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Oat phyA containing transgenic tobacco: an examination of oat phyA mRNA stability and the role of oat phyA in germination of tobacco seed

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Oat *phy*A containing transgenic tobacco: An examination of oat *phy*A mRNA stability and the role of oat *phy*A in germination of tobacco seed

by

Linda Joanne Barnes

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ABSTRACT

The regulation of oat phytochrome A (*phy*A) mRNA stability and the photoregulation of germination of tobacco seeds containing the oat *phy*A gene was examined using oat *phy*A containing transgenic tobacco. Oat *phy*A mRNA has been previously shown to be a relatively unstable mRNA. The intent of this research was to identify elements within the *phy*A mRNA responsible for rendering the message unstable. The approach taken was to use tobacco transformed with either the full-length or truncated versions of the oat *phy*A gene and examine the half-lives of the resulting oat *phy*A mRNAs in transgenic tobacco. RNA blot analysis of oat *phy*A in both oats and in tobacco transformed with the oat *phy*A gene showed a pattern of oat *phy*A hybridizable fragments smaller than the full-length mRNA band. These fragments were thought to be degradation products indicative of a short-lived message. The half-lives of the truncated oat *phy*A mRNAs were found to be between 90 minutes and four hours. The full-length oat *phy*A mRNA had a half-life of about four hours in transgenic tobacco. Determination of the half-life of both truncated and full-length oat *phy*A mRNAs in transgenic tobacco suggests that the amount of oat *phy*A hybridizable fragments does not correlate with mRNA half-life. Germination of seeds from wild-type tobacco plants requires light. Approximately 20% of the seeds from transgenic tobacco plants containing the oat *phy*A gene and grown under fluorescent lighting were found to germinate in the dark. Seeds from transgenic tobacco grown under fluorescent and incandescent lighting did not germinate in darkness. A higher percentage of seeds from both wild-type and transgenic plants were found to germinate when imbibed in the presence of nitrate. About 50% of the seeds from transgenic plants grown in the presence of incandescent lighting and imbibed in the presence of nitrate germinated in darkness. The higher level of phytochrome in the transgenic seedlings promoted germination and was sensitive to the light conditions under which the parent plants were grown. These findings suggest that *phy*A plays a role in promoting germination in tobacco seeds.

CHAPTER 1 INTRODUCTION

Phytochrome Gene Families and Function

Phytochrome is a chromoprotein that plants use to sense their light environment. Phytochrome is important in various plant species for eliciting responses such as induction of flowering, seed germination, de-etiolation, and shade avoidance. Phytochrome is a protein dimer that has two photoreversible forms: Pr and Pfr. Pr, the red light absorbing phytochrome, absorbs red light maximally at 666 nm. Pfr, the far-red light absorbing form, absorbs far-red light maximally at 730 nm (Colbert et al., 1991). The molecular weight of the phytochrome monomer ranges from 114 to 125 kDa (Abe et al., 1985; López-Juez et al., 1992; Wang et al., 1992) depending on plant species. One type of phytochrome, phytochrome A (*phy*A), is known to be a soluble, cytosolic protein. Pfr, formed from Pr upon irradiation with red light, is the active form of the molecule that functions to promote photoresponses. Phytochrome is known to regulate the expression of a number of genes including protochlorophyllide reductase, chlorophyll a/b binding protein (Mösinger et al., 1985), small subunit of ribulose bisphosphate carboxylase (Silverthorn and Tobin, 1987), ferredoxin (Dickey et al., 1992), glutamine synthase (Tingey et al., 1988), and phytochrome itself (Colbert, 1988).

Multiple phytochrome genes have been found to exist in plants (Furuya, 1993). In *Arabidopsis* five phytochrome genes have been identified. These have been designated as *phy*A, *phy*B, *phy*C, *phy*D, and *phy*E (Sharrock and Quail, 1989; Furuya, 1993). The fact that amino acid identity among these phytochrome genes is as little as 50% (Sharrock and Quail, 1989) has raised the possibility that these gene products function to elicit different photoresponses at distinct developmental times or in response to specific photostimuli. *Phy*A and *phy*B are the best characterized phytochrome gene families. They are present in both monocot and dicot species (for review see Furuya, 1993). The structure of the oat *phy*A3 gene is shown in Figure 1.1 (Hershey et al., 1987). *Phy*A is found at a high level in etiolated seedlings, while in green plants it is present at a much lower level (Furuya, 1989). *Phy*B is expressed at relatively low levels in both etiolated and light grown plants (Dehesh et al., 1991). The precise roles of *phy*A and *phy*B in plant development and response to the light environment is now being more carefully examined. Phytochrome deficient mutants and phytochrome overexpressing transgenic plants have been used to elucidate the function of these different genes.

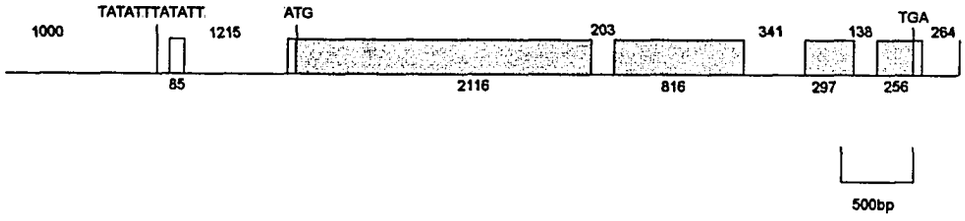


Figure 1.1 Structure of the oat *phy*A3 gene (Hershey et al., 1987). The translation start site is represented by the ATG and the stop codon is represented by the TGA. Numbers indicate the number of base pairs in each feature. Exons are represented by boxes. Shaded boxes represent the coding regions, while open boxes indicate untranslated regions. Lines represent introns and flanking sequences.

Phytochrome deficient mutants

Phytochrome deficient mutants have been isolated in *Arabidopsis*, tomato, and cucumber. *Hy8* and *fre*1 are lines of ethylmethane sulfonate-induced *Arabidopsis* mutants that have been found to be defective in their ability to respond to continuous far-red light (Parks and Quail, 1993; Nagatani et al., 1993). These mutants produce long hypocotyls when seedlings are grown under far-red light. Wild-type plants respond to far-red light by growing very short hypocotyls. In two of the *hy8* lines, *phy*A was immunologically undetectable whereas *phy*B was present at normal levels (Parks and Quail, 1993). The long hypocotyl phenotype under far-red conditions was attributed to a mutation in *phy*A. These mutants maintain a normal phenotype when seedlings are grown under white light conditions, suggesting that *phy*A function in light grown seedlings is negligible.

The *au* mutant in tomato has been shown to be selectively depleted in the *phy*A protein in seeds and dark grown seedlings (Lipucci Di Paola et al., 1988; Koornneef et al., 1985; Nagatani et al., 1993). Light grown seedlings were found to produce long hypocotyls during de-etiolation, have reduced chlorophyll accumulation, and reduced anthocyanin accumulation (Adamse et al., 1988). Although tomato seed germination is light insensitive, these mutants were also found to have poor seed germination compared to wild-type (Georghiou and Kendrick, 1991). These findings suggest that *phy*A functions in the de-etiolation response and may be important for seed germination.

*Phy*B deficient mutants have been identified in both cucumber and *Arabidopsis*. Cucumber long hypocotyl mutants (lh) lack immunologically detectable *phy*B (López-Juez et

al., 1992). These mutants are characterized by displaying elongated hypocotyls when seedlings are grown under white light, having a saturated shade-avoidance response, and increased apical dominance (Adamse et al., 1988; López-Juez et al., 1990).

Arabidopsis hy3 mutants have decreased levels of *phy*B. These were identified by screening for mutants having long hypocotyls when grown under white light. *Hy3* plants were found to flower sooner, have reduced levels of chlorophyll accumulation, and develop long hypocotyls when grown under white light. The *hy3* mutants were found to have mutations within the gene coding for *phy*B (Reed et al., 1993). *Phy*B functions in a different capacity than *phy*A as demonstrated by differences in the phytochrome deficient mutants. It remains to be determined whether the additional *phy* genes (*phy*C, *phy*D, etc.) have distinct or overlapping functions.

Phytochrome function in transgenic plants

Studies of transgenic plants have also been used to implicate distinct roles for *phy*A genes. Dicotyledonous plants transformed with the oat *phy*A gene display a light-exaggerated response when grown under light conditions including reduced internodes, more chlorophyll, and for some species, increased anthocyanin biosynthesis (Kay et al., 1989a; Cherry et al., 1991; McCormac et al., 1991; Boylan and Quail, 1989). These findings suggest not only that oat phytochrome is functional in a dicotyledonous system, but when transcribed from a constitutive promoter, is present and able to function in green, non-etiolated plants (Whitelam et al., 1992).

Studies in phytochrome over-expressing tobacco can help define the specific roles of *phy*A and *phy*B in seed germination in relation to each other and the other members of the phytochrome gene family. As described earlier, *au* mutants deficient in *phy*A phytochrome have provided evidence that *phy*A is responsible for eliciting germination in tomato (Georghiou and Kendrick, 1991). *Arabidopsis* and tobacco plants transformed with an oat *phy*A cDNA showed no increased levels of germination in the dark (McCormac et al., 1993). Seeds from transgenic *Arabidopsis* containing rice *phy*B or *Arabidopsis phy*B genes germinate at higher levels in darkness compared to wild-type seeds when seed is matured on plants that are grown under low red/far-red light ratios.

Phytochrome function in seed germination

Light responsive germination can be greatly influenced by a number of factors prior to imbibition including, genotype, seedlot (Fielding et al., 1992), light conditions at seed maturation (Getten-Haynes and Klein, 1974), temperature (Roberts and Benjamin, 1979;

Vincent and Roberts, 1979; Fielding et al., 1992), and the location of the seed on the plant (Hilton, 1985). Other factors influence germination during or after imbibition. High temperatures during imbibition can reduce germination even in the presence of red light (Fielding et al., 1992; Kristie et al., 1981; Georghiou and Kendrick, 1991). Photosensitivity in a variety of species is greatly increased by the presence of nitrate (DePetter et al., 1985; Roberts and Benjamin, 1979; Vincent and Roberts, 1979; Hartmann and Nezadal, 1990; Grubisic and Konjevic, 1990; Hilton, 1985), or gibberellic acid (DePetter et al., 1985; Hilhorst et al., 1986). In photosensitive species, nitrate functions to stimulate germination during or after imbibition in the presence of Pfr but not Pr. Nitrate is not effective prior to formation of Pfr by the application of light to imbibing seeds (Grubisic and Konjevic, 1990; Hilhorst et al., 1986).

Phytochrome Gene Regulation

The levels of *phy*A protein are regulated in response to light. In etiolated plants Pfr is labile, it has a half-life of between 60-90 minutes (Furuya, 1993). The stability of the two forms of phytochrome, Pr and Pfr, are quite different. Following irradiation with red light, Pr is photoconverted to Pfr and total phytochrome protein abundance declines. This decline in phytochrome levels is due to the rapid degradation of the Pfr form of the *phy*A protein (Vierstra and Quail, 1986). This allows the plant to rapidly produce the active form of the molecule, trigger the appropriate signal transduction mechanism, and remove the Pfr quickly to avoid an exaggerated response.

Regulation of phytochrome mRNA abundance varies with the phytochrome gene and with the species studied. Following a pulse of red light, oat *phy*A mRNA abundance in etiolated oat seedlings was found to drop by 90% within three hours with an apparent half-life of about one hour (Colbert, 1988). Transcription of *phy*A was found to decrease by 90% after irradiation of etiolated oat seedlings with red light (Lissemore and Quail, 1988). Pfr is thus able to regulate *phy*A transcription. In rice, barley, and peas, *phy*A mRNA levels show a dramatic red light-induced decline (Kay et al., 1989b; Dehesh et al., 1991; Rahim, 1992; and Tomizawa et al., 1989). In cucumber cotyledons, the levels of *phy*A mRNA are down regulated in response to light in a transient manner. The *phy*A mRNA was found to decrease to 20% of the original level by three hours after exposure to light and then reaccumulate to 60% of the original levels within 12 hours (Tirimanne and Colbert, 1991). *Phy*A transcript abundance in tomato and *Arabidopsis* changes little following exposure of plants to red light (Sharrock et al., 1988; Sharrock and Quail, 1989). *Phy*B transcripts in rice and *Arabidopsis*

are present at a constitutively low levels and unaffected by light conditions (Dehesh, et al., 1991; Sharrock and Quail, 1989).

Posttranscriptional regulation of *phy*A mRNA abundance could occur at the level of RNA processing, transport from the nucleus to the cytoplasm, or mRNA stability (Belasco and Brawerman, 1993). The average the half-life of plant mRNA molecule has been estimated to be from several hours up to 30 hours (Silflow and Key, 1979; Green, 1993). The rapid decline in *phy*A mRNA abundance in light could be due to destabilization of the mRNA by Pfr production or to *phy*A mRNA being inherently unstable. Work performed by Seeley et al. (1992) suggests that *phy*A mRNA in oats is inherently unstable. Using cordycepin, an RNA synthesis inhibitor, *phy*A mRNA degradation was found to proceed at similar rates in both the dark and in the light. The half-life of *phy*A was found to be about one hour, considerably shorter than the average half-life of plant mRNAs. These experiments established that the message was not destabilized by the presence of Pfr following exposure to light but rather was rapidly turned over at a high rate regardless of the light conditions. With the knowledge that oat *phy*A mRNA is inherently unstable, the question emerges, how do these transcripts get recognized by the cell as mRNAs that should be rapidly turned over?

mRNA Degradation

The steady-state levels of mRNA are dependent upon the rate of synthesis as well as the rate of degradation (Belasco and Brawerman, 1993). Although transcription plays a significant role in the regulation of gene expression, and more directly, mRNA abundance, it is clear that transcription is not solely responsible for the controlling the abundance of mRNA molecules. Control of mRNA turnover either by maintaining a relatively long-lived or short-lived mRNA, or by altering the stability of mRNA in response to specific developmental or environmental cues, gives an organism precise control of gene expression. Nuclear encoded mRNA molecules can vary widely with respect to the rates at which they are degraded. *c-myc* and *c-fos* have half-lives of about 10 minutes (Dani et al., 1984). Altered expression of these genes can result in inappropriate cell replication and differentiation (Brewer and Ross, 1989). *Phy*A mRNA has a short half-life of about 60 minutes (Seeley et al., 1992). β-globin is a relatively stable mRNA with a half-life of greater than 24 hours (Ross and Pizarro, 1983). Transferrin receptor mRNA is regulated in response to iron. In the presence of high iron this mRNA has a half-life of 1.5 hours. In the presence of low iron the same mRNA has a half-life of 30 hours (Müllner and Kühn, 1988). Vitellogenin mRNA has a most dramatic regulation of

mRNA half-life; in the absence of estrogen the message has a half-life of 16 hours while in the presence of estrogen the half-life is 500 hours (Brock and Shapiro, 1983).

Messenger RNA stability must be dependent upon primary sequence. It is this sequence that cytoplasmic components must bind to, cleave, and/or target a message for degradation. RNA has much greater potential for variety of configurations than DNA. It is single stranded and can easily form stem-loops and other secondary and tertiary structures. There is evidence that elements in the 3' untranslated region (3' UTR), 5' untranslated region (5' UTR), or the coding region of transcripts can function to regulate the stability of mRNA. All regions of the mRNA from the cap to the poly(A) tail can potentially affect mRNA turnover rates. For some genes more than one element is required for full regulation of the mRNA abundance (Shyu et al., 1991; Peltz et al., 1993).

Regulatory elements

Poly(A) tracts, found at the 3'-end of nearly all eukaryotic mRNAs, are thought to play a role in regulating mRNA turnover. Most messages with poly(A) tracts are degraded at a slower rate than those without poly(A) tracts and poly(A) removal precedes degradation of many mRNAs (Peltz et al., 1991). Removal of the poly(A) tract from *c-fos* is believed to be dependent upon instability sequences from either within the coding region or the AU-rich domain of the 3' UTR of the message (Shyu et al., 1991). Because poly(A) tracts are so pervasive it is unlikely that they play a direct role in targeting specific messages for degradation, but may function in a general way to stabilize mRNAs that are not tightly regulated or rapidly turned over.

Iron responsive elements (IRE) are mRNA stem-loop structures that are important for regulation of transferrin receptor gene expression. The IRE acts to regulate iron homeostasis by the destabilizing the transferrin receptor mRNA when available iron is in excess (Müllner and Kühn, 1988). Proteins have been identified that bind with high specificity to IRE sequences possibly regulating mRNA degradation. Binding activity was found to be correlated to the level of intracellular iron (Müllner et al., 1989). Interestingly, the same structure found in tandem repeats in the 3' UTR of the transferrin receptor is located in the 5' UTR of ferritin mRNA. Translation of ferritin mRNA is regulated by the IRE in its 5' UTR (Müllner et al., 1989). IRE sequences have not been found in plant ferritin mRNAs. Soybean ferritin mRNA is not regulated at the level of translation in response to iron. In fact, posttransciptional regulation of soybean ferritin gene expression appears to occur at the level of protein turnover (Kimata and Theil, 1993).

Another sequence element common to more than one gene is the AU-rich element (ARE). The ARE has been found in the 3' UTR of several rapidly turned over messages. *C-myc*, *c-fos*, ß-interferon, and granulocyte-machrophage-colony-stimulating factor (GM-CSF) mRNAs have been found to share a common AUUUA consensus sequence (Shyu et al. 1989; Peltz et al., 1991). When inserted into the 3' UTR of a stable mRNA, for example, ß-globin, this element can be made to confer instability to mRNA (Shyu et al., 1991). The AU-rich elements may function as binding sites for trans-acting factors that either degrade mRNAs themselves or target the message for degradation. Using gel shift assays, a 32-kda protein has been found to bind to these AU-rich elements for *c-fos*, *c-myc*, and GM-CSF mRNAs (Vakalopoulou et al., 1991).

The ability of these AREs to function as destabilizing elements in plants has been examined. Reporter genes containing insertions of the ARE have been transformed into tobacco cells. These ARE containing genes were found to lead to rapid degradation of those transcripts (Ohme-Takagi et al., 1993). The mRNA encoding PvPRP1 in bean has been found to be destabilized by a protein that binds an AU rich region in the 3' UTR (Zhang and Mehdy, 1994). It appears that this destabilizing domain is capable of functioning in both animal and plant cells.

