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Avena phytochrome mRNA: in vivo localization, degradation products, and half-life

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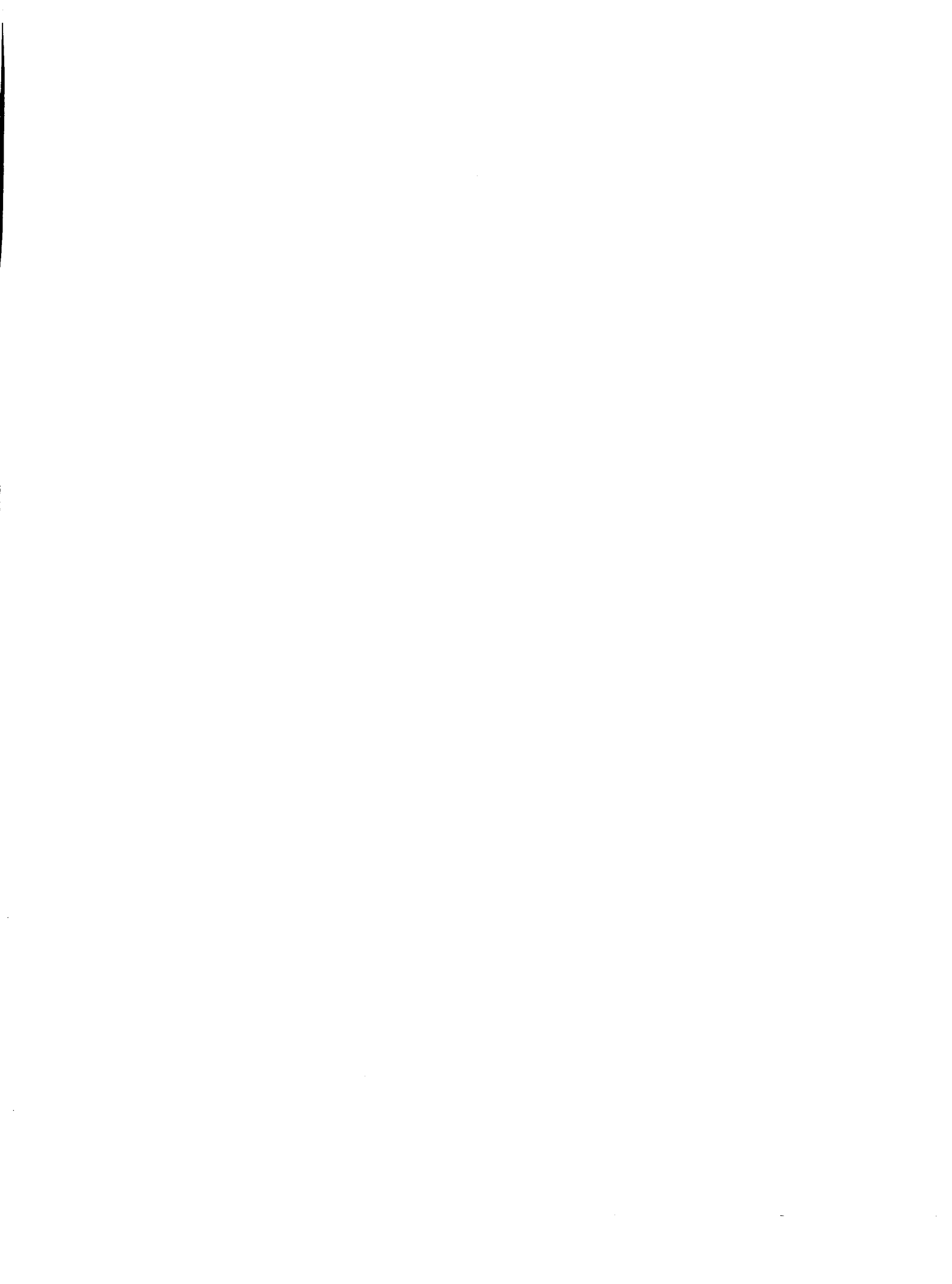
Avena phytochrome mRNA: *In vivo* localization, degradation products, and half-life

Seeley, Kevin Andrew, Ph.D.

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Avena phytochrome mRNA: In vivo localization,

degradation products, and half-life

by

Kevin Andrew Seeley

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TABLE OF CONTENTS

GENERAL INTRODUCTION

Phytochrome and Photomorphogenesis

The best characterized photoreceptor that conveys information to the plant regarding its light environment is phytochrome. Initially the presence of phytochrome was illustrated by measurement of action spectra for the control of flowering in plants, the induction of photomorphogenesis in etiolated pea seedlings, and the initiation of germination in lettuce seeds (Borthwick et al., 1952; Kendrick and Kronenburg, 1986). In all of these experiments red light was found to be most effective at inducing photomorphogenic responses, some of which were reversed by the application of far-red light. Two photointerconvertible phytochrome forms; a red light absorbing (Pr form) and a far-red light absorbing (Pfr form) were proposed to explain the phenomena. Over the past 30 years much research has gone into the isolation and characterization of the phytochrome protein and the regulation of its genes (for review see Jorden et al., 1986; Colbert, 1988)

There are at least three immunologically distinct phytochrome proteins found in oats (Avena sativa L.). The phytochrome (type I) that predominates in dark grown (etiolated) seedlings is a 124 kDa polypeptide (Wang et al., 1991). Type I phytochrome (phyA) protein is almost

undetectable in light grown plants (Wang et al., 1991). At least two other phytochrome proteins are present in detectable quantities in both light grown and dark grown seedlings, but predominate in the light (Tokuhisa et al., 1985; Wang et al., 1991). The latter are distinct proteins of 123 and 125 kDa as differentiated by monoclonal antibodies (Wang et al., 1991).

Three nearly identical type I phytochrome sequences corresponding to the 124 kDa peptide, have been isolated by cDNA cloning from etiolated oat poly(A)$^+$ RNAs (Hershey et al., 1984). The genes for the 123 and 125 kDa oat phytochrome peptides have not been isolated. The three oat phyA mRNA sequences are 96-98% conserved in the coding and 5'-untranslated regions with less sequence homology in the 3'-untranslated regions. PhyA probes made from the isolated cDNA constructs hybridize to a 4.2 kb message in oat poly(A)$^+$ and total RNA (Colbert, 1988; Byrne et al., 1990). The DNA sequence coding for the chromophore binding region appeared to be most highly conserved among the other monocot and dicot species tested (Sharrock and Quail, 1989; Sharrock et al., 1987). There have been a number of phytochrome cDNA clones isolated from other plant species using the oat clone containing the chromophore binding region sequence as a heterologous probe. These species include rice (Kay et al., 1989a), zucchini (Lissemore et al., 1987), pea (Otto et al., 1984), and maize (Christensen and Quail, 1989). In

<u>Arabidopsis</u>, three genes were isolated by screening for homology to the zucchini sequences (Sharrock and Quail, 1989). Of the three genes, <u>phyA</u> has highest sequence homology to the oat type I gene. The <u>phyB</u> and <u>phyC</u> genes are likely to encode proteins corresponding to the two other oat proteins but it is not known which gene corresponds to which immunologically distinct oat protein (Sharrock and Quail, 1989; Wang et al., 1991). Overall, the amount of sequence similarity among the <u>Arabidopsis</u> <u>phyA</u>, <u>phyB</u>, and <u>phyC</u> genes is lower than the similarities between the <u>Arabidopsis</u> <u>phyA</u> and oat type I gene (Sharrock and Quail, 1989). In rice, a phytochrome gene has been isolated that has sequence similarity to the type I oat and phyA <u>Arabidopsis</u> genes and shows light repression (Kay et al., 1989a). The <u>Arabidopsis</u> <u>phyB</u> gene is expressed equally in the dark and light (Dehesh et al., 1991).

Mature type I (etiolated seedling predominant) phytochrome is a dimeric chromoprotein with a monomeric molecular weight 124 kDa in oats. The photoreceptor is synthesized in the Pr form, binds to the chromophore and is relatively stable, allowing it to accumulate to high levels in dark-grown plants. Irradiation of Pr phytochrome with red light results in the photoconversion of Pr to the biologically active Pfr form due to the change in bond angle of a single amino acid in the chromophore region (Colbert, 1988; Kendrick and Kronenburg, 1986; Quail et al., 1986). Irradiation of the

Pfr form of phytochrome with far-red light converts Pfr back
to the Pr form and is adequate to reverse most light-induced
responses (Quail et al., 1986). Because of spectral overlap,
the conversion of Pfr back to Pr by far-red light is
incomplete. This may explain why, provided that Pfr is the
active form, some phytochrome-mediated responses are
"irreversible". These latter responses may simply be more
sensitive to low levels of Pfr. Still, there is some debate
as to which photo-active form of phytochrome is responsible
for the light regulation activity (Smith, 1981), but, because
many of the phytochrome-mediated responses are correlated with
small changes in the Pfr concentration, the Pfr is thought by
most researchers to be the active form (Colbert, 1988;
Kendrick and Kronenburg, 1986; Shanklin et al., 1987).

It is known that phytochrome is involved in regulating
gene expression in response to light. Red light-induced
increases in mRNA abundance have been documented for the
light-harvesting chlorophyll a/b binding protein (cab)
(Silverthorn and Tobin, 1987; Kuhlemeier et al., 1987),
glutamine synthase (Tingey et al., 1988) and for the small
subunit of ribulose 1,5-bisphosphate carboxylase-oxygenase
(Silverthorn and Tobin, 1987; Kuhlemeier et al., 1987). Down-
regulation of mRNA abundance by Pfr production (red-light
treatment) has been found for the protochlorophyllide
reductase gene (Batschauer and Apel, 1984), the ß-tubulin gene

(Colbert et al., 1990), and for the type I (etiolated)
phytochrome gene itself in most plant species investigated
(Colbert, 1988; 1991). Much work has gone into trying to
determine the exact mechanism by which the phytochrome dimer
is involved in the light regulated signal transduction
pathway, but as yet the answer is undetermined.

The Pr form of the phytochrome protein, the form that is
synthesized and binds the chromophore, is quite stable, while
the Pfr form is quickly degraded once light is received
(Shanklin et al., 1987). This apparent need to remove the Pfr
form may reflect a regulatory system in plants that removes
the signal transduction initiator once the appropriate signal
is perceived. High levels of Pr in etiolated seedlings could
increase the seedlings sensitivity to light and give the
seedling an advantage in preparing to develop photosynthetic
competence prior to its emergence from the soil. However,
continued high levels of Pr photoconverted to Pfr following
light reception would cause potentially detrimental phenotypic
changes to occur. Phenotypic changes that occur because of
phytochrome over-expression have been demonstrated in
transgenic experiments. When a functional phytochrome gene
with a constitutive promoter was transformed into tomato, the
transformed plants grew as dwarfs and had darker green
pigmentation (Boylan and Quail, 1990).

In addition to decreases in protein abundance, the phyA

mRNA of oats decreases in abundance very rapidly following red light exposure. This rapid down-regulation of the phyA message adundance mirrors the rapid light induced degradation seen at the protein level and again may indicate a requirement to decrease the receptor of the red light signal transduction chain once light is perceived.

Phytochrome Localization

The determination of the location of a protein that is a critical part of a signal transduction chain is an important step towards understanding how a transduction chain operates in the whole organism. Three approaches have been used to determine the abundance of phytochrome in various tissues in etiolated seedlings.

First, the biochemical properties of the protein-chromophore complex give rise to an absorption spectra that is unique to phytochrome. These light absorption qualities have been used in a number of ways to localize phytochrome protein in seedlings. Spectral methods are relatively insensitive to low levels of phytochrome and detect phytochrome independent of its biological activity, however they do allow the determination of photoreversibility. One method involves determining relative phytochrome abundance in an intact seedling along its long axis (Kondo et al., 1973). This technique demonstrated that spectrally active phytochrome is

present at highest concentrations at the tip of the seedling and at the mesocotyl node. This distribution is maintained in oat seedlings 3 to 6 d after germination. Another spectral experiment analyzed excised, pooled etiolated tissue samples with a dual wavelength spectrophotometric assay, which quantitates the relative amounts of spectrally active phytochrome in oats, maize, barley, peas, and beans (Briggs and Siegelman, 1965). Both temporal and longitudinal differences were detected for the relative concentrations of phytochrome in etiolated oat seedlings in the pooled tissue samples. As with the scanning method, these experiments showed that the phytochrome in 5-day-old etiolated oat seedlings is present at highest concentrations in the tip of the coleoptile and mesocotyl node. Although these data are similar to the data found using the scanning method, the second method accounts for more than one seedling at a time. In addition, these data demonstrate that the lowest concentrations of phytochrome occurred in the primary leaf and mesocotyl.

