all *PHYA* mRNA types were used to analyze the putative *PHYA* mRNA degradation products that provided data to propose two distinct degradation pathways. Unlike *PHYA* mRNA, many eukaryotic mRNAs do not produce detectable degradation products, making the investigation of the degradation mechanism more difficult. The hybridization pattern of the putative *PHYA* degradation products in oat total RNA ruled out a simple 3' to 5' or 5' to 3' exoribonucleolytic degradation. The abundant low molecular weight *PHYA* RNA fragments detected in poly(A)+ RNA showed that a significant amount of *PHYA* mRNA was degraded before the removal of the poly(A) tail. This hybridization pattern of putative degradation products was most consistent with a 5' to 3' exoribonucleolytic degradation pathway for polyadenylated *PHYA* mRNA.

In addition to a polyadenylated mRNA degradation pathway, 25% of the apparently full-length *PHYA* mRNA was poly(A)-deficient implying an additional deadenylated *PHYA* mRNA.
mRNA degradation pathway. The presence of poly(A)-deficient PHYA mRNAs and putative degradation products in polysomal RNA samples suggested that deadenylation was occurring on polysomes, as has been previously reported for eukaryotic mRNA. The amount of apparently full-length poly(A)-deficient mRNA was significantly greater for oat PHYA mRNA than for histone, β-tubulin, and actin mRNAs. The presence of poly(A)-deficient PHYA mRNA was consistent with a second pathway of PHYA mRNA degradation that removed the poly(A) tail prior to degradation of the body of the mRNA, a pathway similar to that reported for the majority of known eukaryotic mRNA degradation mechanisms. The analysis of the putative PHYA mRNA degradation products in poly(A)- RNA ruled out a single exoribonucleolytic degradation, and implied a more complex mechanism, such as the simultaneous 3' to 5' and 5' to 3' exoribonucleolytic pathways. Both the polyadenylated and deadenylated pathways of PHYA mRNA degradation seemed to have occurred while associated with polysomes, suggesting that both pathways occurred in the cytoplasm rather than in the nucleus.

A degradation mechanism that involves multiple pathways could increase the overall rate of mRNA degradation, and might be the explanation as to why PHYA mRNA is unstable as compared to other plant mRNAs. If this is the explanation, then inhibition of one of the two degradation pathways would cause an increase in PHYA mRNA stability. An alternative explanation for the occurrence of two degradation pathways might be that the need for a rapid removal of PHYA mRNA from oat cells after a light treatment is sufficient to warrant a redundancy in mRNA degradation pathways. If this is the explanation, then inhibition of one of the two PHYA degradation pathways might not cause a significant increase in PHYA mRNA stability. Very shortly after the publication of the paper dealing with the oat PHYA degradation mechanism, Chapter 4, it was reported that the yeast PGK1 mRNA is degraded by two distinct pathways when an mRNA destabilizing non-sense codon is introduced (Muhlrad and Parker, 1994). One of the PGK1 mRNA degradation pathways is the 5' to 3' exoribonucleolytic degradation of polyadenylated mRNAs, and the second pathway is the 3' to 5' exoribonucleolytic degradation after the removal of the poly(A) tail. It would seem that the yeast PGK1 mRNA is degraded by similar mechanisms to the oat PHYA mRNA. It would also seem possible that other eukaryotic mRNAs are degraded by similar mechanisms, but due to the lack of detectable degradation products it is experimentally difficult to identify these mechanisms.

An instability determinant sequence that triggers 5' to 3' degradation might seem more likely to reside near the 5' end of the PHYA mRNA. Considering the interactions between the
5'- and 3'-ends of an mRNA that have been shown to be important for translation initiation, it 
would seem possible for an mRNA instability determinant located in the coding region or the 3' 
UTR to trigger degradation at the 5'-end of the PHYA mRNA. The conserved sequences in 
the untranslated regions of the PHYA mRNA (PA5, PA3-1, and PA3-2) are candidates for 
putative PHYA mRNA instability determinants. The functional importance of these sequences 
would need to be confirmed by either loss-of-function PHYA mRNA deletions or gain-of-
function PHYA mRNA insertions into a stable mRNA. All modified mRNAs would need to 
be measured in a faithful oat mRNA degradation system to establish sequences critical to 
mRNA degradation. Alternatively, excess amounts of putative instability determinants in the 
form of RNA-oligonucleotides could be added to an in vitro degradation system so as to 
compete for factors that might be important for PHYA mRNA degradation. This could result 
in the specific stabilization of the polysome-bound endogenous PHYA mRNA and support the 
conclusion that a conserved sequence would be a PHYA mRNA instability determinant.

The fourth and final question that I addressed was whether an RNA-binding protein 
could be identified that bound specifically to PHYA mRNA. UV crosslinking experiments 
were established that resulted in the detection of putative RNA-binding proteins that interacted 
with PHYA RNA. The general competitors heparin and tRNA were included in an attempt to 
reduce the non-specific RNA-binding activities. A 25-kD RNA-binding protein from oat total 
protein extracts and a 20-kD RNA-binding protein from EDTA-treated polysomal extracts were 
identified in etiolated oat seedlings. By the addition of non-labeled competitor RNAs and the 
use of labeled non-specific RNAs it was concluded that both the 25-kD and the 20-kD RNA-
binding activities were non-specific. It is possible that either a PHYA specific RNA-binding 
protein does not exist, or that one exists but in the experiments presented here it was not 
identified. Considering the large size of the PHYA RNA used in these UV crosslinking 
experiments and the high background of non-specific RNA-binding proteins, it would seem 
more likely that a PHYA specific RNA-binding was not detected for technical reasons. The 
use of a small portion of the PHYA mRNA would seem to increase the chance of successfully 
identifying a PHYA specific RNA-binding protein. The identification of a PHYA mRNA 
sequence that functions as an instability determinant would be a necessary first step towards 
successfully identifying a PHYA specific RNA-binding protein. Experiments with RNA-
oligonucleotides for each of the three PHYA mRNA conserved sequences found in either the 5' 
UTR (PA5) or the 3' UTR (PA3-1 or PA3-2) would be the logical next step to establish if 
RNA-binding proteins specifically interact with any of these three sequences.
It is possible that a *trans*-acting factor, important for the destabilizing *PHYA* mRNA, is not a protein. One alternative to a proteinaceous *trans*-acting factor would be an *in vivo* antisense *PHYA* mRNA. Such an antisense mRNA might hybridize to *PHYA* mRNA to cause destabilization of *PHYA* mRNA through a double-stranded RNA complex.

Overall, the degradation of oat *PHYA* mRNA is complex due, in part, to the *PHYA* multi-gene-family structure and to the multiple pathways of degradation for *PHYA* mRNAs. Experimental identification of putative *PHYA* mRNA instability determinants has proved difficult due, in part, to the large size of the *PHYA* message and would be important to the continued understanding of *PHYA* mRNA degradation. Continued investigation into cellular and polysomal factors important for *PHYA* mRNA degradation should also be beneficial. In addition, studies that determine the *PHYA* mRNA half-life in different cell and tissue-types of oats might prove interesting with regard to the developmental and spatial expression of presumed factors involved in *PHYA* mRNA degradation. Further investigation into the oat *PHYA* mRNA degradation mechanism should provide additional insight into eukaryotic mRNA degradation and the regulation of gene expression at the mRNA stability level.
LITERATURE CITED


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