Small auxin-up regulated RNAs (SAURs) in soybean have been found to be highly unstable messages (McClure et al., 1989). The half-lives of these mRNAs vary between 10 minutes and 50 minutes (McClure et al., 1989; Franco et al., 1990). SAUR transcripts contain a conserved down stream consensus sequence (DST) in their 3' UTRs that was found to confer instability to *GUS* and globin transcripts in transgenic tobacco (Newman et al.,1993).

Another example of a plant mRNA sequence that may be involved in mRNA turnover is the ferredoxin I (*fed-1*) gene. In peas, the expression of the *fed-1* gene is light regulated. Accumulation of transcripts is dependent upon the presence of active phytochrome (Kaufman et al., 1986; Elliot et al., 1989). Studies using chimeric transcripts containing portions of the *fed-1* gene from peas have been used in transgenic plants to determine regulatory elements within the *fed-1* mRNA (Dickey et al., 1992). In light treated plants, transcripts containing the 5' UTR of the *fed-1* gene were found to accumulate at levels 4.6-fold greater than those of dark adapted plants. A light responsive element (LRE) has been identified in the first 230 nt of the transcript. The LRE has been shown to confer light responsiveness to reporter genes in transgenic tobacco. The LRE spans both the 5' UTR and a portion of the coding region of the gene. It is not known if the LRE alters the stability of the *fed-1* as the half-life of the mRNA has not been determined in the light or in the dark.

Yeast poly(A)$^+$ RNA has an average half-life of 17 minutes. This is much shorter than that of plant or mammalian mRNAs. The PPR1 gene in yeast encodes a very unstable message; it has a half-life of about one minute (Pierrat et al., 1993). Sequences responsible for conferring instability were identified using chimeric transcripts constructed from pieces of the PPR1 gene fused to the relatively stable yeast mRNA, URA3. It was determined that the 5' UTR of this gene can confer instability to the URA3 message. It was concluded that the sequences within the 5' UTR contain sequence(s) that can promote rapid mRNA turnover (Pierrat et al., 1993).

Sub-cellular localization of mRNA degradation

The location of mRNA turnover could occur at several sub-cellular locations. In the nucleus, mRNA may be turned over as is the majority of hnRNA. mRNA could be targeted to the vacuole for degradation. RNA oligonucleotides have been found in the vacuole of tomato indicating the possible role of the vacuole in mRNA degradation (Abel et al., 1990). In the cytosol, soluble mRNA could be degraded or turnover could occur when the mRNA is bound to polysomes. There is evidence that translation is required for turnover of some messages.

Translation has been found to be critical for regulation of the stability of a variety of mRNAs in both plant and animal systems. ß-tubulin mRNA in animal cells shows a decreased stability in response to increased levels of unpolymerized tubulin subunits (Cleveland et al., 1981). This alteration of stability is dependent upon the first 13 translated nucleotides. Translation of the first four amino acids has been found to be necessary for this control to occur (Gay et al., 1989). The binding of a cellular factor to the ß-tubulin nascent peptide during translation has been found to occur suggesting that this factor be important for rendering the transcript unstable (Theodorakis and Cleveland, 1992).

Frameshift mutations can result in mRNAs that are destabilized (Voelker et al., 1990; Jofuku et al., 1989). A mutant trypsin inhibitor gene, KTi3-, in soybeans was found by Jofuku et al. (1989) to have a 100-fold lower level of RNA than the wild-type mRNA. The mutation was found to be the result of a frameshift. Transcription rates were found to be similar for both the mutant and the wild-type, suggesting reduced level of KTi3- is due to posttranscriptional events.

For a number of rapidly degraded RNAs, inhibition of protein synthesis results in accumulation of mRNAs. This phenomenon is referred to as super-induction and suggests that a relationship exists between mRNA degradation and protein synthesis. A number of different protein synthesis inhibitors have been found to have the same effect, indicating that it

is not the mode of protein synthesis inhibition that is responsible but the halting of translation itself (Altus and Nagamine, 1991; Koeller et al., 1991). Two possible explanations have been proposed for super-induction. First, when protein synthesis is inhibited a labile protein factor required for RNA turnover may no longer be synthesized. Second, is the possiblity that translation itself is required for RNA turnover and when translation is halted, the mRNA no longer decays at the same rate. In soybean, SAURs are a rapidly turned over class of mRNAs. These mRNAs have been found to accumulate, to be super-induced, when treated with a variety of protein synthesis inhibitors (Franco et al., 1990). Tubulin RNA stability is autoregulated by the abundance of tubulin subunits and requires translational elongation for autoregulation. Use of protein synthesis inhibitors disrupts this autoregulated degradation. It has been proposed that during translation, binding of a cellular factor to the nascent tubulin polypeptide activates a ribosome-bound RNase or that ribosomal stalling when a cellular factor binds to the nascent peptide results in increase susceptibility of the RNA to nucleases (Gay et al., 1989).

Sub-cellular localization of mRNA turnover has been examined using cell-free systems. *In vitro* decay systems allow a more specific examination of components or mechanisms involved in mRNA turnover in association with polysomes. Polysome associated *c-myc* mRNA was found to be regulated by a labile factor *in vitro* (Brewer and Ross, 1989). The half-lives of three mRNAs from etiolated oat shoots have been measured using an *in vitro* polysome system (Byrne et al., 1993). Oat *phy*A, β-tubulin, and actin mRNA half-lives were compared both before and after a pulse of red light. The rank order of degradation was found to be the same *in vivo* and *in vitro*. Red light was found to decrease the half-life of ß-tubulin (Byrne et al., 1993). Oat *phy*A mRNA was found to have the same half-life in dark grown as well as light treated seedlings supporting the results from Seeley et al. (1992) using cordycepin treated, dark grown oat seedlings.

Transgenic plants and mRNA turnover

Current evidence suggests that organisms have a great variety of mechanisms by which they can regulate mRNA turnover. Oat *phy*A mRNA is known to be inherently unstable (Seeley et al., 1992); how this mRNA is recognized by the plant as one that should be rapidly turned over is not known. Putative degradation products from *phy*A mRNA have been seen as *phy*A RNA fragments present in total oat mRNA (Seeley et al., 1992) and in the *in vitro* polysome system (Byrne et al., 1993). These *phy*A RNA fragments may be indicative of a rapidly degraded message. Examination of total RNA from transgenic tobacco containing the

coding region of oat *phy*A cDNA displayed this same pattern of oat *phy*A hybridizable material (Barnes and Colbert, 1993). This observation suggested that tobacco may recognize and/or process oat *phy*A mRNA in a manner similar to the way oats recognize and process the same message.

In this study, oat *phy*A mRNA stability is examined in transgenic tobacco plants as a means to identify sequences important in *phy*A mRNA degradation. All work with oat *phy*A containing transgenic tobacco, up until this point, has been using *phy*A cDNAs that did not contain either the complete 5' UTR or the 3' UTR without introns (Keller et al., 1989; Cherry et al., 1991). No measurement of the mRNA half-lives had been made using these transgenic tobacco plants. For this study, plants containing coding region deletions of the oat *phy*A gene are used to measure the half-life of truncated mRNAs. A full-length oat *phy*A mRNA was constructed and transformed into tobacco. These transgenic plants were used to measure the half-life of full-length oat *phy*A mRNA in tobacco.

CHAPTER 2 DETERMINATION OF THE HALF-LIFE OF TRUNCATED OAT *PHY*A mRNAS IN TRANSGENIC TOBACCO

Introduction

A number of specific mRNA sequences have been proposed to function by destabilizing mRNA molecules (Müllner and Kühn, 1988; Shyu et al., 1989; Peltz et al., 1991; Newman et al., 1993). For some of these sequence elements, protein binding factors have been identified (Vakalopoulou et al., 1991; Müllner et al., 1989). These proteins have been implicated in targeting messages for degradation (Peltz et al., 1993). Identification of the destabilizing sequence element(s) in a given mRNA necessitates an alteration or removal of those sequence elements and subsequent *in vivo* or *in vitro* observation of the mRNA's half-life.

It is difficult to alter sequences within an organism and to observe the consequences of that alteration in the organism for which the gene is endogenous. One approach to study mRNA stability in plants is to use transgenic plants. Transgenic plants provide a living system in which to place heterologous genes that would produce altered or truncated mRNAs in transformed plants. This approach is attractive because it allows for direct manipulation of the gene by deleting, or altering specific regions of the gene prior to transformation. The half-life of the unaltered mRNA in transgenic plants could then be compared to the altered message in the same host species presumably revealing sequences necessary for conferring instability to that message. This approach also has the advantage of using whole, live plants, which may be more likely to provide an authentic representation of mRNA turnover than protoplasts or *in vitro* systems.

The half-life of oat *phy*A mRNA is about 60 minutes in etiolated oat seedlings (Seeley et al., 1992). In addition to its relatively short half-life, oat *phy*A mRNA has putative degradation products seen as *phy*A RNA fragments present in both total oat mRNA and in the *in vitro* polysome system (Seeley et al., 1992; Byrne et al., 1993). Seedlings generated from tobacco plants transformed with the oat *phy*A gene were studied. Each of the oat *phy*A genes used to produce transgenic tobacco contained all or a portion of the gene's coding region, an incomplete 5' UTR, and had two *phy*A introns in the 3' UTR (Keller et al., 1989; Cherry et al., 1991). Seedlings from plants that contained oat *phy*A genes with sequential truncations from the 3' coding region were also examined (Cherry et al., 1993).

Work presented in this chapter suggests that oat *phy*A mRNA in transgenic tobacco produces putative oat *phy*A degradation products like those seen in oats, and that deletions

from the 3'-end of the coding region do alter the pattern of these putative degradation products. The half-life of the oat *phy*A mRNA is altered by deletions in oat *phy*A gene in transgenic tobacco, but mRNA half-life does not correlate with the amount of oat *phy*A RNA fragments.

Methods and Materials

Growth of seedlings

Nicotiana tobaccum cv Xanthi transgenic tobacco seeds (R.D. Vierstra, University of Wisconsin) were sterilized in a 50% commercial bleach solution for five minutes and rinsed two times with sterile water. For initial RNA isolations, sterile seeds were sown in petri dishes on cellophane placed over medium containing MS salts, 1 mg/l nicotinic acid, 10 mg/l thiamine, 1 mg/l pyridoxine, 50 mg/l inositol and 6 g/l agar. Plates were placed in absolute darkness for seven days. For inhibition experiments, sterile seeds were sown in petri dishes on two pieces of sterile Whatman 3 MM paper soaked with 5 ml MS salts. Plates were sealed with parafilm and placed in complete darkness for six days. Seedlings were harvested and frozen in liquid nitrogen. Frozen seedlings were stored at -80°C.

Seeds used for poly(A)$^+$ RNA isolations were sown onto Whatman 3 MM paper soaked with 5 ml MS salts, sealed with parafilm and placed in complete darkness. Sixteen hours after imbibition, seeds were irradiated with continuous white light for one hour to stimulate germination and returned to darkness for the remainder of six days. Prior to harvest, one-half of the seedlings were irradiated with continuous white light for four hours. Seedlings were harvested, frozen in liquid nitrogen, and stored at -80°C.

Cordycepin inhibition assay

To determine the cordycepin concentration necessary to effectively inhibit RNA synthesis in tobacco, various concentrations were tested. Etiolated tobacco seedlings were treated with 7 ml of H_20, 0.1, 0.5, or 1 mg of cordycepin per ml of H_20 and allowed to incubate in darkness for 15 minutes with gentle agitation. Following incubation, seedlings were either harvested in darkness or subjected to four hours continuous white light with gentle agitation and then harvested. The cordycepin concentration determined to be adequately inhibit *cab* RNA synthesis was 1 mg/ml. All subsequent cordycepin assays were performed with cordycepin at a concentration of 1 mg/ml, seedlings were treated with shaking for 15 minutes in darkness with 7 ml of H_20 or the inhibitor as a pretreatment and then irradiated with

continuous white light with shaking and harvested at time zero, 60 minutes, and 240 minutes after pretreatment.

RNA isolation and blotting

Total RNA was isolated from seedlings using aurin tricarboxylic acid as described by Seeley et al. (1992). Between 3 and 5 ug of total RNA was electrophoresed on a 1% agarose/3% formaldehyde gel. The RNA from the gel was transferred onto Genescreen nylon membrane using a 25 mM phosphate buffer. RNA blots were prehybridized in 50% deionized formamide, 0.7 % NaCl, 10 % dextran sulfate, 1% SDS for 3-4 hours at 65°C with shaking. Antisense RNA probe was added at a concentration of 5 x 10^5 cpm per ml prehybridization buffer. Sonicated salmon sperm DNA was added to the prehybridization buffer after blots had been prehybridized. Blots were hybridized with the probe overnight at 65°C with shaking. Following hybridization, blot was washed in 2XSSC/0.1% SDS twice at room temperature for three minutes. The blots were then washed under more stringent conditions of 0.1XSSC/0.1% SDS for 15 minutes at 65°C with shaking. Autoradiographs were made from X-ray film exposed to the blot at -80°C and developed.

Total RNA needed for poly(A)$^+$ RNA isolation, was generated from tobacco seedlings using phenol-SDS, large scale RNA isolation (Dean et al., 1985). Poly(U) sephadex chromatography was performed as described by Lissemore et al. (1987).

Probes and probe synthesis

Probe synthesis was performed using linearized plasmid templates and the Promega *in vitro* transcription kit. The probes were labeled with ^{32}UTP. Reactions using T7 RNA polymerase were incubated at 37°C for one hour, while those using SP6 polymerase were incubated at 40°C for one hour. An antisense probe synthesized from pGAB0.7 (Lisssemore and Quail, 1988) linearized with HindIII, using T7 polymerase, was used as a template to make probe specific for *cab* mRNA. Template made from plasmid pAPSX2.7 (Seeley et al., 1992) linearized with EcoRI and using SP6 RNA polymerase was used to make the antisense oat phytochrome probe. Antisense *Arabidopsis phy*A probe was synthesized from plasmid pSP18/8 (Sharrock and Quail, 1989) linearized with EcoRI and using SP6 RNA polymerase.

Quantitation of RNA and half-life determination

To determine the oat *phy*A mRNA half-life for different tobacco transformants, autoradiographs of RNA from cordycepin treated seedlings were placed over RNA blots and used as a guide to excise the desired area. The radioactivity of the excised portion of the blot

was measured using liquid scintillation spectrometry. Background radioactivity was subtracted from these quantitations.

Results

To ask whether *phy*A mRNA abundance in tobacco is dramatically down regulated by light, as it is in oats, a determination of how tobacco *phy*A mRNA is regulated in response to light was performed. Poly(A)$^+$ RNAs were isolated from six-day-old tobacco seedlings grown in darkness and irradiated with four hours of continuous white light prior to harvest. Poly(A)$^+$ RNA was isolated and probed with an *Arabidopsis phy*A probe. RNA blots showed that *phy*A mRNA from etiolated seedlings exposed to white light had a 54% decline in *phy*A mRNA compared to dark treated etiolated seedlings. As a control, the same poly(A)$^+$ RNA was probed with a chlorophyll a/b binding protein (*cab*) RNA probe. As expected, *cab* mRNA was found to be induced 3-fold in response to light (Figure 2.1).

Transgenic tobacco line 9A4 contains a single copy and is homozygous for the oat *phy*A gene (Keller et al., 1989). Transcription of oat *phy*A gene in 9A4 plants is under the control of the CaMV35S promoter (Figure 2.2). Preliminary analysis of RNA isolated from transgenic tobacco 9A4 suggested that oat *phy*A mRNA fragments, similar in appearance to those seen in total RNA isolated from oats, were present (Figure 2.3, lane 4). The *phy*A RNA fragments were as abundant as those in oats. The ethidium bromide stained gel of the same RNA indicated that the transgenic tobacco RNA had not degraded during isolation (data not shown). Wild-type tobacco phytochrome did not hybridize with the antisense oat phytochrome probe (Figure 2.3). In oats, *phy*A transcription is controlled by phytochrome itself and is down-regulated by light (Lissemore and Quail, 1988). The CaMV35S promoter is considered to be constitutive. Total RNA from white and red light treated 9A4 seedlings was isolated and compared to a dark control to establish that the stability of oat *phy*A mRNA from 9A4 was not light regulated (Figure 2.3). The levels of mRNA did not appear to vary in response to red or white light conditions.

A series of oat *phy*A cDNA deletions, BPCA, BPCB, BPCC, BPCD, BPCE, and BPCF were transformed into tobacco (Cherry et al., 1993). Deletions from the 3' end of the coding region, in a 3' to 5' direction, were sequential the smallest for BPCA and greatest for BFCF (Figure 2.4). BPWT, like 9A4, contained the entire coding region of the oat *phy*A gene. The gene construct used to make these contains an incomplete oat *phy*A 5' untranslated region.

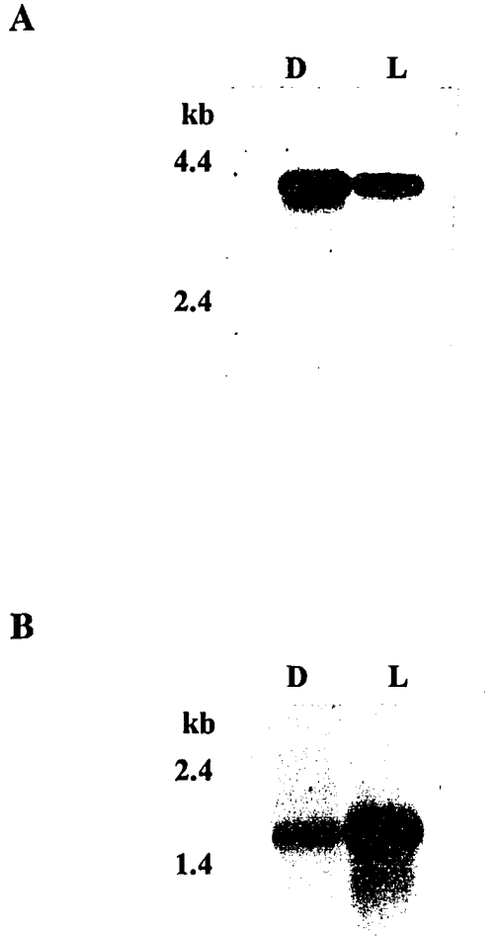


Figure 2.1 Poly $(A)^+$ RNA from wild-type tobacco seedlings grown for six days in darkness (D) or for six ays in darkness and then treated with four hours of continuous white light (L).
A tobacco poly $(A)^+$ RNA probed with an antisense *Arabidopsis phy*A RNA probe.
B tobacco poly $(A)^+$ RNA probed with an antisense maize *cab* RNA probe.