Using a second set of techniques type I phytochrome protein was detected with polyclonal phytochrome-specific antibodies. Immunological methods offer greater sensitivity but detects phytochrome regardless of its biological or spectral activities. In addition, it is more difficult to quantitate the amount of phytochrome present in an organ or

whole tissue using immunological techniques. As seen with spectral techniques, the immunological technique showed that phytochrome protein is localized principally in the coleoptile tip and the mesocotyl node in oat, maize and barley sections (Pratt and Coleman, 1974). In addition to the longitudinal distribution, the immunohistochemical techniques have demonstrated some tissue specificity in the expression of the phytochrome protein. Phytochrome was found to be present in highest concentrations in subepidermal cortical cells and guard cells (Pratt, 1986).

The third method to localize phytochrome in etiolated seedlings involves determining the activity of phytochrome through the gene expression changes that occur over a whole seedling that has had a pin-point area irradiated with red light. This method allows the detection of biologically active phytochrome, but the resolution is low and the data are much more difficult to interpret. This method shows that the approximate location of biologically active phytochrome activity for the low fluence and very low fluence growth responses is below the mesocotyl node (Mandoli and Briggs, 1982). This localization is different from that shown for spectral and immunological methods and may be the result of light scattering in the plant (Mandoli and Briggs, 1982), allowing the phytochrome to be present in a location at a distance from the light entry point.

To date there are no available data to indicate whether the mRNA encoding phyA protein is present in the same tissue and organ specific manner as the protein. It is possible that phyA transcription is constitutive in all etiolated tissues and that the relative differences in protein levels are due to the differences in the translatability of the phyA mRNA in different tissues. Another possibility is that the phyA promoter is regulated in a tissue specific manner. Current research has shown that there are a number of genes that are transcriptionally regulated in a tissue specific manner (Okamuro and Goldberg, 1989).

mRNA Stability

In eukaryotic cells there are thousands of messenger RNAs being synthesized, translated, and degraded at any one time. Investigation of the methods by which the cells are able to control protein concentrations have previously focused primarily on transcriptional regulation. However, this level is not adequate to explain the complexity of gene regulation that is seen in eukaryotes (Okamuro and Goldberg, 1989). Genetic control can involve one or more of the following regulatory levels: transcription, RNA processing, mRNA transport, mRNA stability, translation, post-translational modification, and protein transport and activation. To date there have been few data available on mRNA stability.

Steady-state mRNA concentrations are a product of input (transcription) and output (degradation). If either process changes, the level of that RNA will increase or decrease, resulting in a change in the concentration of that specific message. Among the thousands of distinct messages present in a cell there can be a wide range of stability among different mRNA species. Pulse-chase mRNA radiolabel incorporation experiments using Xenopus liver cells gave an average half-life for mRNA molecules of 16 h (Brock and Shapiro, 1983). The only data demonstrating the average plant mRNA half-life come from studies with soybean suspension cell cultures (Silflow and Key, 1979). These pulse-chase data gave an average cellular mRNA half-life of about 30 hr. An mRNA with a long half-life could have a lower transcription rate and still maintain a high steady-state level, but, because of the high stability, down-regulation of gene expression would be slower following the shut off of transcription. An mRNA with a short half-life would require continuous transcription if high steady-state levels of mRNA were to be maintained. It would, however, be able to decrease mRNA levels very quickly, once transcription has ceased. On the other hand, a third pathway of down-regulation could involve an mRNA whose stability is modified (from long to short half-life) in response to some developmental or environmental cue. In this way, low mRNA levels of transcription could still maintain

high steady-state levels prior to signal reception. After the reception of the environmental/developmental cue, the mRNA would be destabilized to a short half-life and its abundance rapidly decreased. This type of regulation, although less wasteful in terms of transcription, would require a number of other factors to be present to control the cells mRNA degradation machinery and detect when to shift to a different half-life.

Genes both from animal and plant systems have been characterized in relation to their mRNA stability. Many viral mRNAs and oncogene messages have been examined, and a number of them have very short half lives in comparison to the average mRNAs. This may indicate a biological need for tight control of viral gene expression since mutations that increase mRNA abundance lead to cell transformation and tumor production (Shapiro et al., 1986). In plants, data with small auxin up-regulated mRNAs (SAUR) have pointed to rapid decay rates, perhaps for the same reasons (Franco et al., 1990). There are also examples of stable messages (>100 hr), in plants. The pinto bean phaseolin mRNA has an estimated half-life of several days (Chappell and Chrispeels, 1986). In barley, alpha-amylase mRNA has a half-life of about 100 hr (Belanger et al., 1986). In addition to mRNAs with consistently long or short half-lives there are now a number of genes analyzed in both animal and plant systems that show

some form of modulated mRNA stability. A histone mRNA stability is modified in response to stages in the cell cycle (Graves et al., 1987; Peltz and Ross, 1987). Certain types of animal virus infections are able to selectively destabilize all host cellular mRNA's while the viral mRNA's remain stable (Shapiro et al., 1986). Some Dictyostelium mRNA's are destabilized upon disaggregation of the amoeboid cells (Mangiarotti et al., 1989). Increased iron concentrations stabilize transferrin receptor mRNA (Mullner and Kuhn, 1989). In duckweed, cab mRNA is stabilized in the presence of cytokinin (Flores and Tobin, 1988). Legumin mRNA is stabilized in pea plants in the presence of sulphur (Beach et al., 1985). In both potato and soybean the ribulose-bisphosphate carboxylase/oxygenase mRNA is more stable in light irradiated plants (Shirley and Meagher, 1990).

Light-induced decrease in phyA transcription has been demonstrated by nuclear run-on transcription experiments. They show that phyA transcription is reduced to 5% of the dark level within 30 min of Pfr production (Colbert et al, 1985). However, the rapid rate of decrease in phyA mRNA steady-state levels leads to speculation that mRNA instability is involved in down-regulating phyA mRNA, provided that the 30 hr average half-life reported for soybean cell cultures (Silflow and Key, 1979) applies to oat mRNAs. PhyA mRNA abundance rapidly decreases when etiolated oat seedlings are given a saturating

pulse of red light (Colbert et al., 1988; Byrne et al., 1990).
Previous studies with oat seedlings show that there is a 90%
decline in the amount of phyA mRNA within 5 hr after exposure
to red light (Byrne et al., 1990; Colbert et al., 1991;
Gottman and Schafer, 1983). However, the rapid decrease in
phyA transcription seen in nuclear run-on experiments does
not, by itself, explain completely the rapid decrease in phyA
mRNA steady-state levels (Colbert, 1991). Given that the
steady-state levels drop to one half of their original levels
within 90 min (Byrne et al., 1990), the phyA message must
either always have a short half-life (Kay et al., 1989a) or
the stability of the message must be decreased after a
saturating red-light pulse (Quail et al., 1986). No direct
measurements have been made with transcription inhibitors to
estimate the half-life of the phyA mRNA prior to light
treatment of etiolated seedlings.

Approaches to Measure mRNA Stability

One of the major reasons that there is very little direct
evidence regarding the stability of mRNAs relates to the
difficulties in developing techniques for measuring mRNA
stability in vivo under different conditions. Using a pulse-
chase labelling method would be the best way to determine an
mRNA half-life and has been used with some success with the
vitellogenin gene in Xenopus (Brock and Shapiro, 1983). In

whole organs, tissues or organisms, problems with label
uptake, equilibration with the intercellular nucleoside pool
and low sensitivity make it difficult to interpret the data
from pulse-chase experiments (Atwater et al., 1990). The
remaining techniques for determining mRNA stability rely on
some method to alter the transcription of the corresponding
gene. All of these methods provide a maximal half-life value
since any residual transcription or disruption of the mRNA
degradation system would cause the message to appear to be
more stable.

Transcriptional regulation of some genes involves the
addition of hormones or other chemicals to shut off
transcription. This has the advantage of being able to
regulate one gene out of the gene pool without stopping the
transcription of other genes that may be involved in cellular
processes. The chemical forskolin, causes the c-jun mRNA in
nerve cells to decrease 20-fold in steady-state levels, giving
a half-life of about 5 min (Atwater et al., 1990). This
induced half-life may not necessarily reflect the actual
stability under conditions when the chemical is not present.
Another way to moderate transcription of a specific gene is to
make a gene fusion of the target message to a promoter that
can be regulated with some sort of inductive stimulus. For
the c-fos promoter transcription is decreased by the addition
of serum to the growth medium (Shyu et al., 1989). Again,

this does not indicate what the stability of the mRNA is in all conditions.

The main disadvantage of non-specifically modifying transcription is that since all transcription is stopped any labile RNA or protein involved in mRNA degradation would not be synthesized to replace those components lost. Wheat embryos treated with alpha-amanitin show an inhibition of transcription but not a disruption of translation or polysome formation (Jendrisak, 1980). The fungal toxin cordycepin, which is an RNA synthesis inhibitor at high concentrations and a poly(A) tail addition inhibitor at low concentrations, was effectively used to inhibit the auxin-induced elongation in epidermal peels of oat, coleoptile segments of oat, and abraded maize coleoptiles (Cline and Rhem, 1974; Edelmann and Schopfer, 1989; Schopfer, 1989). Concentrations of 20 to 40 uM (50 to 100 ug/ml) cordycepin caused the auxin-induced elongation to be completely inhibited if given a 10 to 20 min pretreatment prior to auxin addition (Cline and Rhem, 1974; Edelmann and Schopfer, 1988).

One of the biggest problems found using transcription inhibitors is the difficulty of ensuring that the inhibitor is entering the desired tissue. A number of techniques have been developed to help to get the inhibitors into the desired tissues of plants. One way to get inhibitor uptake is to use small tissue sections. mRNA isolated from pea segments

floated on a buffer containing 5 ug/ml alpha-amanitin show that 5 ug/ml is sufficient to completely block synthesis of auxin up-regulated mRNA's (Theologis, 1989). In cereal grains the first method involves excising the very tip of the coleoptile, forcing air over the cut seedling, and simply allowing the evaporation-transpiration stream to carry the inhibitor up into the seedling through the cut submerged end (Lissemore and Quail, 1988). A second method involves using a polishing cloth as a gentle abrasive material to scrape away the cuticle without damaging the underlying cells (Edelmann and Schopfer, 1989). Experiments with the uptake of $[^3H]$-leucine showed that the level of labeled amino acid absorbed into all of the tissues was ten-fold higher in abraded seedlings than for tissues distant from the cut ends of non-abraded seedlings. The abrasion technique was used for Zea mays coleoptiles to allow access of transcription and translation inhibitors (Edelmann and Schopfer, 1989; Schopfer, 1989). One way to check on how effective the abrasion is on allowing uptake is to use a vital and non-vital dye to stain abraded and non-abraded coleoptiles. If the abraded coleoptiles were stained with Evans Blue, any cells that were killed would take up the stain and appear blue (Schopfer et al., 1989), acting as an indicator of the amount of damage to the tissue. Knowing the extent of damage is important, since excess damage to the epidermis releases the contents of the

cells potentially increasing nuclease activities or stimulating wound induction pathways. If the abraded coleoptile is stained with neutral red, the living cells rapidly take up this vital dye (Schopfer et al., 1989). The degree of staining with the dye gives an indication of the level of removal of the waxy cuticle and indicates the proportion of cells that are still viable.

The method for inhibitor uptake must be chosen using the considerations of phyA mRNA localization. For example, it would not be effective to remove the tip of the coleoptile if that is where most of the phyA message is located. Based on protein localization data, removal of the tip could remove a high proportion of the phyA mRNA if it has the same distribution pattern as the protein. Other considerations for designing an experiment to determine the half-life of the phyA message include: being able to get adequate uptake of the inhibitor, being able to harvest sufficient tissue that is uniformly treated to isolate enough RNA to analyze, and being able to perform all of the manipulations in total darkness.