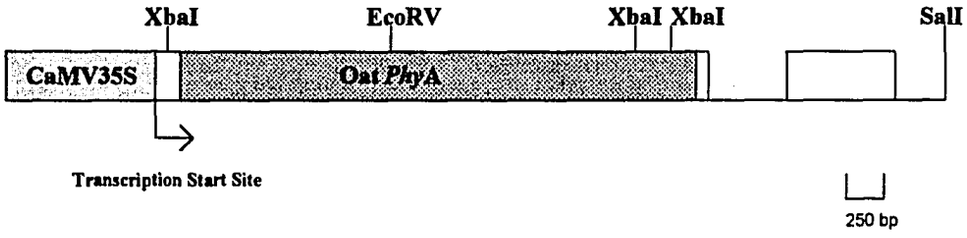


Figure 2.2 Chimeric oat *phy*A gene pCY35phyt. pCY35phyt was used to produce the oat *phy*A containing transgenic tobacco line 9A4. Boxes represent exons and the promoter. The lighter shaded box represents the CaMV35S promoter and the darker shaded box represents coding sequence from the oat *phy*A gene. Open boxes indicate untranslated regions. The line represents control regions in the gene not present in the RNA (Keller et al., 1989).

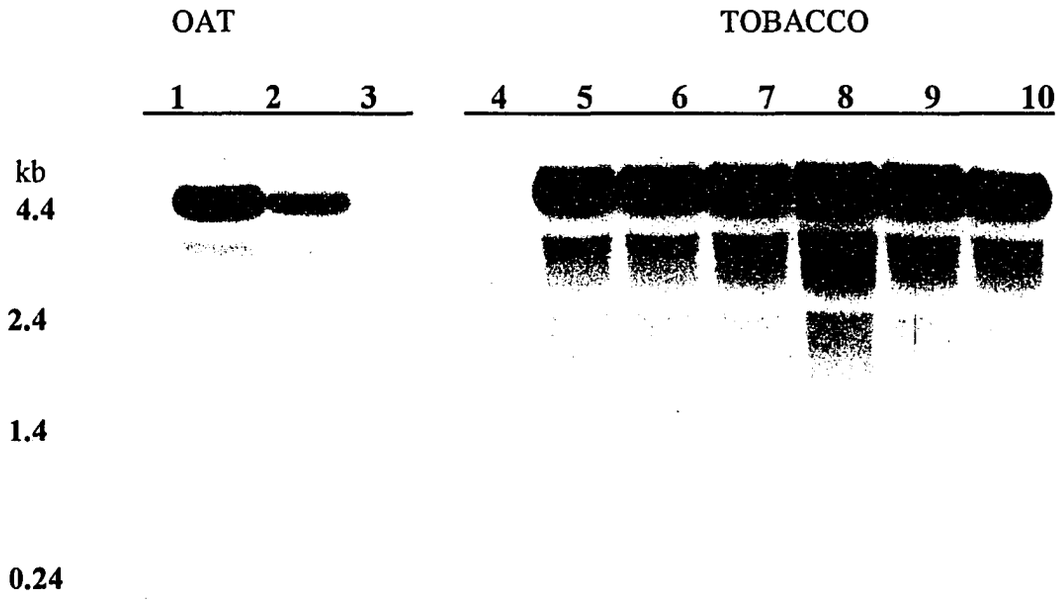


Figure 2.3 RNA blot of total RNA from oat and transgenic tobacco. Lanes 1 through 3 contain RNA from etiolated oat seedlings. Lane 1 is etiolated oat RNA at time zero. Lane 2 is RNA isolated two hours after etiolated oat seedlings were treated with a pulse of red light. Lane 3 is RNA isolated four hours after etiolated oat seedlings were treated with a pulse of red light. Lane 4 is RNA from etiolated wild-type tobacco seedlings. Lanes 5 through 10 contain total RNA isolated from etiolated oat *phy*A containing transgenic tobacco seedlings (9A4). Lane 5 is RNA from seedlings maintained in darkness. Lane 6 is from seedlings placed in red light for 70 minutes. Lane 7 is RNA from seedlings placed in red light for 180 minutes. Lane 8 is RNA from seedlings placed in continuous white light for 85 minutes. Lane 9 is from seedlings placed in continuous white light for 180 minutes. Lane 10 is from seedlings placed in continuous white light for 240 minutes. All RNAs were probed with the oat *phy*A cDNA pAPSX2.7 (Seeley et al., 1992).

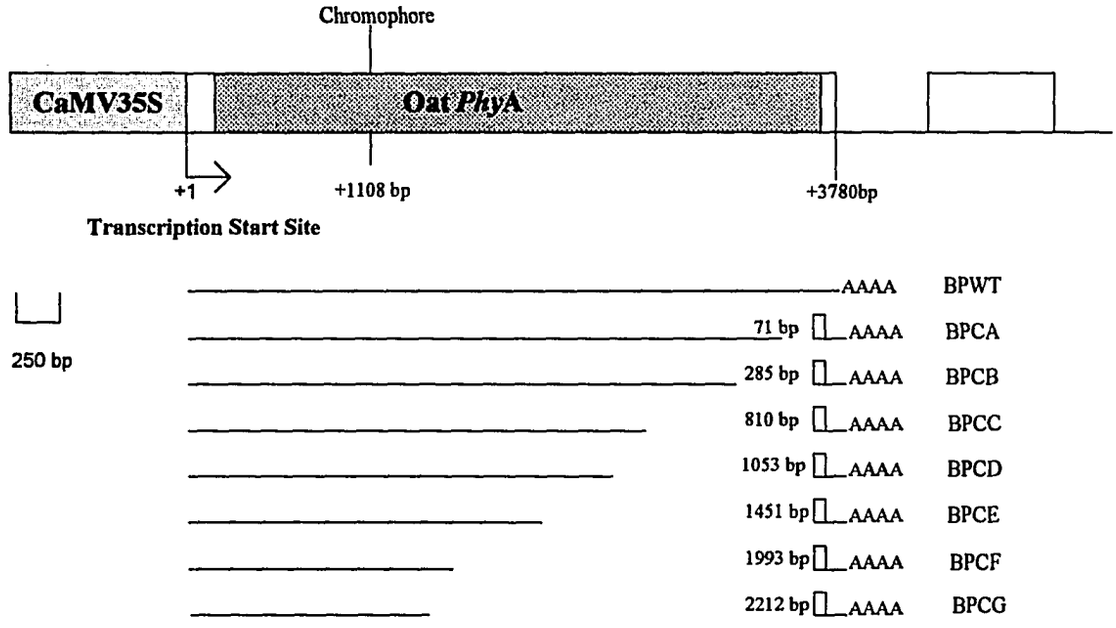


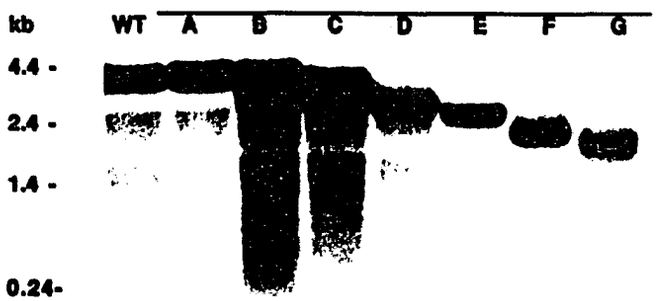
Figure 2.4 The full-length oat *phy*A structural gene and truncated RNAs made from deletions of the gene. The full-length oat *phy*A gene is represented by the boxes. A description of the boxes representing the gene can be attained from Figure 2.2. The chromophore binding site is indicated. BPWT is the RNA produced from the full-length gene. BPCA-BPCG are truncated versions of the gene. Numbers indicate the number of nucleotides removed from the 3'-end of the coding region. Small boxes represent the non-*phy* 45 nt segment containing stop codons in every frame added to each of the deleted constructs (Cherry et al., 1993).

These constructs also contain two small oat phytochrome introns in their 3' untranslated region. For *phy*A genes missing portions of their 3' coding regions, a 45 bp oligonucleotide containing stop codons in all three reading frames was inserted 5' of the oat *phy*A 3' UTR (Cherry et al., 1993). Total RNA from these transgenic plants was isolated and probed with an antisense oat *phy*A RNA probe. The bands visualized by autoradiography were of the size expected (Figure 2.5, panel A). The patterns of *phy*A RNA fragments were varied, not all were similar to that of the oat *phy*A in oats or the 9A4 transgenic plant *phy*A RNA. The full-length band and *phy*A RNA fragments were quantitated and expressed as a band to *phy*A RNA fragment ratio (Figure 2.5, panel B). BPCB and BPCC were found to have lower band to *phy*A RNA fragment ratios than BPWT, the construct possessing the complete 3' end of the coding region; that is they had more *phy*A RNA fragments. BPCA and BPCD had a ratio comparable to BPWT. BPCE and BPCF had high band to *phy*A RNA fragment ratios. The differences in the ratios suggest that the plants are not treating these various truncated RNAs in a like manner. We sought to correlate the half-life of the mRNA of these deleted oat *phy*A sequences with the amount of *phy*A RNA fragments beneath the full-length band.

Cordycepin was used to estimate the half-lives of the deleted oat *phy*A mRNA. Cordycepin is a chain-terminating adenosine analog. To determine what concentrations would be most effective in inhibiting RNA synthesis in tobacco seedlings, six-day-old etiolated seedlings were treated with increasing concentrations of cordycepin and irradiated with continuous white light for three hours. *Cab* mRNA is rapidly up-regulated by light at the level of transcription (Lissemore and Quail, 1988). The level of *cab* mRNA induction was measured using a water control and inhibition of transcription measured as a percent of the control (Figure 2.6). At 1 mg/ml cordycepin, levels of *cab* mRNA were found to be approximately the same as the dark controls. This concentration was used for all subsequent experiments.

The half-lives of three of the truncated oat *phy*A mRNAs were estimated. BPWT, BPCB, and BPCF were chosen because they exhibited the most distinct patterns of *phy*A RNA fragment hybridization. BPWT displayed a pattern similar to that seen in oats. BPCB had a greater amount of *phy*A RNA fragments than BPWT; it had a higher band to fragment ratio. BPCE had a much smaller amount *phy*A RNA fragments and had a high band to RNA fragment ratio. Seedlings from BPWT, BPCB, and BPCE were grown in complete darkness for five days and then treated with water or cordycepin. These seedlings were placed in the light during cordycepin treatment to enable the use of *cab* as a control to measure inhibition of RNA synthesis. Seedlings were harvested at zero, 60, and 240 minutes after pretreatment.

A



B

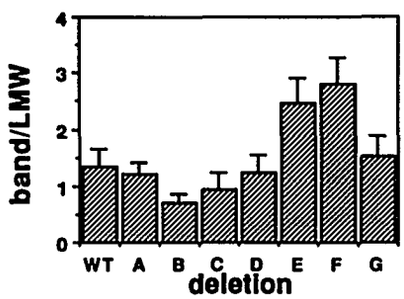


Figure 2.5. Tobacco transformed with the coding region and truncated portions of the oat *phy*A gene.
A Total RNA isolated from oat *phy*A containing tobacco BPWT (WT), BPCA (A), BPCB (B), BPCC (C), BPCD (D), BPCE (E), and BPCG (G). RNA blot was probed with pAPSX2.7 (Seeley et al, 1992) and autoradiographed.
B Quantitation of the band and oat *phy*A fragments from panel **A** by liquid scintillation spectometry. This is the average and standard error of at least three experiments.

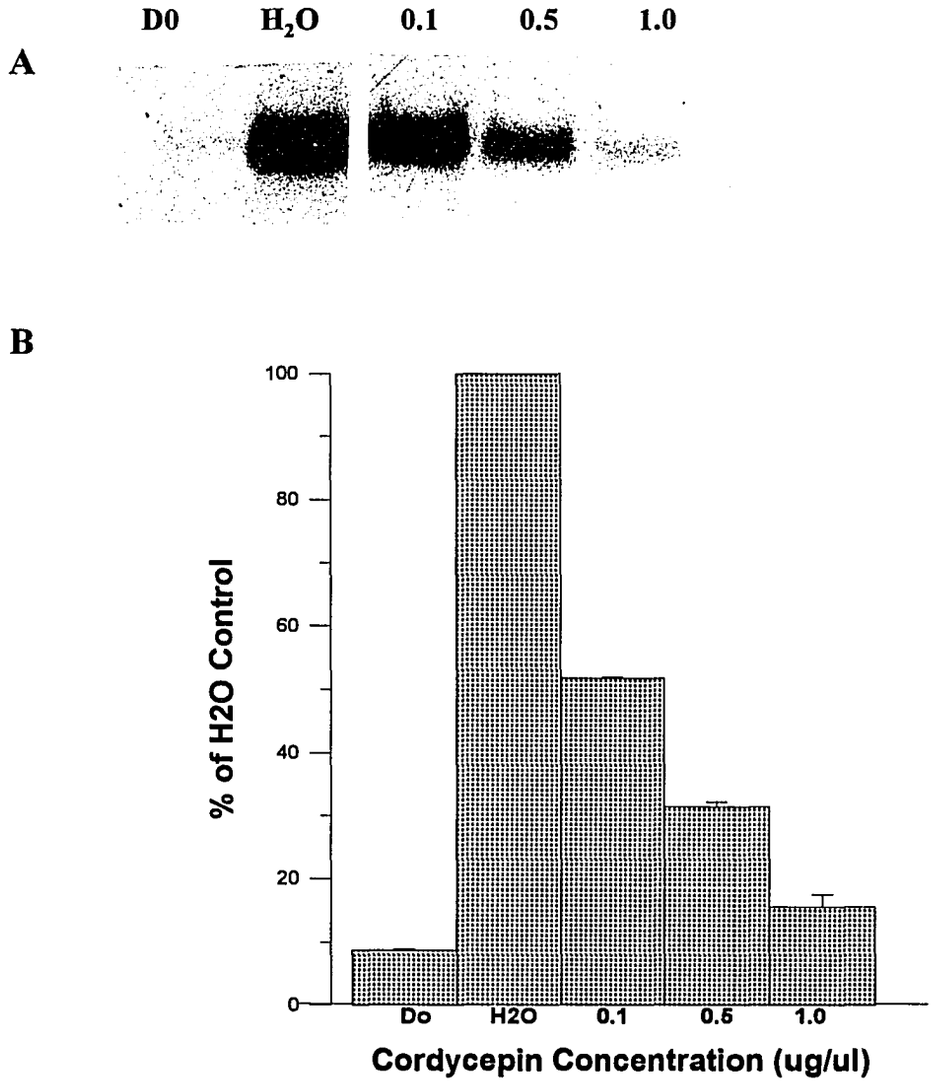


Figure 2.6 Effect of increasing concentrations of cordycepin on *cab* mRNA accumulation in tobacco. Five-day-old etiolated tobacco seedlings were placed in water or cordycepin at 0, 0.1, 0.5, and 1 mg/ml cordycepin for 30 minutes in darkness. Seedlings were then placed in continuous white light and harvested after two hours. Total RNA was isolated.
A Representative blot of RNA from seedlings probed for *cab* mRNA following cordycepin treatment. Blot was exposed to X-ray film for three hours.
B The bands corresponding to the full-length *cab* RNA were excised, and the amount of radioactivity was determined by liquid scintillation spectrometry. Data show the mean and standard error of three independent experiments, including the blot shown in panel **A**.

Total mRNA was isolated from these seedlings, electrophoresed, blotted, and probed with antisense oat *phy*A RNA probe. Quantitation of these blots generated an estimate of the mRNA half-life (Figure 2.7). BPWT and BPCB were found to have a half-life of greater than 300 minutes, much longer than the 60 minutes reported for oat *phy*A mRNA (Seeley et al., 1992). BPCE, the shortest construct, had a half-life of about 90 minutes.

Discussion

It is not known if tobacco *phy*A mRNA is as unstable as oat *phy*A mRNA. Tobacco does, however, regulate the levels of its *phy*A mRNA in response to light. Using poly(A)$^+$ RNA from dark and light treated tobacco seedlings, tobacco *phy*A RNA was found to decrease by about half after four hours of continuous white light. This suggests that tobacco may provide a reasonable system to use for study of oat *phy*A mRNA stability. However, the extent of *phy*A mRNA down-regulation in tobacco is much less dramatic than in oats.

Transgenic tobacco 9A4, transformed with the entire coding region of oat *phy*A cDNA, shows a pattern of oat *phy*A hybridizable mRNA similar to that of the same message in oats. This suggests that tobacco and oats recognize and process this message in a similar manner. The levels of the oat *phy*A message in tobacco do not change in response to treatment of the seedlings with red or white light. As in oats, it is likely that this message has the same half-life in light-treated tobacco seedlings as is does in dark-treated tobacco seedlings.

Truncated versions of the oat *phy*A mRNA in transgenic tobacco seedlings display patterns of *phy*A fragments that differ from that of oat or transgenic tobacco plant 9A4. BPCB and BPCC were found to display more *phy*A RNA fragments than BPWT. BPCA and BPCD had a fragment pattern similar to BPWT. BPCE and BPCF had less fragments than did the other constructs. If oat *phy*A RNA fragments found in the pool of total RNA in oats are produced as a result of rapid degradation of the full-length message, then it seems possible that the absence of this low molecular weight RNA could indicate that the message had been stabilized.