Once an inhibitor has gained access to a given tissue, there can still be artifacts that arise from the action of the inhibitor itself. Since we are interested in observing an enzymatic activity in the cell, i.e., mRNA degradation, concerns arise over the long term use of an inhibitor that may decrease the effectiveness of the degradation pathway by

preventing the synthesis of some of the components of the mRNA degradation pathway. In addition to inhibiting the mRNA degradation pathways, a message may have to be bound to a highly labile factor that 'tags' it as being a short half-life message in order to be degraded quickly. If, because of inhibitor treatment, the labile factor is not synthesized then the half-life determination will be inaccurate. The two most important ways to prevent these types of generated artifacts that are seen with general RNA synthesis inhibitors is to use the lowest possible concentration for the shortest possible preincubation that still achieves maximal inhibition. Abraded maize coleoptiles given a pretreatment of 30 min with 40 uM (100 ug/ml) cordycepin show an inhibition of auxin-induced elongation (Schopfer, 1989).

Questions to be Addressed

The two main questions to be addressed by this research are addressed in the two chapters that follow. The first involves the determination of the localization of the phyA mRNA in the oat seedling. This is critical to the decision as to which type of inhibitor uptake protocol to use. The second is to determine the half-life of phyA mRNA in the dark. In addition to using transcription inhibitors to determine the half-life, I intend to accumulate other evidence for rapid

degradation such as detection of phyA mRNA degradation products.

Explanation of Thesis Format

This thesis has been prepared with two parts that are related very closely but make up two separate publications. You will note that some of the reference, figure and layout styles differ between the two sections. This is because PART I was prepared for publication in Planta while PART II was prepared for, submitted, and accepted for publication, in The Plant Cell. Any references listed in the GENERAL INTRODUCTION or GENERAL SUMMARY are found in the LITERATURE CITED section that follows the GENERAL SUMMARY.

The research presented in PARTS I and II result from work that I performed as a student in Jim Colbert's lab. The research reported in PART I resulted from experiments that I designed and executed. Although Dennis Byrne is a coauthor of PART II, which was submitted to The Plant Cell, his contribution was limited to preliminary experiments relating to the ribonuclease activity experiments and to his help in editing the submission copy.

PART I. DISTRIBUTION OF PHYTOCHROME AND
CHLOROPHYLL A/B BINDING PROTEIN mRNAS
IN ETIOLATED _AVENA_ SEEDLINGS

Distribution of phytochrome and chlorophyll a/b binding protein mRNAs in etiolated _Avena_ seedlings

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ABSTRACT

In 4-day-old dark grown oat seedlings, the majority of the etiolated phytochrome (phyA) mRNA was found within 10 mm of the tip of the coleoptile sheath and in the mesocotyl node. Almost no phyA mRNA was detected in the enclosed primary leaf. In contrast, chlorophyll a/b binding protein (cab) mRNAs were found almost exclusively in the enclosed primary leaf and were barely detectable in the coleoptile sheath or mesocotyl node from red-light treated etiolated seedlings. Separated, dark-grown primary leaves responded to a red light treatment by increasing cab mRNA abundance in the absence of the coleoptile sheath or mesocotyl node tissues.

INTRODUCTION

The transcription of numerous genes, including those encoding type-I phytochrome (phyA) protein and chlorophyll a/b binding protein (cab), is regulated through the phytochrome signal transduction pathway (Kendrick and Kronenberg, 1986; Colbert, 1988; 1991). However, the specific mechanism by which phytochrome regulates transcription is not as yet understood. Production of Pfr (the putative active form of phytochrome) in etiolated seedlings by red light results in an increased turnover rate for the phyA protein (Shanklin et al., 1987) and a decrease in phyA gene transcription (Lissemore and Quail, 1988). The decrease in phyA transcription is followed by a rapid decline in phyA mRNA abundance due to the inherent instability of phyA mRNA (Seeley et al., 1991).

The phyA protein is present at high levels in the coleoptile tip and the mesocotyl node of etiolated oat seedlings as determined by immunohistochemical and spectral methods. The middle-to-lower regions of the coleoptile and the whole primary leaf enclosed within the coleoptile have relatively little phyA protein (Briggs and Siegelman, 1965; Kondo et al., 1973; Pratt and Coleman, 1974). The protein localization data are consistent with the possibility that either phyA transcription and mRNA accumulation occur only in those regions where phyA protein has been detected, or that

phyA transcription and mRNA accumulation occur at high levels ·
in all regions. In the latter, a translational or post-
translational regulatory step could explain the lack of
accumulation of phyA protein in specific tissues. No previous
reports have appeared regarding the localization of phyA mRNA
in etiolated oat seedlings.

The production of the Pfr in etiolated oat seedlings
leads to increased transcription and accumulation of cab mRNA
(Lissemore and Quail, 1988; Edwards and Colbert, 1990; Seeley
et al., in press) as it does in etiolated seedlings of other
plant species (Silverthorne and Tobin, 1987; Kuhlemeier et
al., 1987). Given that it is the leaf enclosed within the
coleoptile that accumulates high levels of chlorophyll
pigments and pigment-protein complexes during
photomorphogenesis, it seems likely that the bulk of the cab
mRNA accumulation would occur in the leaf rather than the
coleoptile. However, the question has not been directly
addressed. Whether the majority of the phyA protein (present
in the coleoptile) plays a major role in the red-light-induced
increase in cab mRNA abundance in the leaf has not been
investigated.

The data presented here support the conclusion that phyA
mRNA accumulates in the same regions of etiolated oat
seedlings as the phyA protein (Pratt, 1986), suggesting that
translational or post-translational controls are not an

important factor in regulating the distribution of phyA
protein. In addition, the phyA protein present in the
coleoptile of etiolated oat seedlings is not required for red-
light induction of cab mRNA accumulation.

MATERIALS AND METHODS

Growth and Light Treatment of Plants

Four-day-old dark-grown oat (Avena sativa cv. Garry)
seedlings were harvested by excision just below the mesocotyl
node. Saturating red light treatments (10 to 20 sec) were
provided as described previously (Colbert et al., 1983).
Etiolated oat seedlings that were either dark-incubated or
dissected 4 hr after a red light pulse and that had a tip-to-
node length of 25 to 30 mm, were cut into 5 mm sections in
room light and placed in liquid nitrogen within 10 min after
transfer from the dark growth room. Dissection of about 100
seedlings gave enough tissue for RNA isolation (described
below). Different organ-enriched regions were isolated by
dissecting 25 to 30 mm long seedlings under room light. The
seedlings were divided into three organ-enriched regions. The
'node' samples were cut 5 mm above and below the mesocotyl
node. The leaf and coleoptile regions were separated by
making a shallow slit within about 5 mm of the cut end of the
coleoptile and gently pulling the enclosed leaf out of the
surrounding coleoptile with a razor blade. The isolated
regions were frozen in liquid nitrogen within 10 min after
transfer from the dark growth room.

The cab induction experiment was performed under infrared
light using an infrared viewing system (FJW Optical Systems).

Isolated leaves or excised seedlings were floated in a
treatment buffer (1 mM PIPES, pH 6.25, 1 mM Na-citrate, 1 mM
KCl, 15 mM sucrose). The excised seedlings (about 10 per 60 X
15 mm plastic dish, 200 mg) or isolated primary leaves (about
30, 100 mg) were given a red-light pulse after a 30 min
pretreatment or incubated in the dark, then the mixture was
swirled at 75 RPM in total darkness for 3.5 hr. The treated
seedlings and leaves were harvested, frozen in liquid nitrogen
and stored at -80°C.

RNA Isolation, Hybridization and Quantification

The RNA isolation procedure (Seeley et al., 1991) was a
modification of the rapid mini-scale RNA isolation described
by Wadsworth et al. (1988). Frozen tissue samples (100-200
mg) were ground with a small mortar and pestle in liquid
nitrogen and thawed in the presence of the ribonuclease
inhibitor aurintricarboxylic acid (Sigma A-1895). Total RNA
(5 to 10 ug) was electrophoresed and analyzed as described
previously (Seeley et al., in 1991). In vitro transcription
templates were prepared from plasmids pAPSX2.7 (phyA probe)
and pGABO.7 (cab probe, as previously described, Seeley et
al., 1991).

RESULTS

In order to determine the localization of the phyA and cab mRNAs in oat seedlings, two types of experiments were performed. The first used 5 mm-long segments from four-day-old etiolated oat seedlings. Because the seedlings tended to vary in the length between the mesocotyl node and tip, only seedlings that had tip to node lengths of 25 to 30 mm were selected. Previous data (Colbert, 1988; Seeley et al., 1991) demonstrated that phyA mRNA levels do not begin to decrease until at least 15 min after exposure of etiolated seedlings to light. Subsequently, we were able to dissect seedlings under standard flourescent room light. Total RNA yields ranged from 300 to 800 ug/gfw for whole seedlings and longitudinal segment samples, only 50 ug/gfw for excised coleoptiles, while primary leaves yielded 1500 ug/gfw. phyA mRNA abundance varied along the longitudinal axis of the etiolated seedlings.

The highest levels of phyA mRNA were found within 10 mm of the tip and at the mesocotyl node (Figure 1A). The phyA-specific hydridization below the mature band was due to phyA RNA fragments produced in vivo (Seeley et al., 1991). Quantitation of three independant dissection experiments shows that the highest amount of phyA mRNA occurs within 10 mm of the tip and within 5 mm of the
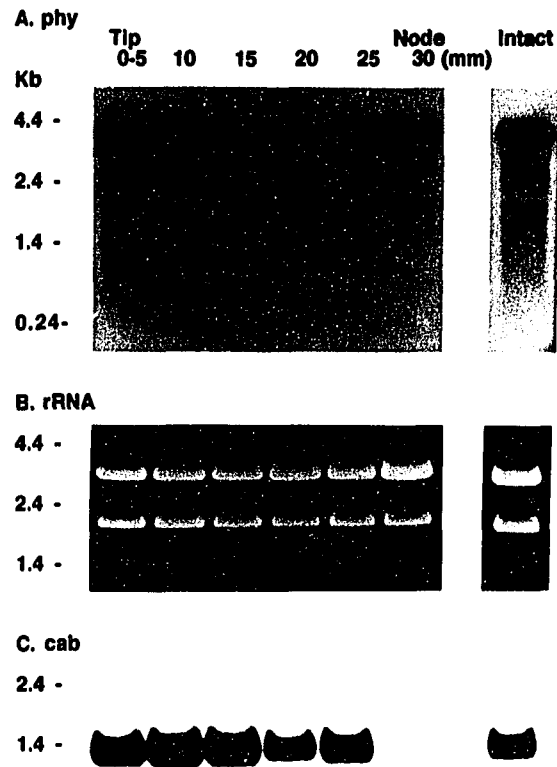
Fig. 1A-C. Determination of the longitudinal distribution of phyA and cab mRNA in oat seedlings. **A** Etiolated seedling segments were cut every 5 mm from the coleoptile tip through the mesocotyl node (left to right) of etiolated seedlings. The total RNA (10 ug per lane) was electrophoresed, blotted, probed for phyA mRNA (pAPSX2.7, antisense) and exposed to x-ray film for 4 h. **B** Ethidium bromide stained rRNA demonstrated that equivalent masses of RNA were analyzed in all lanes. **C** RNA gel blot of the 5 mm segments excised from etiolated seedlings that had been exposed to a red light pulse and incubated in the dark for 4 h. The total RNA (10 ug per lane) blots were probed for cab mRNA (pGAB0.7, antisense) and exposed to x-ray film for 8 h. RNA size markers are indicated.

mesocotyl node (Figure 2). Ethidium bromide staining of rRNA (Figure 1B) indicated that approximately equal amounts of total RNA were electrophoresed in each lane and that the regions of little hybridization in the phyA RNA fragment smear (Fig. 1A) were due to the 18S and 25S rRNAs. In red light-pretreated, etiolated seedlings, cab mRNA was more or less equally distributed with the exception of the mesocotyl node, which had no detectable cab mRNA (Fig. 1C).