To test this hypothesis, the half-lives of the truncated oat *phy*A mRNAs from transgenic tobacco were measured using the RNA synthesis inhibitor cordycepin. Cordycepin was found to inhibit *cab* RNA synthesis during induction with white light at a concentration of 1 mg/ml. BPCE, having the greatest band to *phy*A RNA fragment ratio, was expected to have the longest half-life. It was found to have the shortest half-life, of about 90 minutes. BPWT and

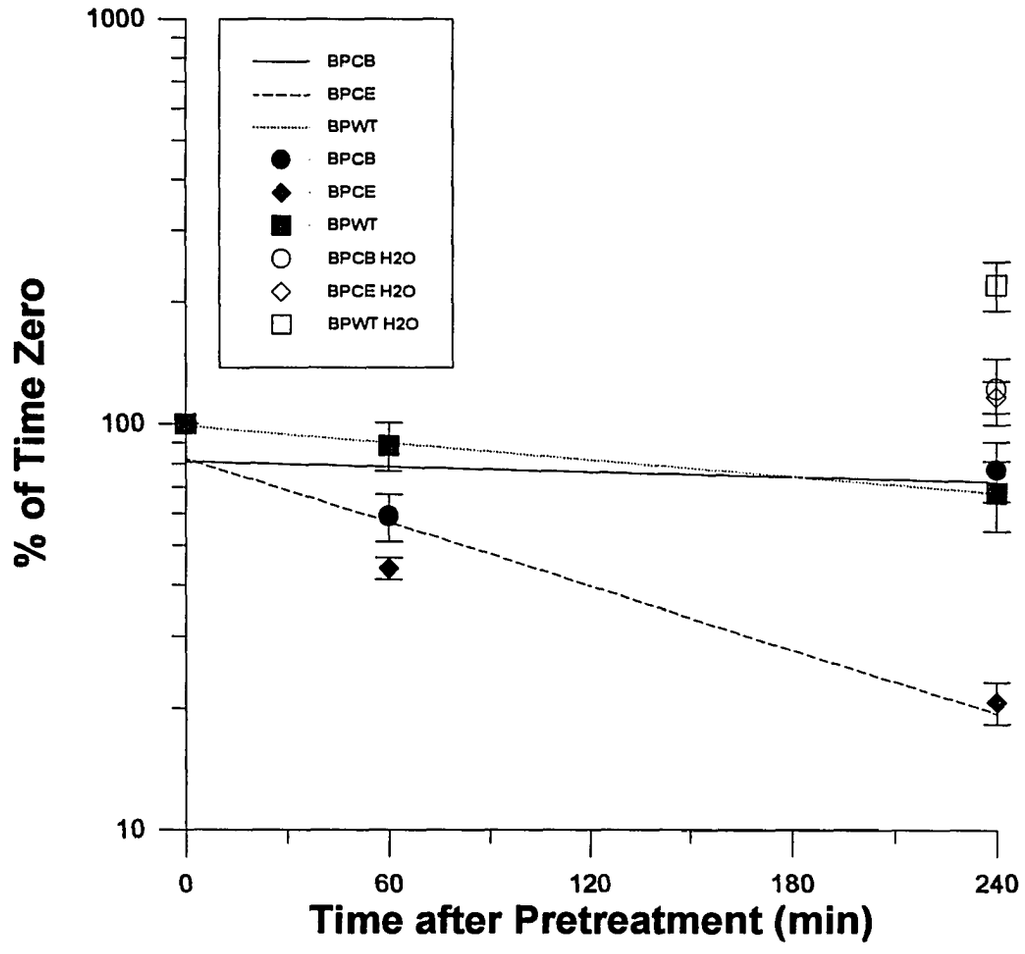


Figure 2.7. Half-life determination of truncated oat *phy*A mRNA in transgenic tobacco. Half-lives of oat *phy*A containing tobacco were determined using five to seven-day-old etiolated seedlings. RNA was isolated from seedlings treated with 1 mg/ml cordycepin for 15 min. in darkness and then harvested (time 0), or placed under continuous white light and harvested after 60 and 240 minutes. Total RNA was probed for *phy*A mRNA and full-length bands excised from the RNA gel blots. The amount of radioactivity for each band was measured using liquid scintillation spectrometry. Data points (plot symbols) are the means of three or more independent experiments and standard error. Linear best fit curves (lines) were used to estimate the half-lives for oat *phy*A mRNAs. ■ BPWT (containing the entire coding region, no 5' untranslated region, and some but not all of the 3' untranslated region), ●BPCB, and ◆ BPCE. mRNA levels of the same tobacco lines treated to water instead of cordycepin and placed in light for four hours prior to harvest ◊ BPCB, 0 BPCE, BPWT.

BPCB, containing a lower band to *phy*A RNA fragment ratio, had the longest half-lives. The half-lives of these messages were not found to correlate with the amount of *phy*A RNA fragments.

The four hour half-life of the incomplete oat *phy*A mRNA in the BPWT transgenic tobacco line suggests that oat *phy*A mRNA is not degraded in tobacco as rapidly as it is in oats. This does not preclude the possibility that a full-length oat *phy*A mRNA may be treated differently in the same system. The fact the half-lives do vary may suggest that what is different about these mRNAs may include sequences responsible for regulating mRNA stability. It is made more complicated by the fact that these transgenic tobacco contain oat *phy*A genes that have an incomplete 5' UTR. These constructs also contain two introns in their 3' UTR. Monocot introns are known to be inefficiently spliced in dicot systems (Keith and Chua, 1986). It is possible that tobacco *phy*A mRNA might not be inherently unstable, and therefore tobacco cells do not rapidly degrade oat *phy*A mRNA. Another possibility is that sequences that are important for rendering oat *phy*A mRNA unstable in oats, are not recognized in tobacco.

CHAPTER 3 CONSTRUCTION OF A FULL-LENGTH OAT *PHYA* GENE

Introduction

Determination of the half-lives of truncated oat *phy*A mRNA in transgenic tobacco have been inconclusive with respect to identification of elements that may important for *phy*A mRNA turnover. The 5' UTR and the 3' UTR of some genes have been shown to be important for regulation of mRNA stability (Müllner and Kühn, 1988; Shyu et al., 1989; Peltz et al., 1991; Dickey et al., 1992; Pierrat et al., 1993; McClure et al., 1989). The genes used to produce the oat *phy*A containing transgenic tobacco discussed in chapter two were not genuine oat *phy*A genes for three reasons. First, none of these genes had a complete 5' UTR. Second, the 3' end of their coding regions contained an oligonucleotide with stop codons in every frame. Third, the oat *phy*A genes had a 3' UTR that contained two introns. The sequences deleted or altered in the 5' UTR or the 3' UTR of the oat *phy*A gene constructs could have resulted in messages that are unable to form necessary secondary structures that allow the mRNA to be recognized as one that should be unstable. Synthesis of a full-length oat *phy*A gene was necessary to make a more accurate estimate of the half-life of oat *phy*A mRNA in transgenic tobacco.

Although a single plasmid containing the full-length oat *phy*A gene was unavailable, most of the gene was available as genomic or cDNA clones. All but the first four base pairs of the 5'-most end of the gene was represented by the cDNA pAP5.2 (Hershey et al., 1985). The chimeric cDNA, pFY122 (Boylan and Quail, 1989) represented the middle of the gene, up to but not including the 3' UTR. A cDNA library was synthesized to generate pPhy11A, a cDNA containing the 3' UTR from the gene. From these three clones it was possible join the pieces together using polymerase chain reaction (PCR) and common cloning techniques to form a full-length oat *phy*A gene.

The full-length oat *phy*A cDNA was cloned into a Ti vector and transformed into *Agrobacterium tumefaciens*. *Agrobacterium* transformed with a Ti vector that includes a full-length *phy*A cDNA was then used to transform tobacco. Seeds from regenerated transgenic tobacco plants were used to determine insert copy number. Single copy, homozygous lines were identified and increased. Tobacco plants containing a partial oat *phy*A gene were found to have shortened internodes and increased chlorophyll content (Keller et al., 1989;

McCormac et al., 1991). Tobacco plants transformed with the full-length oat *phy*A gene were found to display a similar phenotype.

Methods and Materials

cDNA library synthesis

cDNA library synthesis was carried out using poly(A)$^+$ RNA isolated from four-day-old etiolated oat seedlings. First and second strand DNA synthesis was performed using cDNA Synthesis System Plus (Amersham) as described by the manufacturer. Adapters from λGT10 cDNA cloning system were ligated to the double-stranded DNA (Amersham). λZapII arms (Stratagene) were then ligated to the adapter-cDNA and packaged *in vitro* using Gigapak Gold packaging extract (Stratagene). These phage particles were then used to infect *E. coli* cells.

The cDNA library was titered and found to contain 1.38×10^6 plaque forming units (PFU). The cDNA library was amplified as described by Sambrook et al. (1989). The amplified library was found to contain 1.3×10^7 PFU.

cDNA library screening and in vivo excision

Primary screening of the cDNA library was made by plating at a density of about 10,000 PFU per plate. Nitrocellulose filter disks were used to lift plaques from these plates for screening (Sambrook et al., 1989). For hybridization, filter discs were allowed to pre-hybridize overnight in pre-hybridization buffer at 65°C with gentle shaking. Screening of the cDNA library was done using a random primed oat *phy*A DNA probe prepared from pGAP1.7 (Edwards and Colbert, 1990) and labeled with α-^{32}P-CTP. Lifts were hybridized with 5.0×10^5 cpm DNA probe per ml hybridization buffer for 42 hours at 65°C with gentle shaking. The plaque lifts were then washed in 0.3XSSPE/0.1% SDS two times for 20 minutes each at room temperature with gentle shaking for four hours. Positive plaques were visualized using autoradiography and compared with duplicate lifts. Positive plaques were cored and used for further screening. Secondary, tertiary, and quaternary screens were performed in the same manner as the primary screen but at PFU densities of approximately 250, 100, and 50. Plasmids were excised from cored plaques using the *in vivo* excision procedure with helper phage R408 from the Amersham predigested lambda ZapII/EcoRI cloning kit.

An oat actin clone was isolated from the cDNA library using a probe prepared from the soybean actin clone, pSAC3 (Hightower and Meagher, 1985). The oat clone, pOA24,

was found to be about 1.1 kb long. A partial sequence, restriction map, and sequence comparisons were made (see Appendix).

***Restriction digests of plasmid DNA, transformation of* E. coli *and* Agrobacterium**

Restriction digests of plasmid DNA were carried out as described by Sambrook et al. (1989). Generally, one unit or more of restriction enzyme per ug of DNA was used for digestion of plasmid DNA. Digestions were conducted for 4 to 16 hours at 37°C. Calf Intestinal Phosphatase and ligation reactions were as described by Sambrook et al. (1989).

Transformation of *E. coli* was performed as described by Sambrook et al. (1989) using frozen competent cells or using electroporation as described by Ausebel et al. (1989). *E. coli* strain XL1-blue was used to generate all of plasmids described here. *E. coli* was grown in LB media with 50 ug/ul ampicillin at 37°C approximately 16 hours.

Agrobacterium tumefaciens, strain LBA4404 was purchased from Clontech. These cells were grown in LB media and 12.5 ug/ul streptomycin at 28°C for three to six days. LBA4404 contains a disarmed Ti plasmid that has the *vir* region which is required for incorporation of the DNA into the plant genome.

DNA isolation

DNA isolation was performed using Qiagen DNA isolation kits. Mini or Midi columns were used as described by the manufacturer. To quantify the DNA, following isolation, absorbance was measured at A260 nm.

Polymerase chain reactions

Polymerase chain reactions were performed using the protocol and reagents from Perkin-Elmer. Concentrations of 1.25 mM, 2.25 mM, and 3.0 mM $MgCl_2$ were used to determine the best reaction conditions for each PCR reaction. PCR primers were synthesized by the Iowa State University Nucleic Acid Facility and purified by HPLC. The cycling parameters were as follows: One minute at 94°C to denature template DNA, one minute at 40°C for the primers to anneal, and one minute at 72°C to allow *Taq* polymerase to synthesize product. These parameters were followed for five cycles. The annealing temperature was then raised to 50°C to increase the annealing stringency for 35 cycles. DNA synthesized from these reactions was pooled and electrophoresed on a 1% agarose gel. The DNA product was visualized by ethidium bromide staining and removed with a razor blade. Each DNA fragment was isolated using Gene Clean from Bio101. This allowed the separation of the DNA from components of the PCR reaction. The DNA could then be used directly for ligation with other pieces of the oat *phy*A cDNA.

NcoI linker addition to pBluescript

An NcoI linker from New England Biolabs was purchased and used to facilitate directional cloning of the 5'-end of the gene into pBluescript. The linker was phosphorylated, double-stranded, and blunt-ended. To add the NcoI site to the vector, pBluescript was cut with SmaI, which generates a blunt-ended linear piece of DNA. The NcoI linker was ligated into this site, the resulting plasmid was named pBSN

In vitro transcription of pFLII

In vitro transcription of pFLII was performed using a low specific activity synthesis using α-^{32}P-UTP and an *in vitro* transcription kit (Promega). Template consisted of pFLII linearized with XhoI. T7 RNA polymerase was used to make transcript. RNA blot analysis of *in vitro* transcribed pFLII was performed as described earlier (Chapter 2).

Tobacco transformation

Tobacco transformations were carried out as described by Horsch (1988). All plant material was *Nicotiana tobaccum cv* Xanthi. Leaves that were young and green, about 10-15 cm long, were used to generate leaf discs. Leaf discs were taken after leaves were sterilized for five minutes in a 50% commercial bleach solution and rinsed two times with sterile water. Leaf discs were punched from of leaves using a sterile paper punch. Discs were then placed abaxial side down on MS104 medium. A liquid culture of *Agrobacterium* transformed with either pBI121 and pBFLI were grown in LB media for 24 hours. These were diluted 1:10 with MS liquid medium prior to inoculation. Inoculated discs were allowed to become fully infected with the *Agrobacterium* on MS104 medium lacking selective antibiotics for two days and then placed on MS104 medium containing cefotaxime or carbanicillin to kill the *Agrobacterium*. The medium also contained 50 mg/l kanamycin to select for transformed cells. After shoots had formed and were about 1 cm in height, shoots were excised and placed on rooting medium. When plants had generated roots they were transferred to soil and allowed to slowly adjust to the lower humidity. Plants were grown in a growth chamber with a day/night light cycle of 16 hours light followed by 8 hours of darkness.

Determination of copy number and identification of homozygous plants

R0 plants were identified as transgenic by examination of their seed. Seeds were surface sterilized in a 50% commercial bleach solution for five minutes. Seeds were then rinsed twice with sterile water. One hundred sterile seeds were plated onto petri dishes containing a medium of MS salts, 9 g/l agar, and 100 ug/ml kanamycin. Dishes were sealed with parafilm to prevent desiccation and placed in a growth chamber with continuous fluorescent lighting.

After 28 days, seedlings were assessed as either white or green. White plants were considered kanamycin sensitive and green plants were considered transgenic and expressing the NPTII gene that confers kanamycin resistance (Hobbs et al., 1990). This assay was used to identify transgenic tobacco, assess copy number, and to identify plants homozygous for the trait.

Results

Construction of pFLII

Construction of pFLII, the full-length oat *phy*A cDNA required the joining of a 5'-end genomic clone, pAP5.2 with a cDNA, pFY122, and finally the addition of the 3'-end from cDNA pPhy11A (Figure 3.1). The cloning vector chosen for the full-length oat *phy*A cDNA was pBluescript (KS) $^{+}$ because it could be used for *in vitro* synthesis of sense or antisense RNA using the T7 or the T3 RNA polymerase and because its polylinker contained convenient restriction sites. The plasmid did lack the NcoI site necessary for cloning of the pAP5.2 PCR product into the vector. An NcoI linker was purchased from Stratagene that had the following sequence: CATGCCATGGCATG, the NcoI site is underlined. When cut with BamHI and NcoI this vector provided directional cloning of the 880 base pair pAP5.2 PCR product into the vector.

The new clone, containing a complete 5'-end and a 5' BamHI site, was named pPHY20 (Figure 3.2).

The middle of the oat *phy*A gene was derived from pFY122 (Boylan and Quail, 1989). This plasmid contains an oat *phy*A cDNA. Its 5'-end is 75 base pairs from the transcription start site and its 3'-end is at an EcoRI site 50 base pairs downstream of the stop codon. It provided the largest piece of the oat *phy*A gene. PCR was utilized to generate a 2.7 kB fragment from pFY122 DNA template to be cloned into pPHY20. The 5' primer, primer number three, was completely identical to pFY122 just 5' of the NcoI site. Its sequence is as follows: 5' TACAAGTTCCATCAACATCAC 3'. The 3' primer, primer number four, was identical to the 3'-end of the clone up to the EcoRI site. An NcoI site was add to the end of the primer to provide a means for cloning the PCR product into pPHY20. The sequence was as follows: 5' TAACCATGGAATTCAAACAGAACATTTG 3'. In bold is the EcoRI site native to the oat *phy*A gene, underlined is the added NcoI site.