The second type of localization experiment involved isolation of total RNA from isolated coleoptiles, leaves, or nodes dissected from dark-incubated and red-light pretreated etiolated seedlings. The dissection process is shown in Fig. 3. Replicate RNA blots of 10 ug total RNA were hybridized with either phyA- or cab-specific RNA probes (Fig. 4). PhyA mRNA is found in high quantities in the node and coleoptile samples but is almost undetectable in the enclosed primary leaf (Figure 4A). In all of the tissues there was 10- to 20-fold more phyA mRNA in dark seedling samples than in red-light-treated samples. Almost twice as much phyA mRNA is present in total RNA from dark coleoptiles than in total RNA from dark nodes and 20-fold as much as found in total RNA from dark leaf tissue (Figure 1A). In contrast to phyA mRNA localization, cab mRNA was found at highest levels in the leaf (Figure 4B). With significantly longer exposures (data not shown), low but detectible levels were found in the
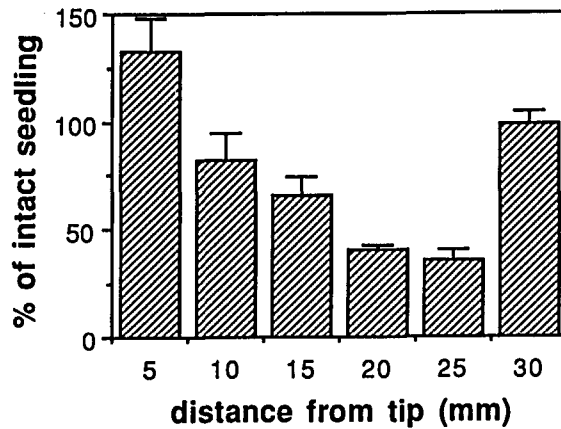
Fig. 2. Quantitation of the longitudinal distribution of phyA mRNA in etiolated oat seedlings. Quantitation of hybridization described in Methods section. The values for the phyA mRNA per unit total RNA have been normalized to total RNA from the whole seedling using intact seedling RNA as 100%. Error bars indicate the standard error of the mean of three independant experiments.
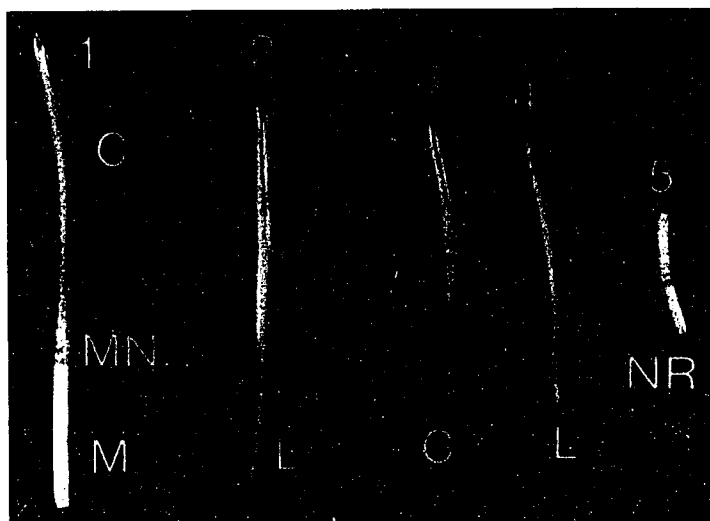
Fig. 3. Dissection process to isolate coleoptile, node region, and primary leaf tissues. Dissection of 4-d-old etiolated seedlings consists of: **1** Excised etiolated seedling, **2** Leaf partially withdrawn from the coleoptile, **3** Separated coleoptile, **4** Separated primary leaf, **5** Node region within 5 mm above and below the node. The regions are: **C** coleoptile, **L** leaf, **M** mesocotyl, **MN** mesocotyl node, **NR** node region.

A. Phy

	Intact		Leaf		Node		Coleoptile	
	L	D	L	D	L	D	L	D

Kb

4.4 -

2.4 -

1.4 -

0.24-

B. Cab

2.4 -

1.4 -

0.24-
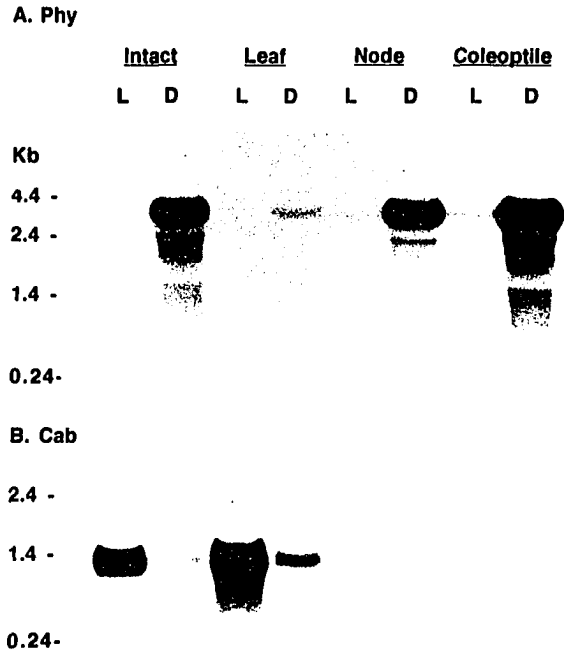
Fig. 4A, B. Measurement of the abundance of phyA and cab mRNA in isolated organs. **A** Total RNA was isolated from intact seedlings (intact), leaves (leaf), coleoptiles (coleoptile), or mesocotyl node regions (node) obtained from 4-d-old etiolated oat seedlings that were either harvested in the dark (D), or 4 h after a red light treatment (L). The total RNA (10 ug/lane) blots were hybridized with an RNA probe derived from the phy cDNA subclone pAPSX2.7 (Seeley et al., in press) and exposed to x-ray film for 4 h. **B** Replicate total RNA blot was hybridized with an RNA probe derived from the maize cab cDNA clone pGAB0.7 (Edwards and Colbert, 1990) and exposed to x-ray film for 8 h. RNA size markers are indicated.

coleoptile and node from dark- or light-treated samples.
Leaves isolated from light-treated seedlings showed a six to
eight fold increase in cab abundance over RNAs isolated from
dark incubated leaves (Figure 4B). Although there were low
levels of cab mRNA in the coleoptile, it still maintained at
least a four-fold red-light induction.

In order to determine whether phyA from the coleoptile is
required for the light-induced increase in cab mRNA abundance
in the leaf, the leaves and coleoptiles were separated prior
to treatment with light and floated in separate dishes in a
treatment buffer. RNA blot analysis showed that the isolated
primary leaf is fully capable of inducing the decrease of the
already low levels of phyA mRNA in the leaf (Fig. 5A) and
increasing levels of cab mRNA about six-fold in response to
red light treatment (Fig. 5B).

A. Phy

Kb

| | 1 | 2 | 3 | 4 | 5 |

4.4 -

2.4 -

1.4 -

0.24-

B. Cab

2.4 -

1.4 -

0.24-
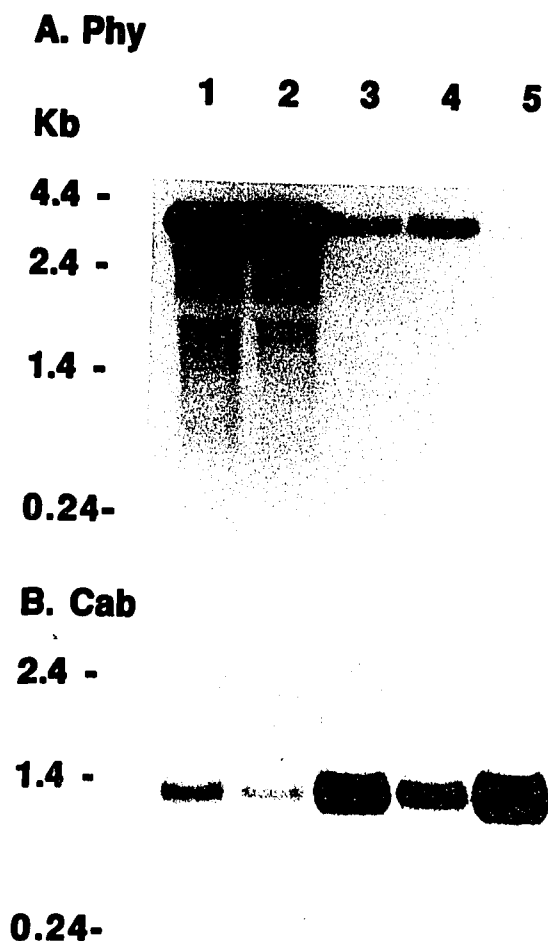
Fig. 5. Red light down-regulation of phyA and induction of cab mRNA in intact seedlings and isolated leaves. A RNA was isolated from: 1 intact etiolated seedlings, 2 excised seedlings floated for 4 h in treatment buffer in the dark, 3 excised seedlings given a red light pulse then floated in treatment buffer for 4 h in the dark, 4 isolated leaves floated for 4 h in treatment buffer and incubated in the dark, 5 isolated leaves given a red light pulse then floated in treatment buffer for 4 h in the dark. RNA gel blots were hybridized with a phy specific probe (pAPSX2.7) and exposed to x-ray film for 6 h. B Replicate RNA gel blot hybridized with a cab probe (pGAB0.7) and exposed to x-ray film for 4 h. RNA size markers are indicated.

DISCUSSION

Analysis of RNA gel blots of total RNA isolated from dissected segments of etiolated dark or light treated oat seedlings demonstrated that the distribution of phyA mRNA was similar to the patterns of the spectral activity and immunodetection of the phyA protein (Pratt, 1986). The high correlation between protein and mRNA data relative to organ specific and longitudinal distribution (Fig. 1 and 4) lead us to believe that the phyA mRNA would most likely be found in the same cells as reported earlier for in situ immunodetectible protein (Pratt, 1986). Since phyA mRNA and the phyA protein are localized to roughly the same tissues, we conclude that translational nor post-translational controls do not play a significant role determining the distribution of phyA protein in etiolated oat seedlings.

There are at least two possible explanations for the differences in phyA mRNA abundance seen in different tissues and organs. First, the phyA gene could be transcribed in all cells, but the mRNA could be less stable in certain cell types. Second, and most likely, the phyA gene is only transcribed at significant levels in those cells where the phyA mRNA and proteins accumulate.

In addition to the localization data for the phyA mRNA, we analyzed the same RNAs for abundance of phytochrome-induced

<u>cab</u> mRNA. <u>Cab</u> mRNA is found in the primary leaves of light-treated seedlings. The phytochrome signal transduction system is clearly responsible for the increase in abundance of <u>cab</u> mRNA following light treatment (Silverthorne and Tobin, 1987; Kuhlemeier et al., 1987; Lissemore and Quail, 1998). However, we found that the increase in abundance of <u>cab</u> mRNA in primary leaves can be induced fully in isolated primary leaves in the absence of the coleoptile sheath and mesocotyl node tissues. Since these tissues contain the majority of the <u>phyA</u> mRNA and the immunodetectible protein (Pratt, 1986), this pool of phytochrome is clearly not required for <u>cab</u> induction. It is possible that either <u>cab</u> gene induction requires only the low quantities of <u>phyA</u> protein present in the leaf or that <u>cab</u> induction is due to another species of the phytochrome family of proteins (<u>phyB</u>, <u>phyC</u>, or others) (Sharrock et al., 1989).

Previous studies have shown that although there is a significantly smaller quantity of <u>cab</u> mRNA in the coleoptile, it increased by the same amount after red light as the primary leaf (Seeley et al., 1991). Perhaps the phytochrome protein in the coleoptile is responsible for induction of genes involved in some of the other developmental processes of seedling development during photomorphogenesis. For example the <u>phyA</u> protein could be responsible for inducing the transient increase in elongation of the coleoptile and arresting the elongation of the mesocotyl (Schaer et al.,

1983). Both of these processes have been reported to be under phytochrome control and are, because of the localization of the phyA protein, good candidates to be regulated by phyA.

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PART II. REGULATION OF TYPE-1 PHYTOCHROME mRNA
ABUNDANCE: EVIDENCE FOR RAPID DEGRADATION
OF PHYTOCHROME mRNA IN VIVO

Regulation of type-I phytochrome mRNA abundance:

Evidence for rapid degradation of

phytochrome mRNA in vivo

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Colbert, J.T., Ph.D.