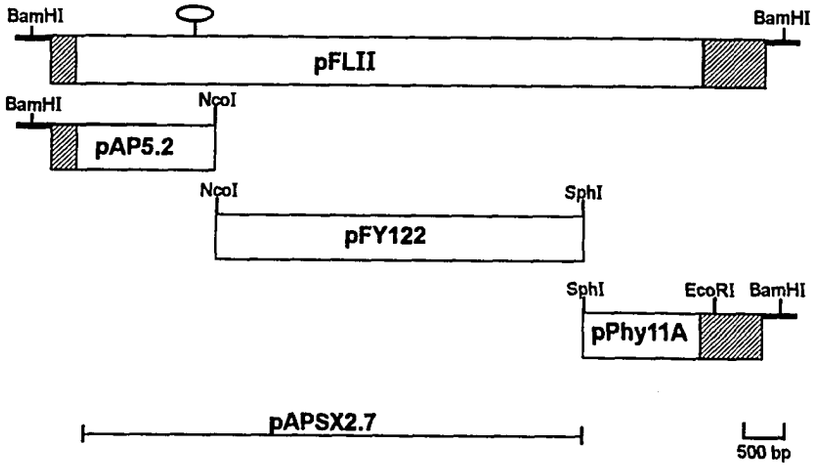


Figure 3.1. Construction of pFLII from fragments of each of three oat phytochrome clones. The 5' end of the gene is represented by pAP5.2, the chimeric cDNA pFY122 makes up the middle portion of the gene, and pPhy11A was used to construct the 3' end of the pFLII construct. Four base pairs were added to the very 5' end of pAP5.2 generating a complete 5' untranslated region. The 5' end primer was 5' AATGGATCCTCCCAGTCCAGACCCCTCAC 3', in bold is first 4bp of the transcript, underlined is a BamHI site added for easy cloning. The 3'-primer for pAP5.2 was 5' TGATTTCGGAGAATACCTCA 3', it is identical to sequences (889bp) just 3' of the NcoI site (883bp). PCR was also used to generate the middle of the gene from the NcoI site (883bp) to just 3' of the EcoRI site (3580bp) of pFY122. The 5' primer used for this construct was 5' TACAAGTTCCATCAACATCAC 3' it is identical to sequences just 5' of the NcoI site (863bp) in pFY122. The 3' primer was 5' TAA*CCATGG*AATTCAAACAGAACATTTG 3', this primer is identical to pFY122 from the 5' end to the EcoRI site (3580bp, bold). An NcoI site has been added (underlined) for ease of cloning. This PCR product was ligated to the 5' fragment from pAP5.2. The 3' end clone was generated from a cDNA library. This cDNA was ligated to the pFY122 PCR fragment at an SphI site (2880bp) thus producing a full-length oat phytochrome gene (pFLII). ⏉ indicates chromophore binding site. Filled boxes indicate untranslated regions. Boxes represent portions of the oat phytochrome clones used for construction of the full-length construct, pFLII. Line labeled pAPSX2.7 (Seeley et al., 1992) represents the 2.7 kB RNA probe used to label oat phytochrome transcripts on RNA blots.

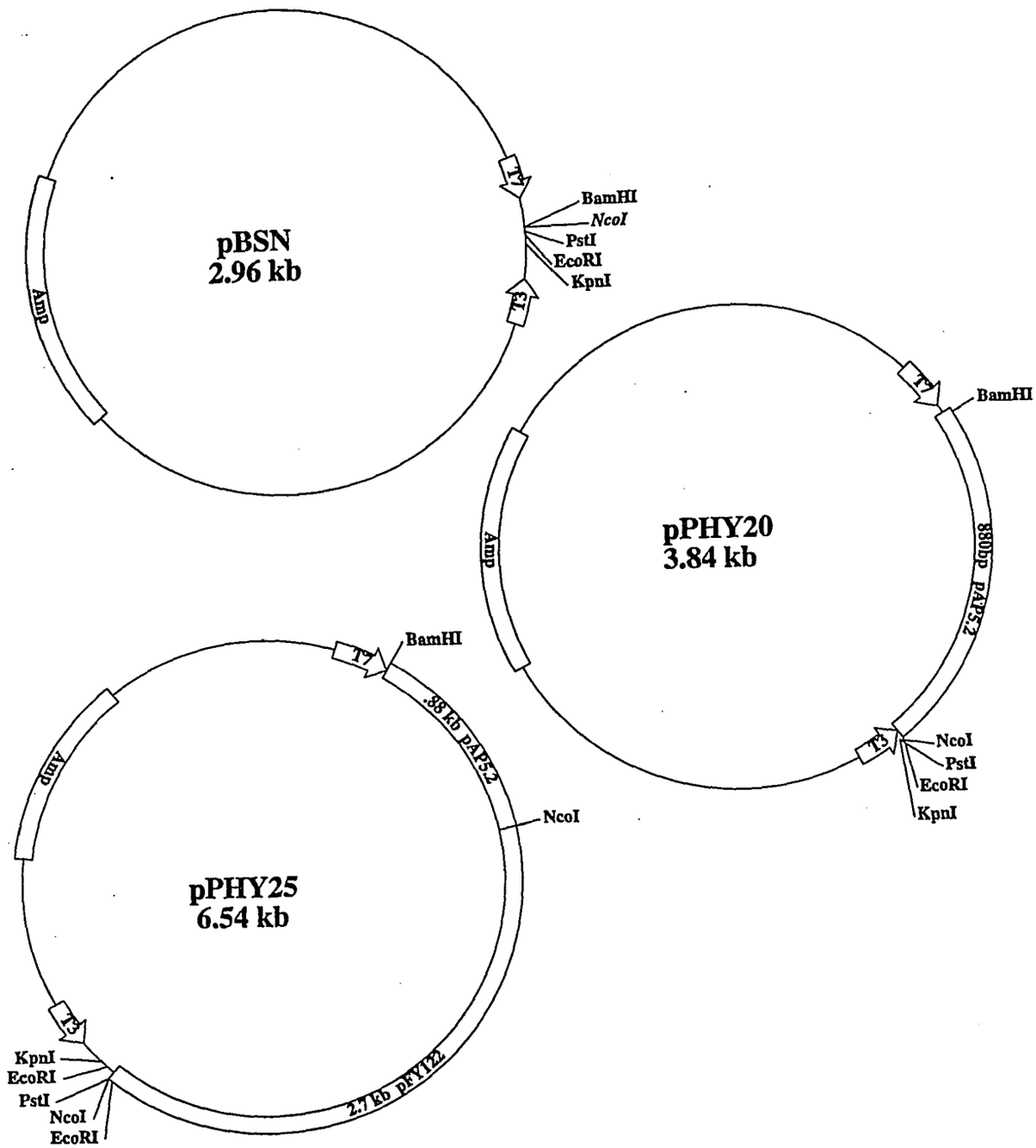


Figure 3.2. Plasmids generated in the construction of pFLII. pBSN was created by adding an NcoI linker into the SmaI site of pBluescript. The 880 PCR generated fragment from pAP5.2, representing the 5'-end of the *oat phy*A gene was cloned into the BamHI and NcoI sites of pBSN creating pPHY20. The 2.7 kB fragment generated from PCR of pFY122 was cloned into the NcoI site of pPHY20 thereby generating pPHY25.

The PCR product from primers three and four using pFY122 as template was isolated and digested with NcoI. pPHY20 was cut with NcoI. The vector was treated with calf intestinal phosphatase ensure that only plasmids ligated to the PCR product could be circularized. The pFY122 PCR product was then ligated into pPHY20. The resulting 6.5 kB plasmid was named pPHY25. Its orientation was determined by digestion with BamHI and EcoRI (Figure 3.2).

A cDNA library was made to in an attempt to attain a full-length oat phytochrome gene. The library was generated from poly(A)$^+$ RNA isolated from etiolated oat seedlings. The library was screened with a random primed DNA probe made from pGAP1.7 (Edwards and Colbert, 1990). Although a full-length *phy*A cDNA was not obtained, the library produced an oat *phy*A cDNA insert 2178 base pairs long in a pBluescript (KS)$^+$ vector. The plasmid generated was called pPhyllA. Partial sequencing of pPhyllA revealed that it contained all of the 3' UTR and included 7 A residues from the poly(A) tail (data not shown).

To combine the 3' untranslated region of pPhy11A with pPHY25, each plasmid was cut with SphI and XhoI. A 1109 base pair fragment was isolated from pPhy11A. This fragment was then ligated to a 5580 base pair fragment from pPHY25. This produced pFLII (Figure 3.3). This plasmid contains the full-length oat *phy*A gene. It has BamHI sites at its 5' and 3' ends and is in the pBluescript vector. The pBluescript vector can be used to produce either sense or antisense RNA using T7 or T3 polymerase. An *in vitro* synthesis of the sense RNA was performed to ensure that it could be transcribed in its full-length form (Figure 3.4).

Tobacco transformation

To transform tobacco with the full-length oat *phy*A gene using *Agrobacterium* mediated transformation, it was first necessary to place the cDNA into a Ti vector. The plasmid pBI121 (Clontech) was used (Figure 3.5). It contains the 35S cauliflower mosaic virus promoter (CaMV 35S), a strong constitutive promoter in tobacco. This plasmid has a β-glucuronidase (*GUS*) gene which could be used to verify transformation of this plasmid into tobacco using a *GUS* assay. It also contains the NPT II gene, which confers kanamycin resistance to the transformed plant, allowing the antibiotic to be used as a selection for transformed plants. pBI121 has a BamHI site just 3' of the CaMV 35S promoter that was used to insert the *phy* BamHI cassette from pFLII. The resulting plasmid was called pBFLI (Figure 3.5).

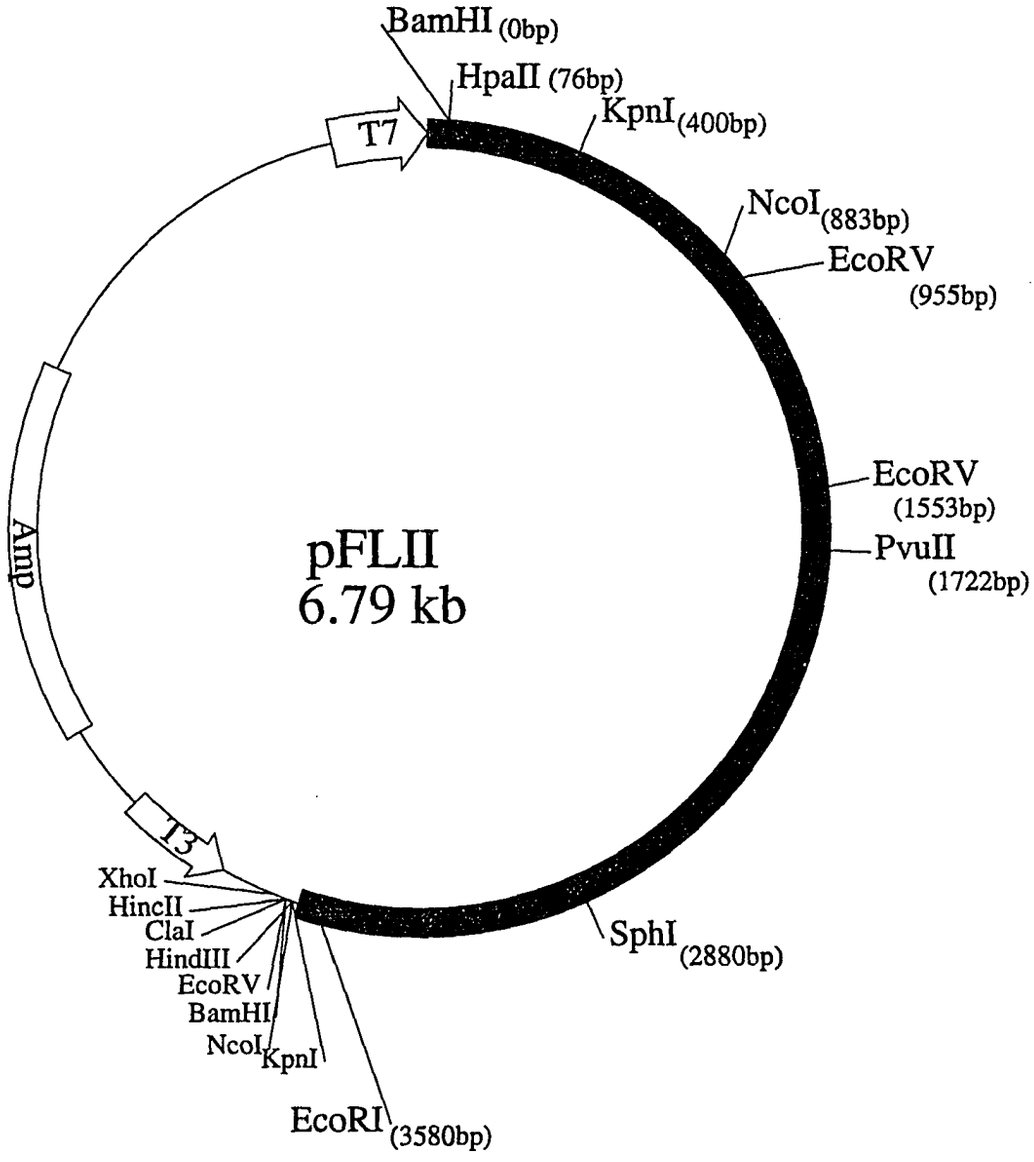


Figure 3.3 pFLII was created from pPHY25 and pPhy11A. pPhy11A was cut with XhoI and SphI generating a 1109 bp fragment. pPHY25 was cut with XhoI and SphI and the 5580 bp fragment from this was isolated. The two fragments were ligated together to produce pFLII. This plasmid map indicates the location of the T7 and the T3 promoters. The restriction sites at either end of the gene are indicated.

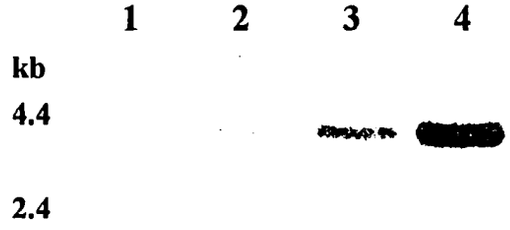


Figure 3.4 *In vitro* synthesis of oat *phy*A mRNA from pFLII. T7 RNA polymerase. pFLII was cut with XhoI to form a linear piece of DNA from which RNA could be synthesized. RNA was loaded on the gel based on CPM: lane 1, 1000 CPM; lane 2, 2000 CPM; lane 3, 3000 CPM; lane 4, 4000 CPM.

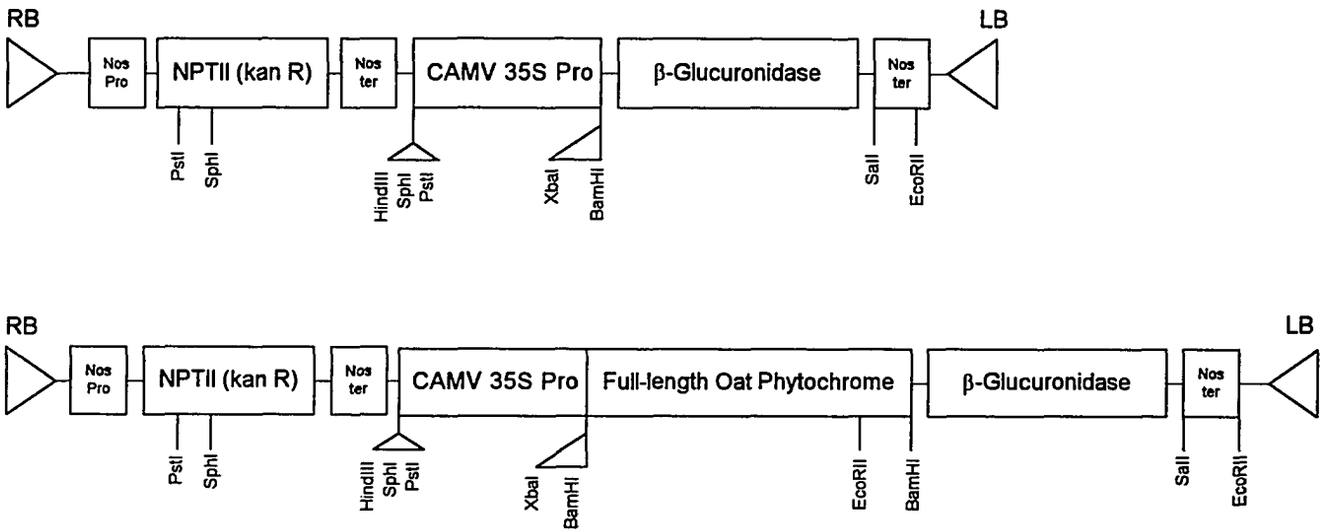


Figure 3.5. Ti vectors containing *GUS* and the complete oat *phy*A gene. pBI121 (Clontech) was used as both a vector for pFLII and as a transformation control. The NPTII gene confers kanamycin resistance. The *GUS* gene is under the control of the CaMV35S promoter. The oat *phy*A containing cassette formed from cutting pFLII with BamHI was ligated into the BamHI site in pBI121 creating pBFL1.

Agrobacterium, strain LB4404, also from Clontech, was transformed with pBFLII using electroporation. As a control, pBI121 alone was also used to transform *Agrobacterium*. This control assured that the changes in the subsequent transgenic plants were attributable to the presence oat *phy*A gene rather than to the transformation procedure or the vector. Leaf discs were grown in the presence of kanamycin to ensure that they had been transformed. Shoots transformed with the pBFLI construct were generated and induced to root with continued use of selection. Following rooting, these plants were placed in soil and grown to maturity in a growth chamber.

Plants transformed with the full-length oat *phy*A gene pBFL1 were screened for kanamycin resistance to identify transgenic individuals resulting from a single insertion. Seeds from regenerated, self-pollinated, plants were sown onto 7% agar containing one half MS salts and 100 ug/ml kanamycin (Hobbs et al., 1990). After 28 days, those plants that remained green were scored as transgenic (containing the NPTII gene) while those that became white were considered to be devoid of the transgene (Figure 3.6). Seeds of transgenic plants were found to segregate in a manner suggesting that the plants contain from zero to four copies of the NPTII gene (Table 3.1). From this screen, seeds from two plants containing single copies of the NPTII gene, BFL1-1 and BFL1-2, were used to generate 15 R1 plants. Fifteen progeny of each plant were grown to maturity and self-pollinated. These seeds were analyzed using the kanamycin resistance assay to identify homozygous plants (Table 3.1). Several plants were found to be homozygous for kanamycin resistance. All subsequent experiments were performed with seeds from the single copy, homozygous R1 plant, BFL1-18.

The phenotype of the oat *phy*A containing transgenics was similar to but not as pronounced as that of oat *phy*A containing tobacco line 9A4 (Keller et al., 1989). Plants were slightly shorter than their wild-type counterparts and were found to contain more chlorophyll (data not shown).

One isogenic line, BFL1-2, had curly leaves (Figure 3.7). This phenotype may be due to an insertional mutation rather than to increased levels of phytochrome as no other transgenic plants exhibited this phenotype.

Discussion

A full-length oat *phy*A gene was generated using two available clones and by synthesizing a third cDNA containing the 3'-end of the gene from a cDNA library. The full-length construct, pFLII, was cloned into the Ti vector, pBI121, forming pBFLI. pBFLI was

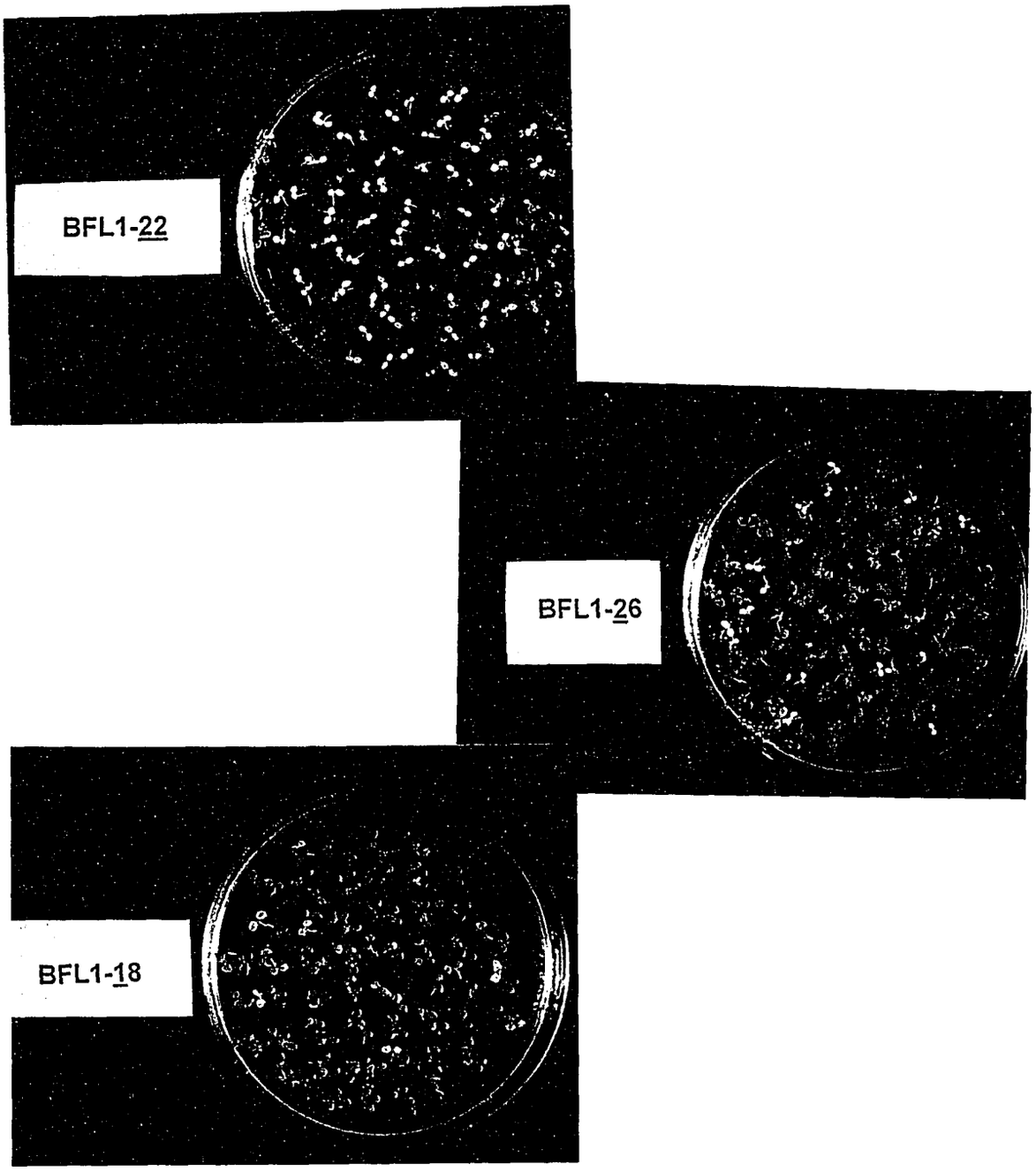


Figure 3.6. Seedlings grown from seeds of R1 plants grown in the presence of 100mg/ml kanamycin. Seeds from plant BFL1-22, plant was null for the NPT II gene. Seeds from plant BFL1-26, plant was hemizygous for the NPT II gene. Seeds from BFL1-18, plant was homozygous for the NPT II gene.

Table 3.1. Determination of copy number of the NPTII gene and identification of isogenic plants using a kanamycin resistance assay. N/D indicates not done.

Plant	Parent plant	% Green seedlings	% White seedlings	Copy Number
BFL1-1	R0	79.6	20.4	1 (Hemizygous)
BFL1-2	R0	77.7	22.3	1 (Hemizygous)
BFL1-4	R0	91.0	9.0	2 (Hemizygous)
BFL1-5	R0	99.0	1.0	4 (Hemizygous)
BFL1-6	R0	79.6	20.4	1 (Hemizygous)
BFL1-7	R0	76.1	23.9	1 (Hemizygous)
BFL1-8	R0	6.1	93.9	? (Hemizygous)
BFL1-11	R1			N/D
BFL1-12	R1			N/D
BFL1-13	R1	78.0	22.0	1 (Hemizygous)
BFL1-14	R1	78.5	21.5	1 (Hemizygous)
BFL1-15	R1	83.0	17.0	1 (Hemizygous)
BFL1-16	R1	27.3	72.7	1 (Hemizygous)
BFL1-17	R1	77.2	22.8	1 (Hemizygous)
BFL1-18	R1	100.0	0.0	1 (Homozygous)
BFL1-19	R1	100.0	0.0	1 (Homozygous)
BFL1-110	R1	0.0	100.0	0 (Null)
BFL1-111	R1	73.7	26.3	1 (Hemizygous)
BFL1-112	R1	100.0	0.0	1 (Homozygous)
BFL1-113	R1	71.9	28.1	1 (Hemizygous)
BFL1-114	R1	76.5	23.5	1 (Hemizygous)
BFL1-115	R1	74.3	25.7	1 (Hemizygous)
BFL1-21	R1			N/D
BFL1-22	R1	0.0	100.0	0 (Null)
BFL1-23	R1	71.8	28.1	1 (Hemizygous)
BFL1-24	R1	0.0	100.0	0 (Null)
BFL1-25	R1	72.1	27.9	N/D
BFL1-26	R1	74.0	26.0	1 (Hemizygous)
BFL1-27	R1	100.0	0.0	1 (Homozygous)
BFL1-28	R1			N/D
BFL1-29	R1			N/D
BFL1-210	R1	90.2	9.8	1 (Hemizygous)
BFL1-211	R1	78.7	21.3	1 (Hemizygous)
BFL1-212	R1	100.0	0.0	1 (Homozygous)
BFL1-213	R1	71.9	28.1	1 (Hemizygous)
BFL1-214	R1			N/D
BFL1-215	R1	73.8	26.2	1 (Hemizygous)

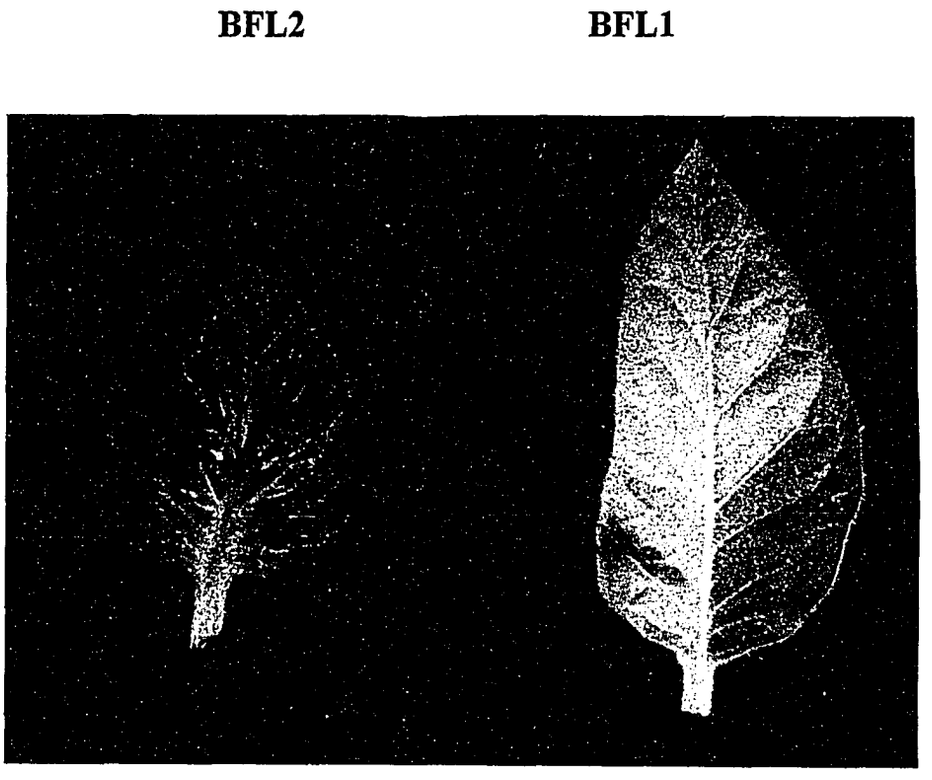


Figure 3.7. Leaves from a tobacco oat *phy*A containing transgenic plant line BFL1 and one that was transformed with BFL2. The transgenic plant, BFL2, and its progeny produce a curly leaf morphology. BFL1 transgenic plant line produces leaves similar in size and morphology to that of wild-type.

electroporated into *Agrobacterium* which was in turn used to infect tobacco leaf discs. The resulting kanamycin resistant transgenic plants were grown to maturity for seed to be used in subsequent experiments.

BFL1 plants are the first transgenic tobacco plants to contain a gene capable of transcribing the full-length oat *phy*A mRNA. All other oat *phy*A containing transgenic tobacco have had oat *phy*A genes with incomplete 5' UTRs, introns in the 3' UTR and/or deletions in the 3' translated region (Keller et al., 1989; Cherry et al., 1993). The phenotype of the BFL1 plants was found to be comparable to that of the oat *phy*A transgenic tobacco previously described (Keller et al., 1989).

CHAPTER 4 DETERMINATION OF THE HALF-LIFE OF THE FULL-LENGTH OAT *PHY*A mRNAS IN TRANSGENIC TOBACCO

Introduction

Comparison of the half-life of *phy*A mRNA in oats to a truncated version of the oat *phy*A gene in transgenic tobacco revealed a much longer half-life of mRNA in the transgenic tobacco. The half-life of oat *phy*A mRNA is about 60 minutes in etiolated oat seedlings (Seeley et al., 1992). The half-life of truncated oat *phy*A mRNA in transgenic tobacco was found to be greater than four hours (Chapter 2). Tobacco does regulate levels of its *phy*A in response to light, although the extent of *phy*A mRNA down-regulation in tobacco is much less dramatic than in oats. Tobacco *phy*A RNA decreases to about one half the dark levels after four hours of continuous white light (Chapter 2).

The gene used to produce oat *phy*A containing transgenic tobacco had the complete oat *phy*A coding region but lacked the 5' UTR and possessed two small introns in its 3' UTR. The 5' UTR is important for regulation of the stability of the PPRI gene in yeast (Pierrat et al., 1993). Monocot introns have been found to be inefficiently spliced in dicots (Keith and Chua, 1986). These factors could affect the mRNA stability. If the sequences rendering the message unstable were located in the 5' UTR of the oat *phy*A message, missing sequences could render the message more stable. If the element(s) necessary for message stability is in the 3' UTR the remaining introns could alter stability. Because of the differences between the oat *phy*A in tobacco and the full-length mRNA in oats, a direct comparison the oat *phy*A mRNA half-life in oats and in tobacco was inconclusive. This led to the need for a full-length oat *phy*A gene to be use for measuring the mRNA half-life in transgenic tobacco.

To examine the stability of the full-length oat *phy*A mRNA in transgenic tobacco, a full-length oat *phy*A gene was constructed (Chapter 3). Transformation of the full-length gene into tobacco has provided oat *phy*A mRNA containing plants. The full-length oat *phy*A construct, pFLII, contains a complete 5' UTR and an authentic 3' UTR. It does not contain any introns nor any non-*phy* sequences within the gene itself.

In addition to examination of oat *phy*A gene in tobacco, the plants used as a control expressed the β-glucoronidase (*GUS*) gene. The half-life of the *GUS* mRNA gene was also measured and compared to other estimates of *GUS* mRNA half-life in oat protoplasts (Higgs and Colbert, 1993) and in transgenic *GUS* containing tobacco suspension cultures (Newman et al., 1993).

Methods and Materials

Growth of seedlings

Seed used to grow seedlings were from oat *phy*A containing transgenic tobacco line BFL1-18. Approximately 200 seeds were sown in petri dishes on two pieces of Whatman 3 MM filter paper soaked with 5 ml of MS salts. Petri dishes were sealed with parafilm and placed in complete darkness for five to seven days.

Cordycepin inhibition assay

Cordycepin assays were performed as described in Chapter 2.

RNA isolation and RNA blotting

Total RNA isolation was performed using the aurin tricarboxylic acid method and RNA blotting was performed as described in Chapter 2.

RNA probe synthesis

RNA probe synthesis was performed using pAPSX2.7 as described in Chapter 2. Antisense *GUS* RNA probe was synthesized from pBSGUS (Higgs and Colbert, 1993). pBSGUS was linearized with BamHI and RNA synthesized using T7 polymerase.

Quantitation of RNA and half-life determination

To determine the half-life of full-length oat *phy*A and *GUS* in transgenic tobacco autoradiographs were used as described in Chapter 2. Radioactivity was measured using liquid scintillation spectrometry.

Results

BFL1, the transgenic tobacco line containing the full-length oat *phy*A cDNA, was determined to contain the oat *phy*A RNA by RNA blot analysis (Figure 4.1, panel A). Total RNA from 6-day-old oat *phy*A containing transgenic tobacco was isolated, electrophoresed, and blotted. When probed with the pAPSX2.7, anti-sense oat *phy*A RNA probe, two bands were visualized. The expected 4.1 kb full-length oat *phy*A mRNA was visible in addition to a larger 5.1 kb band. The most intense band was of the expected 4.1 kb size. The larger band of RNA was also found to hybridize with pBSGUS, an antisense RNA probe for *GUS* (Figure 4.1, panel B). The less intense, higher molecular weight band was interpreted to be an oat *phy*A-*GUS* mRNA hybrid. For half-life measurements the 4.1 kb band was used rather than the 5.1 kb band.

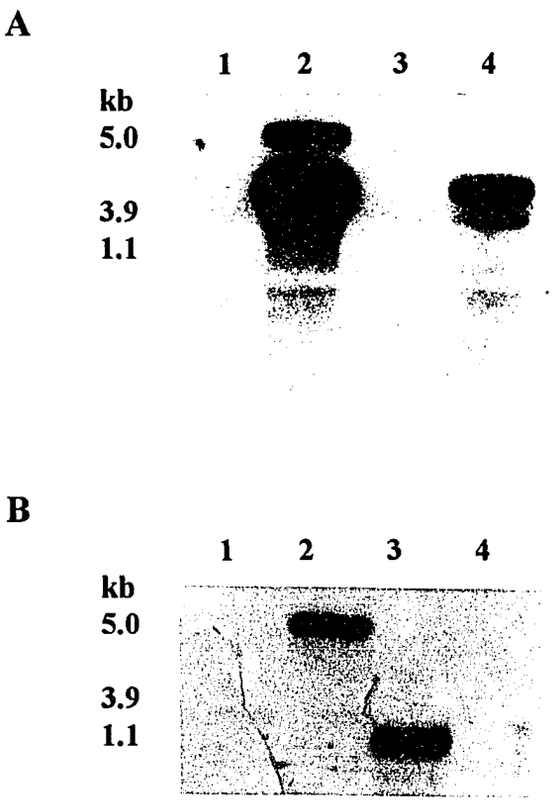


Figure 4.1. Total RNA probed for oat *phy*A or *GUS*.
A RNA blot probed with pAPSX2.7 antisense oat *phy*A RNA probe.
B RNA probed with the pBSGUS antisense *GUS* RNA probe. Lane 1, wild-type tobacco RNA; lane 2, oat *phy*A containing transgenic plant BFL1-18; lane 3, *GUS* containing transgenic plant BI121; lane 4, oat RNA.

Cordycepin, a chain-terminating adenosine analog, was used to estimate the oat *phy*A mRNA half-life in etiolated BFL1 seedlings. Seedlings were grown in complete darkness for five days and then treated with water or cordycepin for a 15 minute pretreatment. Following pretreatment, seedlings were placed in continuous white light. Seedlings were harvested at time zero, 60, and 240 minutes after pretreatment. Total RNA was isolated, electrophoresed, blotted and probed with an antisense oat *phy*A RNA probe. The half-life of the full-length oat *phy*A mRNA was estimated to be about four hours (Figure 4.2).

The half-life of *GUS* mRNA was also measured using cordycepin. Seedlings from tobacco transformed with BI121 were treated with cordycepin and the half-life of the *GUS* mRNA was measured in transgenic tobacco. The GUS mRNA half-life was found to be about three hours (Figure 4.3).

Discussion

Transgenic tobacco plants containing the full-length oat *phy*A mRNA were used to determine the half-life of oat *phy*A mRNA in tobacco. RNA isolated from BFL1-1 plants displayed two bands when hybridized with the oat *phy*A antisense probe. The smallest band, 4.1 kb, was found to be an oat *phy*A mRNA band, the larger band was found to be an oat *phy*A-*GUS* hybrid. It appears that the tobacco transcription machinery inefficiently recognized the poly(A) addition signal located in the 3'-end of the oat *phy*A cDNA (Hershey et al., 1985). The *GUS* gene in the vector pBI121 contains a NOS 3' terminator that functions efficiently in tobacco. Two bands were seen in several independently transformed oat *phy*A containing transgenic plants (data not shown).

The half-life of full-length oat *phy*A mRNA in transgenic tobacco was measured to determine if sequences in the 5' UTR or the 3' UTR are important for conferring instability to the message. Endogenous levels of *phy*A RNA in tobacco decrease by about one half following exposure of seedlings to four hours of continuous white light (Chapter 3). Like the transgenic tobacco containing only the coding region of the gene, a half-life of about four hours was determined for the full-length oat *phy*A mRNA in transgenic tobacco. It can be concluded that whether or not the 5' UTR and/or the 3' UTR of oat *phy*A are important in oat, they do not alter the stability of the message in transgenic tobacco.