ABSTRACT

Type-I phytochrome (phyA) mRNA abundance in total RNA from four-day-old etiolated oat seedlings decreased rapidly following production of Pfr (phytochrome far-red absorbing form) caused by a red light pulse. In these total RNA samples, we detected putative in vivo phyA mRNA degradation products both before and after Pfr production. Cordycepin-treated coleoptiles were unable to accumulate chlorophyll a/b binding protein (cab) mRNA in response to red light, indicating that cordycepin was effective at inhibiting mRNA in the coleoptile. In the presence of cordycepin, phyA mRNA rapidly decreased in abundance, consistent with the hypothesis that it is inherently unstable, rather than being destabilized by Pfr.

INTRODUCTION

Phytochrome plays a crucial role in regulating plant development in response to light. Considerable evidence links the change in expression of specific light-responsive genes to the photoconversion of phytochrome from the Pr (red light-absorbing) to the Pfr (far red light-absorbing) form (for reviews see Kendrick and Kronenberg, 1986; Smith and Whitelam, 1990).

Red light-induced increases in mRNA abundance have been documented for the light-harvesting chlorophyll a/b binding proteins (cab) (Silverthorne and Tobin, 1987; Kuhlemeier et al., 1987), glutamine synthase (Tingey et al., 1988), and for the small subunit of ribulose 1,5-bisphosphate carboxylase-oxygenase (Silverthorne and Tobin, 1987; Kuhlemeier et al., 1987). Down-regulation of mRNA abundance by Pfr production has been found for protochlorophyllide reductase (Batschauer and Apel, 1984), asparagine synthetase (Tsai and Corruzi, 1990), beta-tubulin (Colbert et al., 1990) and for type I (etiolated-seedling-predominant) phytochrome (phyA) itself in most of the plant species that have been investigated (Colbert, 1988; 1991).

In four-day-old etiolated oat seedlings, the expression of type I phytochrome is decreased at several levels after exposure to a red light pulse, including: selective

proteolysis of the Pfr form of the photoreceptor (Shanklin et al., 1987), a decrease in mRNA abundance (Colbert et al., 1985) and a rapid decline in transcription (Lissemore and Quail, 1988). The importance of down-regulation of phyA gene expression is clearly demonstrated by the mutant phenotype (dwarf, dark green) observed in transgenic tobacco and tomato plants that over-express oat type I phytochrome (Boylan and Quail, 1989; Keller et al., 1989)

PhyA mRNA abundance decreases dramatically when etiolated oat seedlings are given a saturating pulse of red light (Byrne et al., 1990; Colbert, 1988; 1991). Previous studies using oat seedlings show a 90% decline in the amount of phyA mRNA present in the poly(A)$^+$ RNA fraction within 3 to 5 h following a red light pulse. Studies with isolated nuclei demonstrate that Pfr formation induces a decrease in transcription initiation to 10% of dark levels within 30 min of a red light pulse (Lissemore and Quail, 1988). The rapid decrease in phyA transcription seen in nuclear run-off experiments does not, by itself, explain the rapid decrease in phyA mRNA steady state levels. If phyA mRNA had a half-life of 30 h in the dark, as estimated for most poly(A)$^+$ RNAs present in soybean suspension culture cells (Silflow and Key, 1979), then phyA mRNA would persist at high levels in the cell long after the Pfr-induced decrease in transcription. Kinetic analysis combining the transcription and mRNA abundance data led to the proposal that

phyA mRNA exhibited a half-life of 16 to 47 h in etiolated oat seedlings prior to light treatment (Quail et al., 1986; Colbert, 1988).

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

Müllner and Kuhn, 1988), beta-tubulin mRNA is destabilized by microtubule depolymerization (Gay et al., 1989; Pachter et al., 1987) and vitellogenin mRNA is stabilized in the presence of estrogen (Brock and Shapiro, 1983; Nielsen and Shapiro, 1990).

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

RESULTS

Effect of Red Light on phyA mRNA Abundance

Figure 1 shows that exposure of four-day-old etiolated oat seedlings to red light resulted in a dramatic decrease in full-length phyA mRNA abundance to less than 10% of the dark level. During this decrease phyA mRNA exhibited an apparent half-life of about 90 min (Figure 1B). In addition, a smear of low molecular weight (LMW) hybridization, from 4.2 kb to 0.2 kb, was found in all total RNA samples that had detectable phyA signal. The LMW phyA smears disappeared at approximately the same rate as the mature mRNA band (Figure 1B). The observation that the abundance of the LMW phyA fragments decreased at about the same rate as the full-length message after light treatment supports the interpretation that the LMW hybridization is phyA specific. The LMW phyA RNA fragments were present in total RNA samples from oat seedlings that had never been exposed to light (Figure 1A; lane D_0, D_6) and from those irradiated with red light (Figure 1A; lane $R_{0.25}$-R_6). An increase in LMW phyA hybridization was observed at short times after red light (Figure 1B; lane $R_{0.25}$ and $R_{0.5}$). The regions of little hybridization at about 3.8 and 2.0 kb in the LMW phyA smears coincided with the 25S and 18S ribosomal RNAs, shown in Figure 2A, indicating that the phyA probe exhibits specific binding under our hybridization conditions.
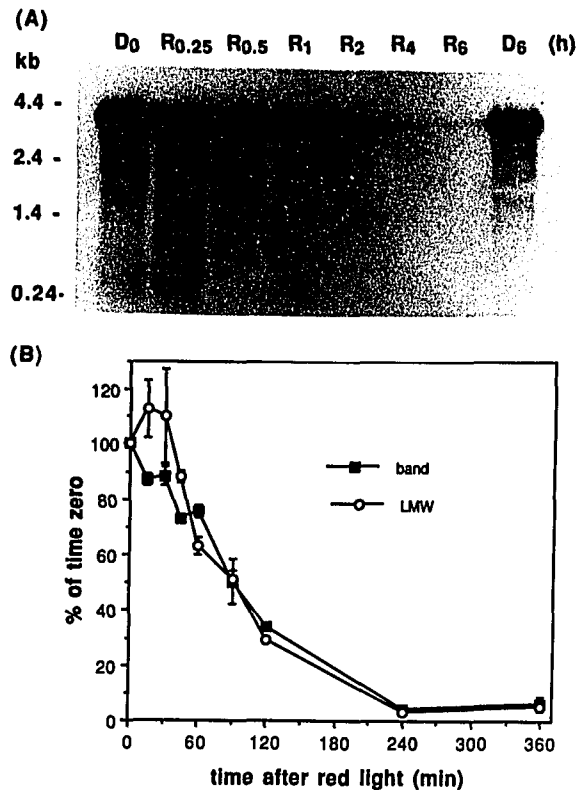
(A)

kb D_0 $R_{0.25}$ $R_{0.5}$ R_1 R_2 R_4 R_6 D_6 (h)

4.4 -

2.4 -

1.4 -

0.24-

(B)

% of time zero (y-axis)

120
100
80 — band
   — LMW
60
40
20
0

0   60   120   180   240   300   360

time after red light (min)

Figure 1.   Red light-induced decrease in phyA mRNA abundance
and detection of putative phyA mRNA degradation products.

(A).   A representative northern blot of total RNA from four-
day-old etiolated oat seedlings harvested at various times
after a saturating red light pulse (R time points), harvested
prior to the red light pulse (D_0), or kept in darkness for six
additional hours before harvest (D_6).   Samples of 5 ug from
each preparation of total RNA were electrophoresed on a 1%
agarose/3% formaldehyde gel, blotted to a nylon membrane,
hybridized with a ^{32}P-labeled antisense RNA probe (pAPSX2.7)
and exposed to x-ray film for 12 h.   Positions of the RNA size
markers are indicated.
(B).   Quantitation of the decrease in phyA abundance after red
light.   The regions corresponding to full-length phyA mRNA and
the LMW smears were excised from the blots and the amount of
radioactivity determined by liquid scintillation counting.
Error bars indicate standard error of the mean of the four
independent experiments, including the blot shown in panel
(A).

LMW phyA mRNA Fragments Are Produced In Vivo

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

To address the possibility of mRNA degradation during
total RNA isolation, several control experiments were
performed. First, replicate northern blots of total RNA
samples from the red light time course experiment, shown in
Figure 1, were hybridized with RNA probes to sequences
complementary to the cab or actin mRNA. Cab mRNA abundance
increased in response to red light as expected (Figure 2B),
whereas the actin mRNA levels remained constant following red
light treatment (Figure 2C). The actin data, taken together
with the ethidium bromide staining of rRNA (Figure 2A)
demonstrate that approximately equal amounts of mRNA and total
RNA were electrophoresed in each lane. Actin mRNA showed none
of the LMW hybridization seen on the phyA blots, indicating
that at least some poly(A)$^+$ mRNAs in these total RNA samples
were intact after isolation and RNA gel analysis (Figure 2C).
In addition, LMW cab RNA hybridization was evident only after
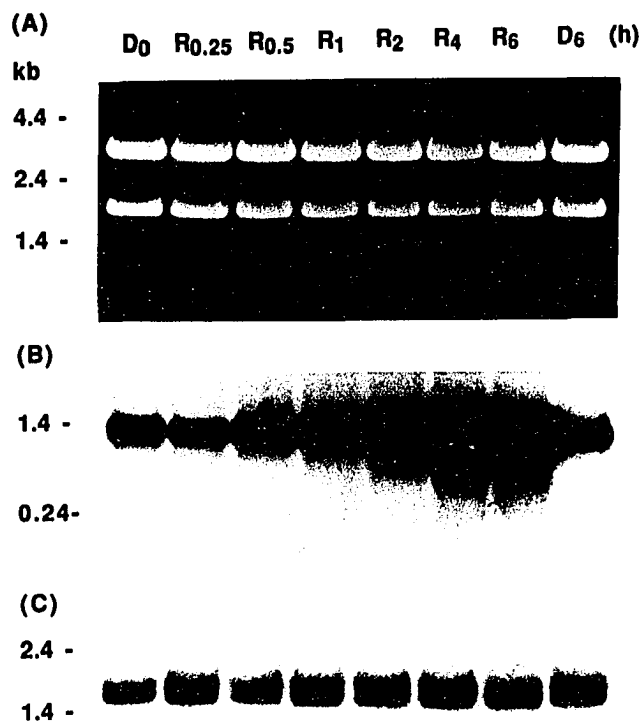
Figure 2. Visualization of rRNA, actin mRNA and cab mRNA in oat total RNA samples.

(A)   Ethidium bromide staining of total RNA samples. After electrophoresis of the ethidium bromide-stained samples, the agarose-formaldehyde gel was photographed on a UV light-emitting transilluminator.
(B)   Replicate northern blot hybridized with the maize cab antisense probe (pGABO.7). This blot was exposed to X-ray film for 6 h. The positions of the RNA size markers are indicated.
(C)   Replicate northern blot hybridized with the antisense soybean actin probe (pACT24). This blot was exposed to X-ray film for 48 h. The positions of the RNA size markers are indicated.

the light treatment had induced large quantities of cab mRNA (Figure 2B).

Figure 3 shows that the LMW phyA fragments were present in total RNA samples isolated using a variety of RNA isolation techniques. These techniques included: Small-scale (ATA), polysomal isolation (Poly), guanidinium thiocyanate centrifugation (GTC), and phenol:SDS large scale preparations (Phe). Each of these techniques employ distinctly different strategies to minimize ribonuclease activity and yet all of these techniques yielded RNA that gave an extensive smear of LMW phyA hybridization in northern blot analysis. The observation of LMW phyA RNA fragments associated with isolated polysomes suggests a cytosolic origin for these fragments.

Finally, two distinct assays were performed to detect degradation of exogenously added RNA during RNA extraction. First, a transcription reaction using pAPSX2.7 produced a radiolabeled 2.7 kb sense strand phyA mRNA fragment that was added to frozen oat seedlings at the start of RNA extraction by the ATA method. In vitro-synthesized pAPSX2.7 sense strand RNA showed no detectable hybridization to total oat RNA on northern blots (data not shown), therefore, ribonuclease protection by complementary RNA sequences in the seedlings was assumed to be insignificant. The in vitro-synthesized sense strand RNA had no 5'-end cap or 3'-end poly(A) tail and would
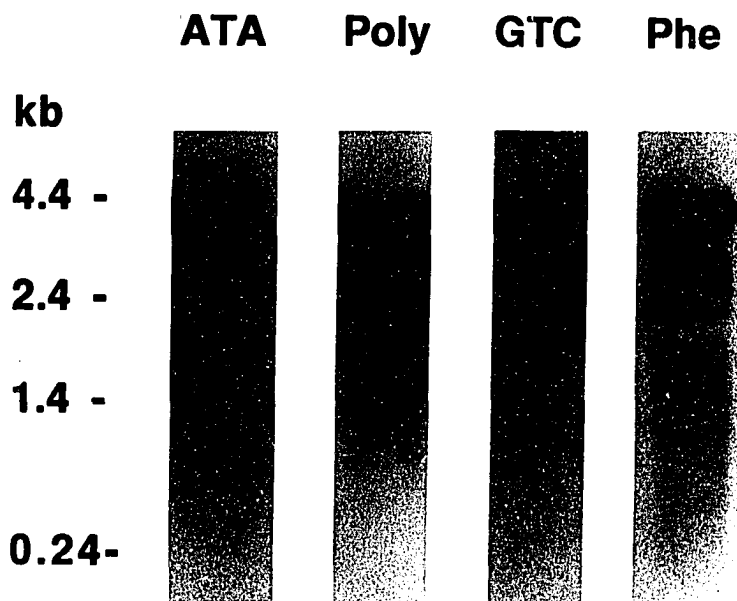
Figure 3.   Visualization of full length phyA mRNA and putative
phyA mRNA degradation products in RNA samples isolated by
various methods.

Total RNA was isolated from four-day-old etiolated oat
seedlings by the small scale (ATA) method , by
phenol:chloroform extraction of purified polysomes (Poly), by
extraction with guanidinium-thiocyanate followed by
centrifugation through CsCl (GTC), or by a phenol:SDS
extraction procedure (Phe).   Total RNA was loaded at 10 ug per
lane, then samples were electrophoresed, blotted onto nylon
membranes, and hybridized with a ^{32}P-labeled phyA probe
(pGAP1.7 or pAPSX2.7).   Positions of the RNA size markers are
indicated.

presumably have been degraded quickly by any general
ribonuclease activity present during the isolation procedure.
As shown in Figure 4; lanes A-C, the phyA RNA fragment was not
degraded, as observed in two independent experiments, when
added at the start of a standard ATA RNA isolation . In
addition to the phyA RNA, a smaller beta-tubulin in vitro-
synthesized radiolabeled sense strand RNA fragment was added
to a separate ATA RNA isolation. The beta-tubulin RNA also
remained intact during RNA isolation (Figure 4; lanes B-D).
Similar results were obtained when in vitro-synthesized RNAs
were added to oat seedlings at the start of RNA extraction by
the phenol:SDS RNA isolation procedure (data not shown).

A second experiment was devised using native mRNA. In
this experiment poly(A)$^+$ RNA extracted from dark-grown
seedlings (phyA mRNA enriched) was added to frozen tissues
from light-treated (6 h after red light) oat seedlings (phyA
mRNA depleted). The dark-grown seedling poly(A)$^+$ RNA was co-
isolated, using the ATA technique, with the total RNA from the
light-treated seedlings and hybridized on northern blots using
antisense pAPSX2.7 RNA transcript as a probe. After co-
isolation with RNA from the light-treated seedlings, no
increase in LMW phyA fragments was observed in two independent
experiments using two separate poly(A)$^+$ RNA samples,
indicating that full-length phyA mRNA was not degraded during
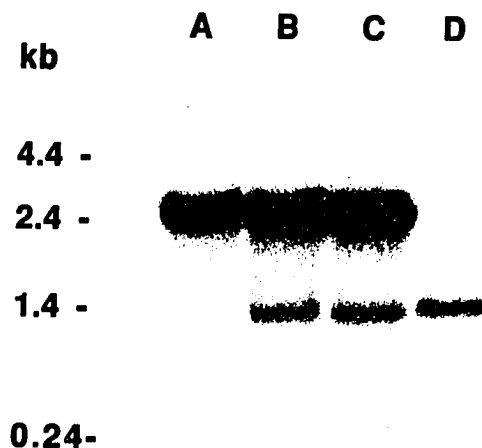the isolation procedure (data not shown).

Figure 4. Absence of ribonuclease activity during the ATA RNA isolation procedure.

Representative gel showing the protection of in vitro synthesized RNAs from general ribonuclease activity during the isolation of total RNA from four-day-old etiolated oat seedlings. ^{32}P-labeled sense strand RNAs were synthesized from an oat phyA cDNA subclone (pAPSX2.7) and from an oat β-tubulin cDNA subclone (pGβ1). The predicted sizes of the in vitro transcripts was 2.65 kb (phyA) and 1.2 kb (tubulin). The transcripts lacked both a poly(A) tail and a 5'7-methyl guanosine cap. The labeled RNAs were electrophoresed, blotted onto a nylon membrane and autoradiographed directly. The expected size of the in vitro- synthesized RNAs was observed either with (lane C) or without (lane B), 5 ug of dark-grown oat total RNA added to the labeled RNA fragment samples. The labeled RNAs were then added separately to etiolated oat seedlings at the time of grinding for RNA isolation by the ATA method. The labeled RNAs were co-isolated with the oat RNA present in each sample of etiolated oat seedlings. Samples of 5 ug of total RNA containing 275 cpm of phyA probe or 75 cpm of β-tubulin probe were electrophoresed in lanes A and D, respectively.

Taken together these data suggest that the LMW phyA hybridization observed in northern blot analysis represent in vivo-produced phyA mRNA degradation products (Figures 1 and 3). The high abundance of these degradation products in light-treated and dark-grown seedlings, relative to the full-length phyA mRNA band, suggests that phyA mRNA is rapidly degraded both before and after Pfr production.

PhyA mRNA Has a Short Half-life in the Dark

To test the prediction that phyA mRNA is rapidly degraded both before and after a light treatment, we used an RNA synthesis inhibitor to estimate the half-life of phyA mRNA in etiolated oat seedlings never exposed to light. This estimate could then be compared to the half-life of 60 to 90 min previously estimated for phyA mRNA after light treatment (Figure 1B; Colbert et al., 1985; Colbert, 1988). An infrared viewing system was used to allow manipulation of the oat seedlings in otherwise total darkness. Experiments with time courses from 1 to 4 h following illumination with IR radiation showed no significant changes in the levels of phyA message (data not shown).

We used cordycepin, a chain terminating adenosine analog (Cline and Rhem, 1974; Schopfer, 1989) to inhibit RNA synthesis. Preliminary studies revealed that virtually none of the phyA mRNA hybridizing to the pAPSX2.7 antisense RNA

probe was localized in the leaves of 4-day-old etiolated oat

seedlings (Fig. 4A, page 33). We also determined that, while

most of the cab mRNA is in the enclosed leaves, detectable

quantities of cab mRNA are induced by light in the coleoptile

as shown in Figure 5. To aid in the uptake of cordycepin by

the coleoptile, we excised oat seedlings, using the IR viewer,

and abraded off the cuticle using a polishing cloth (Edelmann

and Schopfer. 1989). Excision of the seedlings and abrasion

of the cuticle followed by immersion in incubation buffer did

not substantially affect either the light-induced decrease in

phyA mRNA abundance or increase cab mRNA abundance (Figure 5,

lanes 1-3). However, when 100 ug/ml cordycepin was included

in the incubation buffer, beginning 30 min prior to the light

treatment, the induction of cab mRNA accumulation by light was

completely inhibited (Figure 5, lane 4). In Figure 6 the

abraded dark controls (IR- illuminated) demonstrate that the

experimental manipulation of the seedlings did not cause

changes in phyA mRNA levels. In the presence of cordycepin

phyA mRNA abundance decreased after exposure to red light, but

to a lesser extent than in the absence of cordycepin (Figure

5A).

Having demonstrated that 100 ug/ml cordycepin is

effective in preventing cab mRNA synthesis in the coleoptile,

we used the same experimental conditions to estimate the half-

A. Phy
kb

	Dark Light			
	control		cordycepin	
			(-)	(+)
	1	2	3	4

4.4 -

2.4 -

1.4 -

0.24-

B. Cab

1.4 -
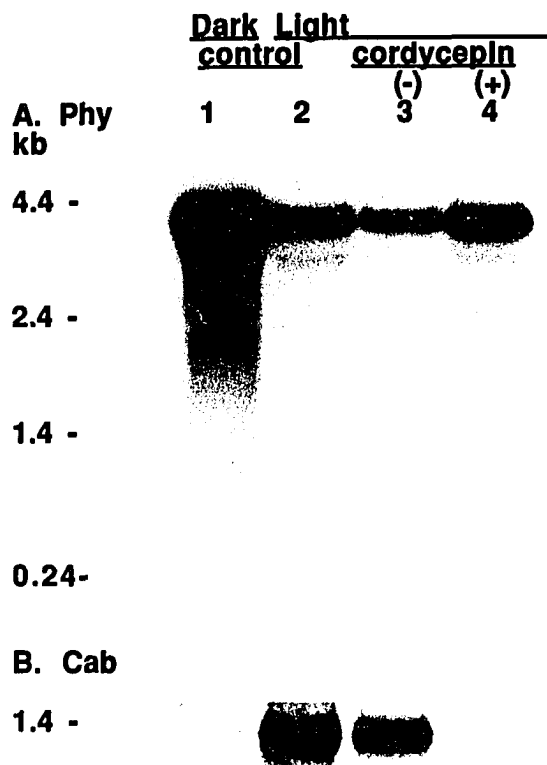
Figure 5. Effect of cordycepin on light regulation of phyA and cab mRNA levels.

Intact etiolated oat seedlings were harvested in the dark (Lane 1) or exposed to a red light pulse and harvested 3.5 h later (Lane 2). Other seedlings (using the IR viewer) were excised, abraded, and floated in buffer without (Lane 3) or with (Lane 4) 100 ug/ml cordycepin for 30 min. After the 30 min pretreatment the excised abraded seedlings were exposed to a red light pulse and incubated an additional 3.5 h in the dark. At harvest the coleoptiles were separated from the enclosed leaves. Total coleoptile RNA (10 ug) was analyzed on replicate northern blots and hybridized to either phyA (A) or cab (B) antisense probes and exposed to x-ray film for 2 and 24 h respectively.

(A)
kb

4.4 -

2.4 -

1.4 -

0.24-

(B)

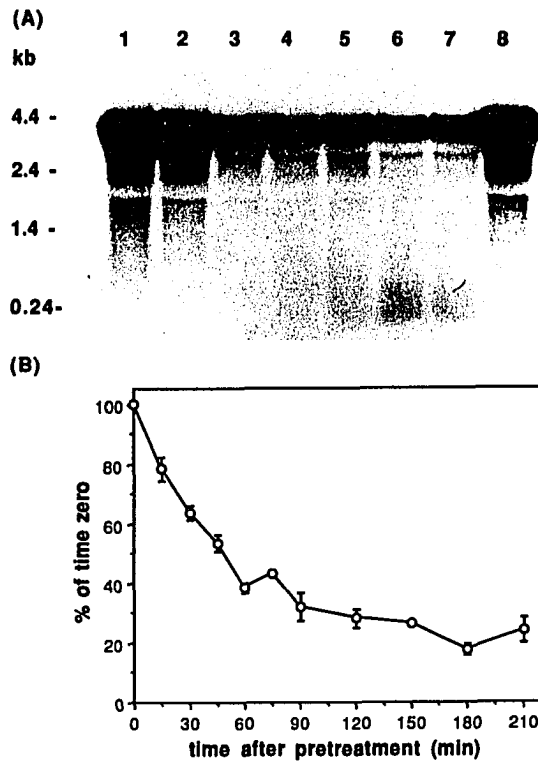Figure 6.   Effect of cordycepin on phyA mRNA abundance.