The half-life determined for oat *phy*A in transgenic tobacco reflects the predicted half-life of the tobacco *phy*A gene if transcription of the gene in tobacco is shut off in response to light as it is in oats. In rice, barley, and peas *phy*A mRNA levels drop dramatically following

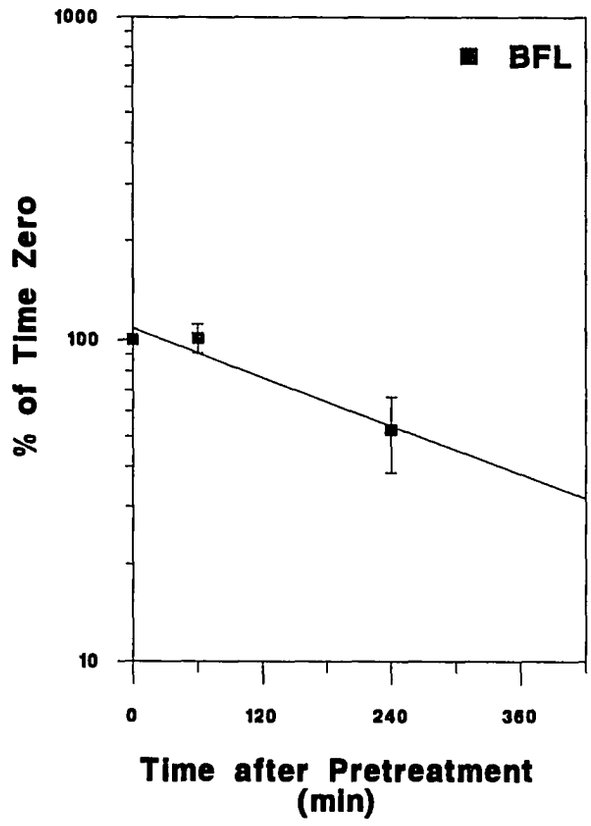


Figure 4.2. Half-life determination of full-length oat *phy*A mRNA in transgenic tobacco. Half-life of oat *phy*A containing tobacco was determined using 6-day-old etiolated seedlings. RNA was isolated from seedlings treated with 1 mg/ml cordycepin for 15 minutes in darkness and then harvested (time 0), or placed under continuous white light and harvested at 60 and 240 minutes. Total RNA was probed for *phy*A mRNA and full-length band excised from the RNA blots. The amount of radioactivity for each band was measured using liquid scintillation spectrometry. Data show the means of three or more independent experiments and standard error. Linear best fit curves were used to estimate the half-lives for *phy*A mRNAs.

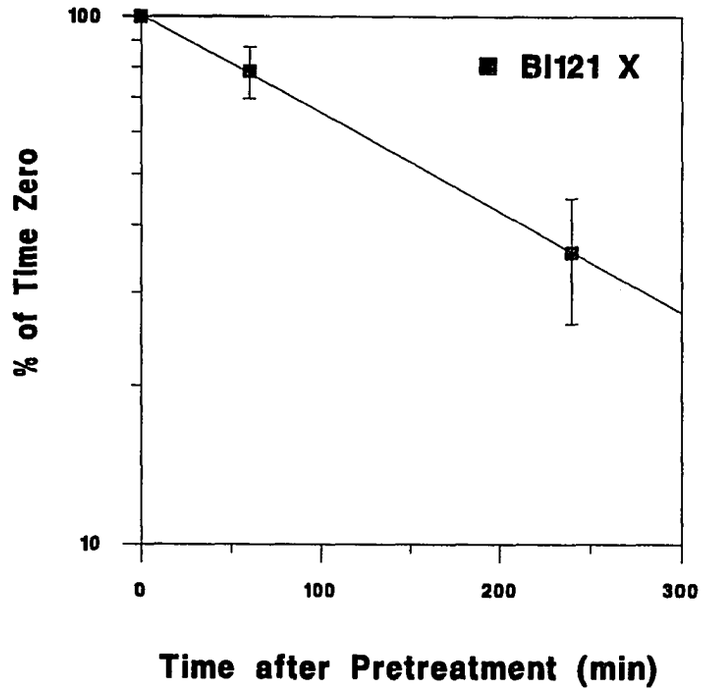


Figure 4.3. Half-life determination of *GUS* mRNA in transgenic tobacco. Determination was made as described for Figure 4.2. Total RNA was probed for *GUS* mRNA and the full-length band was excised from RNA blots. The amount of radioactivity for each band was measured using liquid scintillation spectrometry. Data show the means of three or more independent experiments and standard error.

exposure of plants to red light (Kay et al., 1989a; Rahim, 1992; Tomizawa et al., 1989; Dehesh et al., 1991). Cucumber *phy*A mRNA levels decline in a transient manner in response to light (Tirimanne and Colbert, 1991). Tobacco *phy*A RNA abundance may be more similar to that of *phy*A in *Arabidopsis* or tomato. *Arabidopsis* and tomato *phy*A mRNA abundance change little in response to light (Sharrock et al., 1988; Sharrock and Quail, 1989). Tobacco *phy*A may not be inherently unstable or it may unstable and regulate its own mRNA by a different mechanism or sequence elements than that used by oats.

An examination of *GUS* mRNA in transgenic tobacco can provide a comparison of the same mRNAs in different plants or cell types. *GUS* mRNA was found to have a half-life of about 160 minutes in transgenic tobacco. In oat protoplasts, *GUS* mRNA has been estimated to be about 35 minutes (Higgs and Colbert, 1993). In stably transformed tobacco suspension culture, *GUS* mRNA has been estimated to have a half-life of 80 minutes (Newman et al., 1993). The differences in the half-life estimations for all three systems may reflect the differences in the method for measuring mRNA half-life, or differences in the manner each cell type or species recognizes and degrades *GUS* mRNA.

CHAPTER 5 THE EFFECTS OF OAT *PHY*A ON GERMINATION OF TOBACCO SEEDS

Introduction

The purpose of generating tobacco transformed with oat *phy*A cDNA in this study was to investigate whether tobacco would rapidly degrade oat *phy*A mRNA. Production of transgenic plants also provided an opportunity for examination the effect of oat *phy*A expression on the phenotype of the transgenic plants, specifically seed germination.

Previous studies of oat *phy*A containing transgenic plants have revealed that when present a high levels, for example when transcribed from a constitutive promoter, oat *phy*A is functional in a dicotyledonous system and functions in green, non-etiolated plants (Whitelam et al., 1992). Dark grown transgenic tobacco seedlings containing the coding region of the oat *phy*A were found to contain 2.6 times more spectrally detectable phytochrome per gram fresh weight than did wild-type seedlings. Light grown transgenic plants contained 20 times more spectrally active phytochrome than did the wild-type plants (Keller et al., 1989). Additional studies of transgenic tobacco containing oat or rice *phy*A genes revealed that plants overexpressing the *phy*A protein display a light-exaggerated response when grown under light conditions. Some of these responses included reduced internode length, and increased levels of chlorophyll (Kay et al., 1989b; Cherry et al., 1991; McCormac et al., 1991). An increased level of anthocyanin biosynthesis was found in transgenic tomato containing oat *phy*A (Boylan and Quail, 1989).

Phytochrome deficient mutants have revealed that *phy*A may be important for seed germination in tomato. Germination in the *au phy*A deficient tomato mutants is greatly reduced in spite of the fact that tomato is a light insensitive species with regard to germination (Georghiou and Kendrick, 1991).

Seed germination in response to light or dark is controlled, in part, prior to imbibition. Light conditions during to seed maturation in *Arabidopsis* has been shown to influence phytochrome controlled germination of the seeds. When matured under conditions deficient in far-red light, seeds were found to be capable of germinating in the dark. Seeds matured under conditions rich in far-red light were unable to germinate in dark (Gettens-Hayes and Klein, 1974). Transgenic *Arabidopsis* seeds containing a rice *phy*B gene or an *Arabidopsis* *phy*B gene under the control of the CaMV35S promoter germinate at higher levels in darkness compared to wild-type seeds when seed is matured on plants are grown under low red/far-red

light ratios. Both wild-type and *phy*B containing transgenic seeds germinated at a high levels in the dark when matured under high red to far-red light ratios (McCormac et al., 1993). Oat *phy*A containing transgenic *Arabidopsis* matured under different light treatments did not show significantly altered responses to germination in darkness compared to wild-type. For *Arabidopsis*, it appears that *phy*B is responsible for eliciting germination.

The presence of nitrate has been shown to stimulate germination during or after imbibition (DePetter et al., 1985; Roberts and Benjamin, 1979; Vincent and Roberts, 1979; Hartmann and Nezadal, 1990; Grubisic and Konjevic, 1990; Hilton, 1985). This stimulation will occur in the presence of Pfr but not Pr (Grubisic and Konjevic, 1990; Hilhorst et al., 1986). Nitrate functions to lower the threshold of Pfr required to elicit germination.

Phytochrome has been found in unimbibed seeds. Although cultivated oats are light insensitive with regards to germination, three immunologically detectable phytochromes have been found in unimbibed seeds of *Avena sativa.* Included among these is the 124 kDa *phy*A phytochrome (Wang et al., 1992).

Using transgenic tobacco line BFL1-18, germination was examined from seed generated from plants grown under two different light treatments. Germination of transgenic tobacco was also examined when imbibed in the presence of potassium nitrate.

Materials and Methods

Growth conditions, seed harvest and storage

Plants were grown in a growth chamber under incandescent and fluorescent lights or fluorescent lights alone. The fluence rate was 175 uM/m^2s under fluorescent lighting and 190 uM/m^2s under the combination of fluorescent and incandescent lighting. Lighting was maintained for 16 hours per day followed by 8 hours of darkness. The temperature was maintained at 26^{o}C. Plants were watered daily and fertilized regularly. Self-pollination was maintained by covering newly developed flower buds with a small bag made from Kimwipe toweling. Seeds were harvested when the seed pods were brown and dry. Seeds were stored in darkness, at room temperature, in small glass vials.

Germination assays

All germination assays were performed with seeds from plant BFL1-18 or wild-type plants. Germination assays were performed within four months of seed harvest. Assays were done on two autoclaved pieces of Whatman filter paper in a petri dish soaked with 5 ml of sterile water. Seeds were sown by sprinkling seeds on the surface of the wetted filter paper.

Dishes were sealed with parafilm and placed in complete darkness. Light-treated seeds were exposed to one hour of 190 uM photons/m^2s continuous white light between 16-24 hours after seeds were sown and returned to darkness for the remainder of five days. Germination was scored as positive when radicle emergence could be visualized using a dissecting scope.

Nitrate germination assays

Germination assays were done on two autoclaved pieces of Whatman filter paper in a petri dish soaked with either 5 ml of water, 1 mM, 10 mM, 50 mM, or 100 mM KNO_3. Seeds were sown by sprinkling seeds on the surface of the wetted filter paper, sealing the petri dish with parafilm and placing the seeds in complete darkness. Dark treated seeds were left in complete darkness for five days. Light treated seeds were exposed to one hour of 190 uM photons/m^2s continuous white light between 16-24 hours after seeds were sown and returned to darkness for the remainder of five days. Germination was scored as positive when radicle emergence could be visualized using a dissecting scope.

Results

To examine the effects of oat *phy*A on seed germination, seeds of both wild-type and BFL1-1$\underline{8}$ from plants grown under fluorescent lights were sown on filter paper wetted with water. The seeds were either given a light treatment 16 hours after imbibition or left in total darkness for five days. Percent germination of wild-type and transgenic seeds given a light treatment after imbibition was greater than 95%. When imbibed in total darkness, about 1% of the wild-type germinated while 22% of the BFL1-1$\underline{8}$ seeds germinated (Figure 5.1).

Germination of photosensitive seeds has been shown to be enhanced by nitrate (DePetter et al., 1985; Roberts and Benjamin, 1979; Vincent and Roberts, 1979; Hilton, 1985; Grubisic and Konjevic, 1990). This enhanced germination has been demonstrated to be effective only in the presence of Pfr generated after seeds are imbibed and exposed to light in photosensitive species. To determine if wild-type and transgenic seeds are differentially responsive to nitrate, seeds were imbibed in various concentrations of KNO_3 in complete darkness and germination examined five days later (Figure 5.2). Germination of BFL1-1$\underline{8}$ transgenic tobacco was increased from about 20% to greater than 50% in darkness in the presence of 50 mM KNO_3. Wild-type seeds had an increase in the percent germination from 1% to about 15%. BFL1-1$\underline{8}$ seeds responded to nitrate at a much higher level than did the wild-type seeds suggesting that the transgenic seeds had higher amounts of Pfr prior to imbibition.

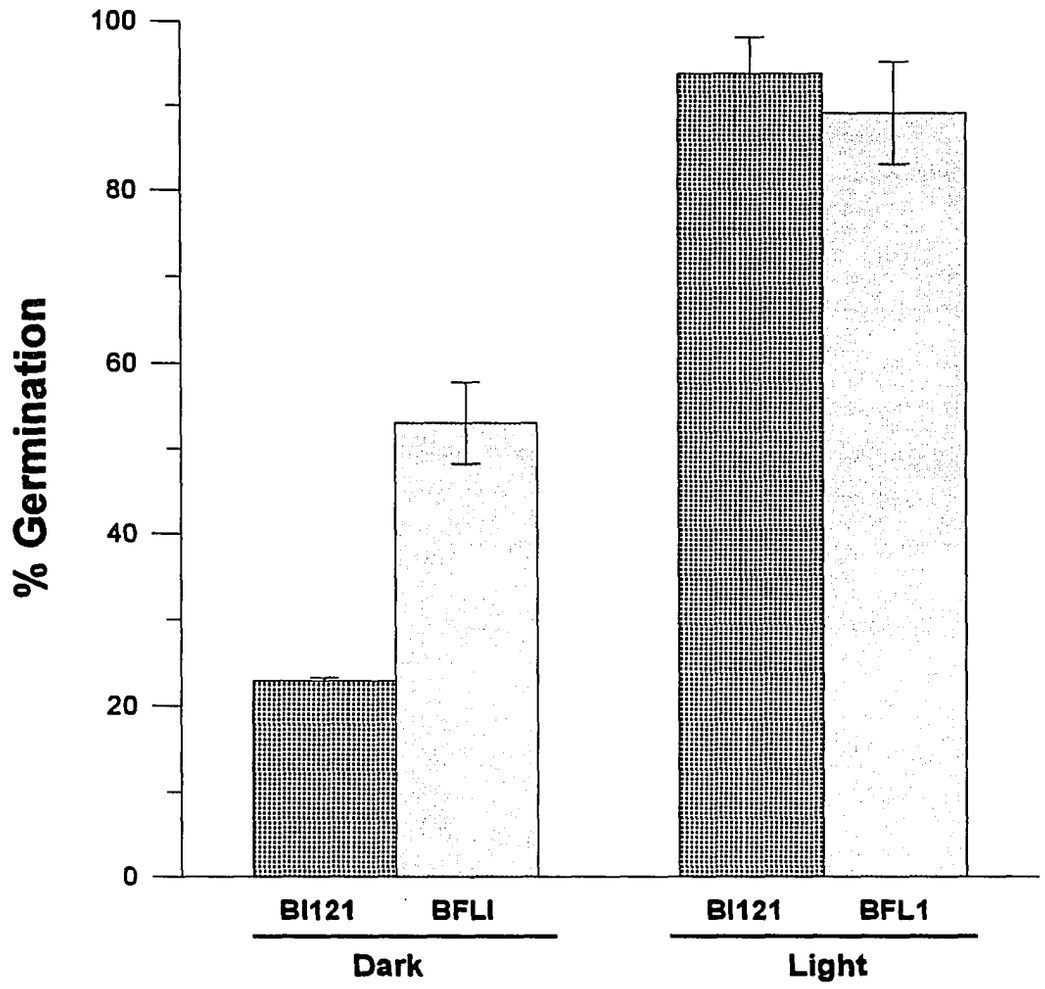


Figure 5.1. Germination rates of BI121 (control) tobacco seeds and oat phytochrome containing BFL1-18 transgenic tobacco seeds in response to dark and light treatments. 100 or more seeds were sown onto petri dishes containing 2 pieces of Whatman filter paper soaked with 5 ml of sterile water. These were sealed and immediately placed in complete darkness. Light treated samples were given a one hour treatment with continuous white light 16-24 hours after sowing and returned to darkness for the remainder of five days. Germination was scored positive with the emergence of the radicle. Data are the average of three experiments and error bars represent standard error.

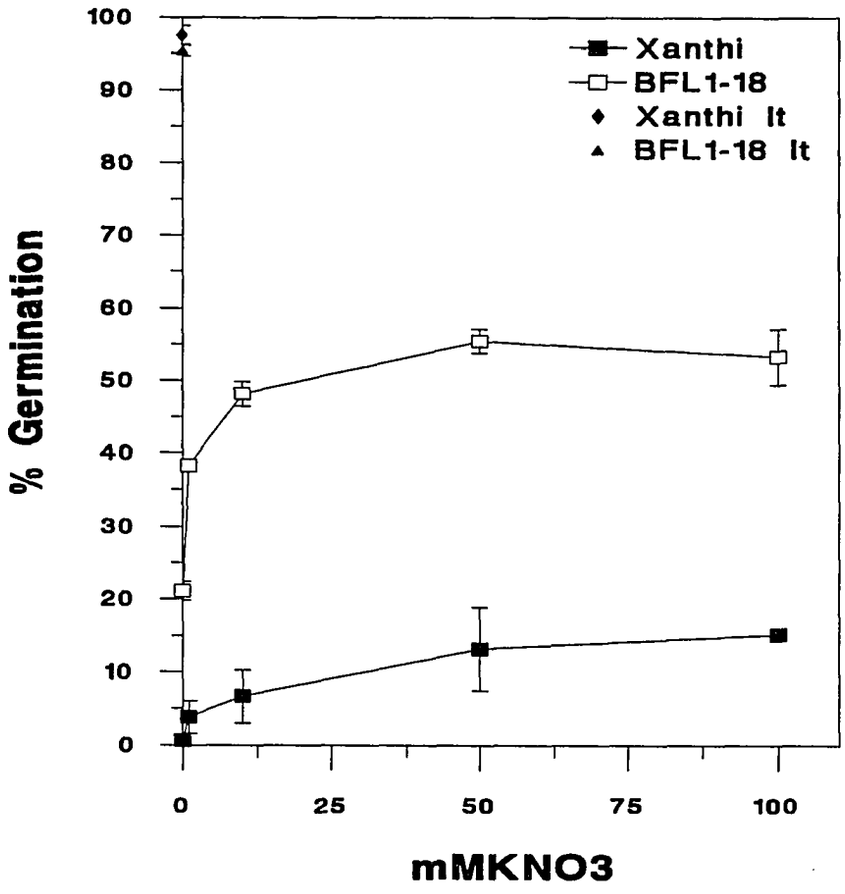


Figure 5.2. Percent germination of both wild-type (Xanthi) and BFL1-18 tobacco seeds as a function of increasing concentrations of KNO_3. 100 or more seeds were sown onto petri dishes containing two pieces of Whatman filter paper soaked with 5 ml of sterile water, 1 mM, 10 mM, 50 mM, or 100 mM KNO_3. Petri dishes were then sealed and seeds were placed in complete darkness for five days and radicle emergence was assessed. Light controls were performed as described for Figure 5.1. Data are the mean of three experiments and error bars represent standard error.