Etiolated oat seedlings were excised, abraded, placed in
control or cordycepin-containing buffers and harvested at
various times following a 30 min pretreatment. All procedures
were done using the IR viewer in otherwise total darkness.
(A). RNA was isolated and phyA mRNA was detected as described
previously. Total RNA (10 ug) was electrophoresed in each
lane, blotted, hybridized and exposed to x-ray film for 3 h.
Lanes 1 and 8 are abrasion and buffer controls (without
cordycepin) at time zero (=100%) and 3.5 h of incubation,
respectively. For this experiment seedlings exposed to
cordycepin (Lanes 2 - 7) were harvested at 30, 60, 120, 150,
180, and 210 min after pretreatment.
(B). The bands corresponding to full length phyA mRNA were
excised from northern blots and the amount of radioactivity
determined by liquid scintillation spectrometry. Data show
the means from four independent experiments for the 0, 30, and
60 min time points, three independent experiments for the 90
and 120 min time points and two independent experiments for
all others. Error bars indicate standard error of the mean of
the independent experiments, including the blot shown in panel
(A).

life of phyA mRNA. In this series of experiments the 30 min

pretreatment with cordycepin was not followed by light; the

excised, abraded seedlings were simply allowed to remain

in total darkness for times up to 3.5 h after pretreatment,

under these conditions phyA mRNA rapidly decreased in

abundance, exhibiting a half-life of about 50 min. Although

phyA mRNA decreased in abundance after cordycepin treatment

with an initial half-life similar to that after P_{fr} production,

phyA mRNA abundance was not decreased as much by cordycepin as

by light. By four hours after red light phyA mRNA abundance

was about 5% of the initial level, whereas by 3.5 h after the

30 min cordycepin pretreatment, phyA mRNA abundance was about

20% of the time zero level.

Experiments employing metabolic inhibitors are

susceptible to artifacts resulting from unintended secondary

effects of the inhibitors. We attempted to minimize the

potential for such artifacts by demonstrating directly that

mRNA synthesis (i.e., cab mRNA synthesis) was inhibited in the

coleoptile and by measuring phyA mRNA abundance at relatively

short times after exposure of the seedlings to cordycepin

(Figure 6). Another strategy to minimize secondary effects is

to use inhibitors at the minimum required concentration.

Therefore, we determined whether 100 ug/ml cordycepin was near

the minimum concentration to needed to cause the maximum

decline in phyA mRNA abundance. Excised and abraded seedlings
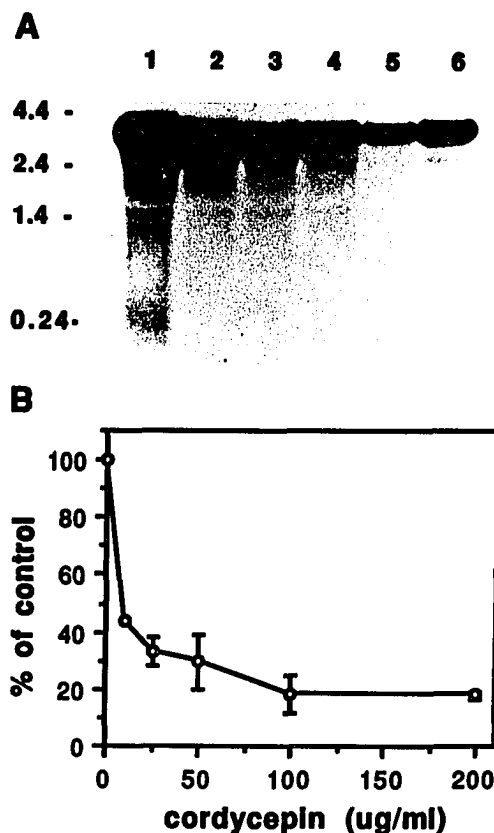
Figure 7. Effect of different concentrations of cordycepin on phyA mRNA abundance.

Etiolated oat seedlings were excised, abraded, placed in control buffer or buffer with cordycepin at various concentrations, and harvested after 4 h. All procedures were done using the IR viewer in otherwise total darkness.
(A). Representative blot of cordycepin concentration experiment. RNA was isolated and phyA mRNA was detected as described previously. Total RNA (10 ug) was loaded into each lane, electrophoresed, blotted, hybridized, and exposed to x-ray film for 3 h.
(B). The bands corresponding to the full-length phyA mRNA were excised and the amount of radioactivity was determined by liquid scintillation spectrometry. Data show the mean and the standard error of the mean of two independent experiments, including the blot shown in panel (A).

were exposed to various concentrations of cordycepin for 4 h. As shown in Figure 7, saturation was observed between 50 and 100 ug/ml cordycepin, indicating that the cordycepin concentration used in the half-life estimation experiments was not far in excess of the required concentration.

DISCUSSION

The 1 h apparent half-life of phyA mRNA in the poly(A)$^+$
RNA fraction following light treatment of oat seedlings is
well documented (Colbert, 1988; 1991). We have estimated the
apparent half-life of phyA message in total RNA to be about 90
minutes in red-light treated seedlings. These data support
preliminary observations (Colbert et al., 1985) suggesting
that the decrease in the amount of polyadenylated full length
phyA mRNA after light treatment of oat seedlings is due to
degradation of phyA mRNA, not only to deadenylation of the
message. The slightly longer estimate of phyA mRNA half-life
obtained from total RNA samples may be due to the time
required for deadenylation of the phyA mRNA prior to further
degradation. Deadenylated, but otherwise full-length mRNA,
would not be observed in the poly(A)$^+$ RNA fraction leading to
a shorter estimate for the half-life of phyA mRNA in those
samples. The phyA mRNA half-life estimated in poly(A)$^+$ RNA
and total RNA are both likely to be overestimates because phyA
transcription does not reach the minimum level until 15 to 30
min after Pfr production (Lissemore and Quail, 1988).

Analysis of phyA mRNA abundance in total RNA samples
allowed the detection of LMW phyA RNA fragments. We have
addressed the question of whether the LMW phyA RNA fragments
seen in RNA blot analysis are due to in vivo events or to

degradation during RNA extraction. There are several lines of evidence that support an in vivo origin of the LMW phyA mRNA fragments: First, total RNAs hybridized with actin or cab probes do not show extensive LMW RNA fragments, except when cab mRNAs are present at high levels. If the LMW phyA mRNA fragments were due to ribonuclease activity during RNA isolation then we would have expected to see extensive degradation of other mRNAs as well. Second, the isolation of total RNA using distinct methods for inhibiting nuclease activity show similar LMW fragment patterns. Third, degradation during RNA extraction was not detected for in vitro-synthesized sense strand phyA RNA, beta-tubulin sense strand RNA or dark-grown seedling poly(A)$^+$ phyA mRNA added at the start of ATA or Phenol:SDS RNA isolation from oat seedlings. If the LMW phyA hybridization was due to ribonuclease activity present during RNA isolation, then the non-capped, non-polyadenylated RNAs or native phyA mRNA presumably would have been extensively degraded. From these data, we conclude that the LMW phyA mRNA fragments are not an artifact of the isolation of RNA from oat seedlings but are present in vivo. In addition, similar LMW phyA hybridization fragments have been observed in cucumber (Tirimanne, T.A., and Colbert J.T., in press), corn, tomato, tobacco, and barley seedlings (Seeley, K.S., Rahim, I., and Colbert, J.T., unpublished results), indicating that the presence of LMW

fragments for the phyA mRNA may not be exclusive to oats. It is noteworthy that similar LMW phyA RNA fragments were evident in RNA blots from transgenic tobacco plants possessing a rice phyA gene that was under the control of a constitutive promoter (Kay et al., 1989b).

There are a number of possible sources of the in vivo produced LMW phyA mRNA fragments, including: a) premature transcription termination products in the nucleus, b) degradation of hnRNA, or c) rapid turnover of mature phyA mRNA in the cytoplasm. A kinetic argument suggests that the LMW fragments are not due to premature transcription termination. Since phyA transcription drops to minimal levels within 15 to 30 min after red light (Lissemore and Quail, 1988), we would expect to see levels of LMW transcription termination fragments to decrease over the same time course as transcription. However, the LMW smears decrease in abundance more slowly, at the same rate as the mature band, suggesting that they are phyA mRNA degradation products and not due to premature transcription termination. Indeed, we have no evidence that phyA transcripts in the preRNA pool contribute substantially to the LMW phyA mRNA hybridization seen on northern blots. Only in zucchini do we see a higher molecular weight phyA band (Tirimanne, T.A., and Colbert J.T., in press). The 5.6 kb zucchini phyA band has been shown previously to be a polyadenylated mRNA (Lissemore et al.,

1987). Total RNA samples from plant species other than oats, or isolated from oats using alternative techniques, show no splicing intermediates, suggesting that hnRNA does not represent a substantial fraction of the hybridizable phyA RNA. Finally, the presence of similar LMW fragments in total RNA samples prepared from isolated polysomes supports a cytosolic origin for the LMW fragments. If hnRNA degradation were responsible for LMW phyA mRNA fragments then we would expect to find few such fragments in the polysome fraction.

If we accept that the LMW phyA mRNA fragments are produced in vivo, in the cytosol, then the most likely explanation for their presence is that they are intermediates of phyA mRNA degradation and that the conversion of phyA degradation intermediates to nucleotides is slow enough to allow detection of the intermediates. The interpretation of the mRNA fragments as in vivo phyA mRNA degradation products leads to the prediction that if phyA mRNA is destabilized after Pfr production, more of these degradation products should be observed after light treatment. However, we detect similar amounts of the degradation products in dark control and light-treated seedlings, suggesting that the rate of phyA mRNA degradation does not dramatically increase after Pfr production. These data support the hypothesis that phyA mRNA is inherently unstable (Kay et al., 1989a) rather than being destabilized by Pfr as previously proposed (Quail et al.,

1986; Colbert, 1988). The significance of the transient increase in LMW phyA mRNA fragments at 15 min post-irradiation is currently unclear, but could indicate a slight increase in the rate of phyA mRNA degradation after Pfr production.

A more direct way to address the question of whether phyA mRNA exhibits inherent instability or Pfr-induced instability is to estimate the half-life of the phyA message in oat seedlings that have never been exposed to light. We used the RNA synthesis inhibitor cordycepin (Cline and Rhem, 1974; Schopfer, 1989) to estimate the half-life of phyA mRNA in etiolated oat seedlings. These experiments show that the half-life of phyA mRNA is about 60 min prior to red light treatment in etiolated oat seedlings. This value is quite similar to the estimated half-life of phyA mRNA in both total RNA (Figure 1) and poly(A)$^+$ RNA after Pfr production (Colbert et al., 1985).

A half-life of about 1 h is substantially shorter than the average half-life of 30 h reported for most poly(A)$^+$ RNAs in soybean suspension culture cells (Silflow and Key, 1979), and the average half-life of 16 h reported for most poly(A)$^+$ RNAs in Xenopus liver cells (Brock and Shapiro, 1983). A 1 h half-life is also much shorter than the 16 to 47 h half-life previously predicted for phyA mRNA in etiolated seedlings (Quail et al., 1986; Colbert, 1988). The possibility of artifacts resulting from the use of a metabolic inhibitor was

minimized by using a cordycepin concentration near that required for maximal inhibition, and limiting the treatment with cordycepin to 4 h or less. Furthermore, use of an RNA synthesis inhibitor is more likely to lead to an overestimation, rather than an underestimation, of the half-life of an mRNA due to potentially incomplete inhibition of RNA synthesis. Therefore, we conclude that phyA mRNA is inherently unstable with a half-life of about 1 h, similar to the short-lived histone, (Graves et al., 1987; Marzluff and Pandey, 1988) c-myc (Dani et al., 1984) and c-fos (Wilson and Treisman, 1988; Shyu et al., 1991) mRNAs. In addition, we conclude, unlike earlier proposals (Quail et al., 1986; Colbert, 1988), the instability of phyA mRNA is Pfr-independent. The observation of a short half-life for phyA mRNA in etiolated oat seedlings is consistent with the interpretation of the LMW phyA mRNA fragments being due to the rapid degradation of phyA mRNA in the seedlings. Phytochrome mRNA also appears to be relatively unstable in a cell-free mRNA degradation system (Ross and Kobs, 1986) that we are developing for use with plant mRNAs (Byrne et al., 1990; Byrne, D.H., Seeley, K.A., and Colbert, J.T., unpublished results).

Although the half-life of phyA mRNA is similar after cordycepin treatment or Pfr production, cordycepin does not lead to as great a decrease in phyA mRNA abundance as does red

light. This could be due to incomplete inhibition of phyA mRNA synthesis by cordycepin. However, cab mRNA synthesis appears to be completely inhibited under the same conditions. In addition, several experiments have shown that the production of Pfr in the presence of cordycepin leads to a smaller decrease in phyA mRNA abundance than in the absence of cordycepin. These observations suggest that a labile factor required for phyA mRNA degradation is depleted during cordycepin treatment, inhibiting degradation of phyA mRNA. This factor could be envisioned to be either a phyA-specific ribonuclease or a molecule that targets phyA mRNA for degradation by a more general nuclease. The factor could either be a protein or an RNA molecule. Preliminary experiments show that the protein synthesis inhibitor cycloheximide inhibits the ability of etiolated oat seedlings to decrease phyA mRNA abundance after Pfr production (Colbert, 1991). Exposure of seedlings to cycloheximide in darkness resulted in accumulation of phyA mRNA to 125% of control levels over 4 h (Seeley, K.A., and Colbert, J.T., unpublished results). Taken together with the cordycepin data these observations are consistent with the possibility that an unstable protein factor plays an essential role in phyA mRNA degradation. However, the available data do not rule out the possibility that the two inhibitors are working by distinct mechanisms.

The pathway by which phyA mRNA is degraded rapidly is unknown. Other nuclear-encoded mRNAs have been reported to be deadenylated prior to degradation (Binder et al., 1989; Brewer and Ross, 1988; Shyu et al., 1991). This may also be true for the phyA mRNA. RNA blot analysis of phyA mRNA in poly(A)$^+$ RNA reveals little LMW hybridization (Cotton et al., 1990) or polyadenylated phyA RNA fragments that are larger than about 1.5 kb (Colbert et al., 1985). Whether the phyA RNA fragments observed in some poly(A)$^+$ RNA samples are produced in vivo or during poly(A)$^+$ RNA isolation remains to be established. We have observed that poly(A)$^-$ RNA possesses both LMW phyA RNA fragments and detectible quantities of apparently full-length phyA mRNA (Seeley, K.A., and Colbert, J.T., unpublished results). The greater amount and size distribution of LMW phyA RNA fragments in total RNA, together with the presence of poly(A)$^-$ but otherwise full-length phyA mRNA, is consistent with the hypothesis that phyA mRNA is deadenylated at an early stage in the degradation pathway. It is important to note that the phyA mRNA degradation products do not show, on agarose gels, any distinct intermediate-sized bands suggesting that the pattern of degradation does not involve sequence specific cleavage. However, preliminary experiments using phyA probes specific for the 3'-end and 5'-end of the mRNA have not supported a simple unidirectional exonuclease

digestion model (Seeley, K.A., and Colbert, J.T., unpublished results).

The significance of the rapid degradation of phyA mRNA to plant development is still in question. The possibility that oat seedlings need to decrease their capacity to synthesize type I phyA protein after light is perceived in order to properly induce photomorphogenesis is supported by the observation that over expression of oat phytochrome in transgenic tobacco (Keller et al., 1989) and tomato (Boylan and Quail, 1989) leads to developmental abnormalities. Decreases in phytochrome expression occur, in part, at the protein level because the Pfr form of the protein is rapidly degraded via the ubiquitin pathway (Shanklin et al., 1987). An inherently unstable mRNA would further decrease expression of the type I phyA protein by rapidly leading to diminished capacity for translation of type I phyA protein after the Pfr-induced decrease in transcription. We have observed potential phyA mRNA degradation products in other plant species suggesting that phyA mRNA may be inherently unstable in these species as well. However, whether phyA mRNA is inherently unstable in all plant species remains to be determined. Exposure of etiolated tomato seedlings to red light results in little or no decrease in phyA mRNA abundance (Sharrock et al., 1988). This observation could be due either to no effect of Pfr on phyA transcription in tomato, or to

tomato phyA mRNA being relatively stable. In either situation, the regulation of phyA expression during photomorphogenesis must occur principally at the translational or post-translational level in tomato seedlings.

Understanding the role that mRNA degradation plays in the maintenance of steady-state levels of phyA mRNA will lead to increased understanding of the regulation of gene expression in plants. This is the first report of direct evidence that phyA mRNA is inherently unstable. Little is known about how plant cells recognize mRNAs that are to be rapidly degraded (Okamuro and Goldberg, 1989) Further work is being performed to determine which sequences or secondary structures are required to target phyA mRNA for rapid degradation.

MATERIALS AND METHODS

Growth and Light Treatment of Plants

Four-day-old dark-grown oat (<u>Avena</u> <u>sativa</u> cv. Garry)
seedlings were harvested by excision just below the mesocotyl
node. Harvest of etiolated seedlings was performed under dim
green light. After harvest the plant tissues were frozen in
liquid nitrogen and stored at -80°C until isolation of RNA.
Saturating red light treatments were provided as previously
described (Colbert et al., 1983). After the light treatment
the seedlings were placed back into the dark for the
appropriate time prior to harvest.

RNA Isolation and Quantification

The principle method for RNA isolation was a modification
of the rapid mini-scale RNA isolation described by Wadsworth
et al. (1988). Frozen tissue samples (100-200 mg) were ground
with a small mortar and pestle in liquid nitrogen and thawed
in the presence of aurintricarboxylic acid (ATA, Sigma A-1895)
and phenol-chloroform (1:1). After phenol-chloroform
extraction, the RNA was separated from DNA by differential
precipitation of RNA with 3M LiCl. Total RNA yields ranged
from 300 to 800 ug/gfw for whole seedlings and about 50 ug/gfw
for excised coleoptiles. Other techniques for RNA isolation
included: phenol-SDS, large scale isolation (Dean et al.,
1985), polysome isolation (Larkins and Hurkman, 1978;

Telericio and Chourey, 1985), and guanidinium-thiocyanate/CsCl centrifugation (Colbert et al., 1983). Poly(A)$^+$ RNA was purified from phenol:SDS-isolated total RNA using poly(U)-Sephadex chromatography as described previously (Lissemore et al., 1987).

Total RNA (5 to 10 ug) and poly(A)$^+$ RNA (0.1 to 1.0 ug) were electrophoresed in 1% agarose/3% formaldehyde gels, blotted onto nylon membranes (Gene Screen, Dupont) and hybridized [50% deionized formamide (Nucleic acid grade, BRL), 1.0 M NaCl, 10% dextran sulfate, and 1% SDS (Bio-Rad)] at 65°C with specific, ^{32}P-labeled, in vitro-synthesized, antisense RNA probes (Riboprobe, Promega) added to the hybridization mixture at 500,000 cpm/ml. Autoradiographic exposures were made at - 80°C on XAR5 (Kodak) X-ray film with intensifying screens (Dupont). Quantitation of hybridization was accomplished by overlaying the autoradiogram on the blot, carefully cutting out the membrane and determining the amount of radioactivity bound to the excised membrane fragment using liquid scintillation spectroscopy. Background hybridization was subtracted by the amount of radioactivity bound to a similar sized membrane piece from a region on the same blot to which no RNA was bound.

Plasmid DNA and in vitro RNA synthesis

DNA isolation and modification, in vitro transcription template preparation, agarose gel electrophoresis and subcloning were performed using standard molecular biology techniques or manufacturers instructions (Sambrook et al., 1989; Bio 101; Promega; Stratagene; Qiagen). Fragments from different parent clones were subcloned into pGEM3 (Promega) or pBluescript (Stratagene) transcription vectors. Plasmid pGAP1.7 (Edwards and Colbert, 1990) contains an internal 1.7 kb PstI fragment of the oat phyA cDNA clone pAP3.2 (Hershey et al., 1984) cloned into PGEM3. This fragment includes the sequence for the phyA chomophore binding region. In vitro transcription from the T7 polymerase promoter of NcoI-linearized pGAP1.7 gives antisense (complementary to phyA mRNA) phyA RNA. Plasmid pAPSX2.7 is a 2.7 kb SacI/XbaI fragment from the oat phyA subclone pFY122 (Boylan and Quail, 1989) cloned into pGEM3. This fragment includes the chromophore binding region and 1.2 kb of the phyA gene 3' to the end of pGAP1.7. Probe synthesis using SP6 polymerase on EcoRI-linearized pAPSX2.7 gives antisense phyA mRNA. Sense (identical to the phyA mRNA) strand RNA was transcribed at low specific activity (50 mM concentration of all four unlabelled nucleotides) using T7 polymerase on a XbaI-linearized pAPSX2.7 template. The construct pGβ1 contains a 1.2 kb fragment in pGEM3 from the 3' end of the pβ1 β-tubulin oat cDNA clone

(Colbert et al., 1990). Low specific activity sense strand RNA was transcribed from the SP6 promoter on a HindIII-linearized pGß1 template. Plasmid pAct24 is a 0.14 kb XhoI/EcoRI subclone from the third exon of the pSac3 (Shah et al., 1982) soybean actin gene in pBluescript. Antisense probes were transcribed from the T3 polymerase promoter on a XhoI-linearized pACT24. The subclone pGAB0.7 (Edwards and Colbert, 1990) contains a 0.7 kb PstI fragment from pAB1084, a corn chlorophyll a/b binding protein (cab) cDNA clone in pGEM3 from the 3' end of the cDNA, to include sequences from the 3' untranslated region. Antisense probes were synthesized from the T7 polymerase promoter using HindIII-linearized pGAB0.7.

Coleoptile Abrasion and Inhibitor Treatment

Four-day-old etiolated seedlings were excised 5-10 mm below the mesocotyl node and passed four to six times through a loop of dry polishing cloth as described previously (Edelmann and Schopfer, 1989; Schopfer, 1989). Control experiments were performed using Evans Blue or neutral red dyes to determine the amount of abrasion needed to remove the waxy cuticle but not damage the underlying tissue. All dark experiments used an IR viewer (FJW Optical Systems) to manipulate the seedlings in the absence of phytochrome active radiation. After abrasion, the seedlings (about 10 per dish) were placed in incubation buffer (1 mM PIPES, pH 6.25, 1 mM

Na-citrate, 1 mM KCl, 15 mM sucrose) in a 60 X 15 mm plastic dish, with or without inhibitor (cordycepin, Sigma C-3394). This mixture was then swirled at 75 RPM in total darkness for the desired time. At harvest, excised abraded seedlings were blotted dry on paper towels, frozen in liquid nitrogen, stored at -80°C, and RNA was isolated using the ATA procedure.

Acknowlegements

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GENERAL SUMMARY

In the course of this research I have addressed two major issues relating to the of the role that phytochrome plays in plant development. First, the localization of phyA mRNA in four-day-old etiolated oat seedlings was determined and, second, the in vivo half-life of the phyA mRNA in the dark was determined following RNA synthesis inhibition. Detailed discussions about these results can be found in PART I and PART II. In this section it is my intention to summarize the significance of these findings by relating the experimental results with their potential biological considerations.

Photomorphogenic development in flowering plants is dependent on the plant's ability to detect low levels of light while the seedling is below the soil surface, allowing it to prepare to become photosynthetically competent before emerging from the soil. At the same time the synthesis of the components of the photosynthetic system is repressed until that light is received, preventing the wasteful synthesis of unneeded components. PART I of my work demonstrates that the majority of the phytochrome in four-day-old oat seedlings is not required for light-induced increases in cab mRNA abundance in the primary leaf. The majority of the phytochrome protein and its mRNA are found in cells either near the tip of the coleoptile or in the mesocotyl node. This localization and

signal tranduction pattern suggests that there are other functions for the etiolated phyA protein. PhyA may be involved in the induction of coleoptile elongation and the inhibition of mesocotyl extension, which are responses reported previously for red-light-treated etiolated oat seedlings (Schaer et al., 1983). Further research into the specific mechanism of signal transduction is needed to determine which of the phytochrome proteins is involved in which developmental response.

Transcription of phyA is rapidly down-regulated by red light and the phyA protein, stable in its dark form, is quickly degraded once it perceives light. This hints at the possibility that the plant requires only a transient phytochrome signal to induce photomorphogenesis. PART II of this work demonstrates that the phyA mRNA is rapidly turned over with a half-life close to 1 hr independent of light treatment. This observation correlates well with the hypothesis that the phyA light regulation system is an extremely sensitive system that has a requirement to quickly decrease the amount of the photoreceptor once the signal is perceived. Although it appears to be somewhat wasteful to make large quantities of labile message, it may indicate that there is a selective advantage of being better prepared for photosynthetic development the moment the seedling has emerged from the soil and not before.

Research into the cell's ability to determine which messages should be degraded or stabilized should provide useful insights. Other techniques or areas that could be explored include; mapping degradation products for intermediates, using 3' or 5' end probes to see if a direction of degradation can be determined, finding the sequences responsible for determining stability, finding which proteins preferentially bind to the phyA message, and finally subcloning degradation sequences into stable messages.

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