Seed from plants grown under both fluorescent and incandescent light were examined for altered patterns of seed germination due to increased levels of far-red light from incandescent lighting. The expectation was that the amount of Pfr in the seed would be lower, resulting in reduced germination. Seeds from both wild-type and BFL1-1$\underline{8}$ plants germinated at a rate of less than 1% when placed in complete darkness. To test seed viability, seeds were sown on water and exposed to a brief exposure to white light following imbibition and returned to darkness. These seeds germinated at a rate of nearly 100% (Figure 5.3).

To determine if transgenic seeds had increased levels of phytochrome, wild-type and BFL1-1$\underline{8}$ seeds matured under fluorescent and incandescent lighting were sown on 50 mM KNO_3 and allowed to germinate in complete darkness. These seeds had an increased rate of germination from less than one percent in water to about 48% on KNO_3 (Figure 5.3). This increase indicates that Pfr levels in the transgenics were still higher than in the wild-type plants.

Discussion

In complete darkness, germination of oat *phy*A containing transgenic tobacco, 9A4, has been reported to be less than 10% by McCormac et al. (1993). In the same experiments, wild-type tobacco was reported to germinate at about 15%. About 20% of the BFL1-1$\underline{8}$ seeds from plants matured under the fluorescent lighting described here germinate when in darkness. In addition, we have shown wild-type tobacco seeds to germinate at less than 1% in total darkness. The same cultivar, Xanthi, was used for the present work and McCormac's work. This suggests that the transgenic seed used for the experiments described in the present work contain more Pfr in the unimbibed seeds than those seeds used by McCormac et al. (1993). Higher levels of Pfr stored in the dormant seed would allow the seed to germinate following imbibition in the absence of light. The discrepancy between these two sets of data could be due to differences in the light conditions the plants were grown under during seed maturation. *Arabidopsis* seed germination potential was shown to be enhanced if seeds were matured under fluorescent lights rather than grown under incandescent lights (Gettens-Hayes and Klein, 1974).

McCormac et al. (1993) harvested seed from plants grown under natural daylight supplemented with radiation from sodium-vapor lamps. Our seeds were collected from plants grown under fluorescent lighting in a growth chamber. These conditions would be depleted in far-red light relative to natural daylight supplemented with sodium vapor lamps. This

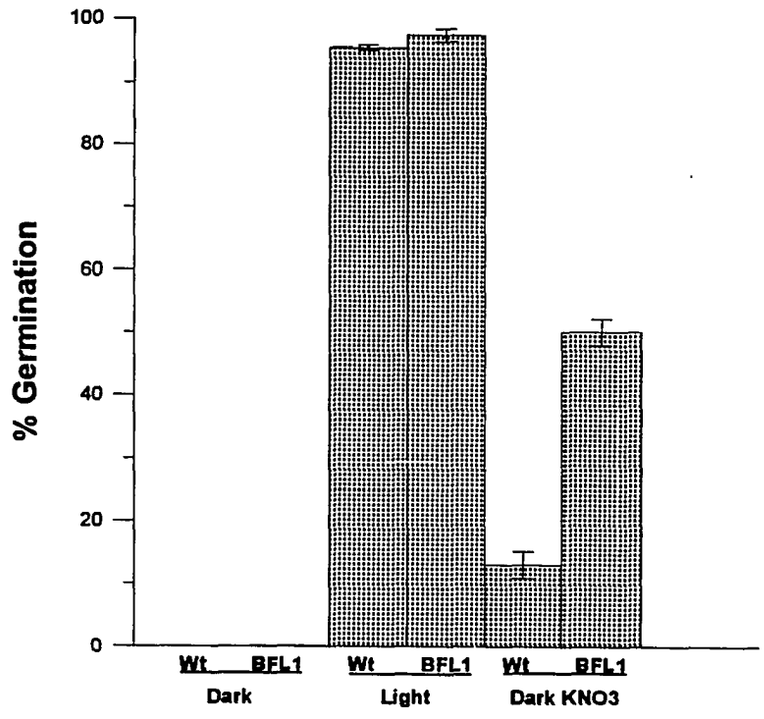


Figure 5.3. Percent germination of BFL1-18 and wild-type (Xanthi) from seeds matured under fluorescent and incandescent lighting on water or 50 mM KNO_3. Germination assay is the same as described for Figure 5.1. Data are the average of three independent experiments and error bars represent standard error.

difference may be sufficient to result in a relative increase in the level of Pfr in seeds used in the experiments reported here. This increase in Pfr likely occurred in wild-type plants as well, but the total amount of phytochrome may have been insufficient to promote germination. The transgenic tobacco is likely to have a higher level of total phytochrome (Keller et al., 1989; Boylan and Quail, 1989) and so, under artificial light, generate levels of Pfr sufficient to promote germination in greater than 20% of the seeds.

To test the hypothesis that the discrepancy between our initial results and those of McCormac et al. (1993) was due to low levels of far-red light during seed maturation, we tried growing plants under far-red enriched conditions. Seeds from wild-type and BFL1-1$\underline{8}$ plants grown under both fluorescent and incandescent light did not germinate on water. It is likely that these seeds are richer in Pr and lower in Pfr than the seeds matured under fluorescent lighting. It is unlikely that the absolute levels of phytochrome are different for these two seed types. If anything, less phytochrome would be present in the seeds grown under fluorescent light as Pfr is degraded more rapidly than Pr. Oat Pfr from *phy*A is degraded more rapidly in the Pfr form than Pr form in transgenic tobacco (Keller et al., 1989).

It has been suggested that it is the Pfr/Ptotal ratio that triggers a phytochrome response rather than the absolute amount of Pfr (Smith, 1981). If the Pfr/Ptotal ratio were responsible for initiating phytochrome responses, then the absolute levels of phytochrome would not be expected to alter the phytochrome response (Kendrick and Kroneberg, 1986). In these investigations the ratios of Pfr/Ptotal in both the wild-type and BFL1-1$\underline{8}$ would be expected to be the same as both were grown under identical conditions, although they do not contain the same total amount of phytochrome. Based on these observations, it appears that absolute levels of Pfr are responsible for eliciting tobacco germination rather then a the ratio of Pfr to Ptotal.

Additional evidence that increased levels of Pfr are present in transgenic tobacco containing the oat *phy*A gene comes from experiments in which seeds were imbibed in the presence of nitrate. Previous studies have shown that nitrate increases the sensitivity of seeds to Pfr (Grubisic and Konjevic, 1990; Hilhorst et al., 1986). It is thought that nitrate lowers the threshold of seed sensitivity to Pfr. BFL1-1$\underline{8}$ seeds sown on 50 mM nitrate and placed in complete darkness were found to germinate more frequently than the wild-type tobacco suggesting that these transgenic tobacco contain more Pfr than wild-type.

To examine the levels of Pfr more closely in the seeds matured under fluorescent and incandescent lighting, BFL1-1$\underline{8}$ and wild-type seeds were germinated in the presence of 50 mM KNO_3. BFL1-1$\underline{8}$ seeds germinated in darkness at a rate of about 48%. Wild-type seed

grown under the same conditions did not have appreciably different germination rates than wild-type seed matured under fluorescent lights alone. These data indicate that Pfr levels in transgenic tobacco were still higher than in wild-type plants, high enough so that when the threshold of Pfr required to germinate was lowered BFL1-18 seeds were able to germinate.

These data suggest that, for tobacco, *phy*A may play a significant role in regulating seed germination. At the very least, the present study does demonstrate that oat *phy*A can function to elicit germination in transgenic tobacco. This is consistent with experiments with the *au* mutant in tomato (Georghiou and Kendrick, 1991). The *au* mutant was found to be *phy*A deficient and unlike the wild-type tomato was found to have poor germination in the dark. The low amount dark germination that did occur was uninhibited by far-red light. Higher levels of dark germination in tobacco overexpressing *phy*A likewise support the idea that *phy*A is responsible for eliciting germination in these light sensitive species.

In conclusion, it appears that oat phytochrome A can function in tobacco to promote germination in transgenic seeds. Transgenic seed germination is dependent upon the light conditions at the time of seed maturation. Potassium nitrate is capable of lowering the threshold level of Pfr required for germination. Finally, it appears that *phy*A may be important for eliciting seed germination in tobacco. Unlike *Arabidopsis*, whose germination is unaltered by increased levels of oat *phy*A, tobacco responds quite dramatically.

CHAPTER 6 CONCLUSIONS

The study of phytochrome gene regulation is complex because gene expression is, in part, regulated at the level of transcription in some species, the rates of mRNA turnover may vary from one species to another, and the level of phytochrome protein is post-translationally regulated in response to light. Likewise, the study of phytochrome function is complex because the different phytochrome genes may have distinct or overlapping functions. In addition, it is now becoming evident that some functions of *phy*A in one species maybe performed by *phy*B in another species. To better understand one aspect of phytochrome gene regulation the study of oat *phy*A mRNA turnover in transgenic plants was performed.

In some cases the elements that function to destabilize a message in one organism can function to destabilize a different message in a different organism. This suggests that there are some elements common to different organisms that function in a like manner. One example of this is the AU-rich element found in the 3' UTR of c-*myc* and c-*fos*. This element has been found to render a globin mRNA unstable in transgenic tobacco (Ohme-Takagi et al., 1993). The AU-rich element has been identified in a destabilized plant mRNA (Zhang and Mehdy, 1994). It seems possible, that RNA destabilization elements may be common to both monocotyledonous and dicotyledonous species.

Transgenic tobacco was used to investigate the possibility that a dicotyledonous system could be used to study the stability of an unstable monocotyledonous mRNA. This study was the first to measure oat *phy*A mRNA half-lives in transgenic tobacco and the first to transform tobacco with the full-length oat *phy*A gene.

In oats, *phy*A mRNA is relatively unstable with a half-life of about 60 minutes. Tobacco *phy*A mRNA was found to decrease by about 50% within four hours after exposure of seedlings to light. Transcriptional regulation of tobacco *phy*A mRNA has not been determined. If *phy*A transcription in tobacco is dramatically decreased by Pfr, this then implies a four hour half-life for tobacco *phy*A mRNA. In transgenic tobacco, the half-life of an oat *phy*A mRNA generated from an oat *phy*A gene containing both an incomplete 5' UTR and processing two introns in the 3'-UTR was found to be about four hours. The half-life of a full-length oat *phy*A mRNA in transgenic tobacco was also found to be about four hours. Tobacco may not recognize sequences in the oat *phy*A gene that are responsible for rendering the message unstable in oats. This lack of recognition may be attributable to differences among monocotyledonous and dicotyledonous plants. It is also possible that tobacco *phy*A

mRNA has a half-life of four hours and tobacco can recognize oat *phy*A mRNA as it does its own *phy*A message.

Analysis of RNA blots show oat *phy*A mRNA hybridizable fragments common to both the oat *phy*A mRNA in oats and in transgenic tobacco. These fragments were proposed to be putative degradation products indicative of a short-lived message (Seeley et al., 1992). The amount of oat *phy*A RNA fragments was found to vary with deletions from the 3' coding region of the gene. The half-life for the oat *phy*A RNA in transgenic plant line BPCE, the line with the lowest amount of oat *phy*A RNA fragments, was found to have the shortest half-life. These findings suggest that the abundance of the oat *phy*A fragments does not correlate with mRNA half-life.

Transgenic tobacco plants expressing the oat *phy*A gene have provided an opportunity to examine some of the physiology of *phy*A function. *Phy*A has been implicated as having a role in germination of tomato seedlings as demonstrated by the reduced germination of the *phy*A deficient *au* mutants (Georghiou and Kendrick, 1991). In *Arabidopsis* plants transformed with a *phy*B gene germination is increased (McCormac et al., 1993). *Arabidopsis* germination is not enhanced by increased amounts of *phy*A (McCormac et al., 1993). These results imply that germination in different species may by controlled by different *phy* genes.

Unlike work reported by McCormac et al. (1993) using tobacco transformed with the oat *phy*A gene, germination experiments performed here with transgenic tobacco implicate *phy*A as important in germination of tobacco seeds. Discrepancies between these two works is likely to be the result of the light conditions under which the seeds were matured. It underlines the importance of light quality in phytochrome experiments and demonstrates that tobacco seeds store phytochrome in their seeds in either the Pr or the Pfr form.

Finally, it is noteworthy that most weed species germinate in response to light. Light sensitivity is relevant from an agronomic perspective, it has been found that simply performing all field work at night will drastically reduce the number of weeds in a field (Hartmann and Nezadal, 1990). This suggests that many species store phytochrome in their seeds in the Pr form and germinate after photoconversion of Pr to Pfr following exposure to light. Most crop species have been bred to be light-insensitive with respect to germination. It is possible that the seeds of crop species have been selected for a signal transduction mechanism that over-rides the need for Pfr, or for storing Pfr in unimbibed seeds allowing them to germinate following imbibition while buried in the soil. In either case a study of the mechanism used by crop species to germinate in the absence of light could prove interesting.

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APPENDIX

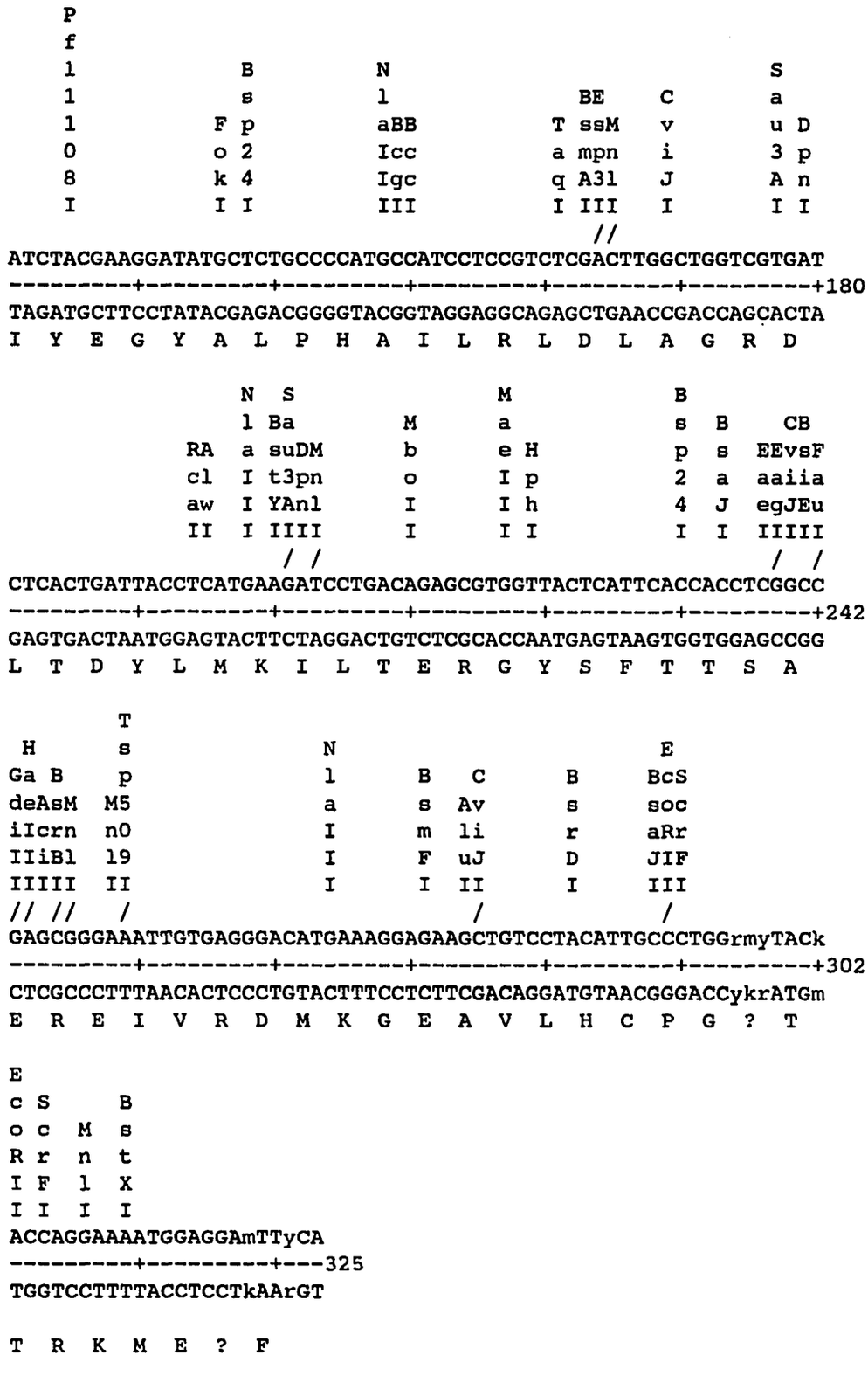
The following is a partial sequence of the cDNA clone pOA24. This is a partial actin clone from a cDNA library prepared from etiolated oat seedlings (Chapter 3). The soybean actin clone pSac3 (Hightower and Meagher, 1985) was used to screen the cDNA library for an actin clone.

The cDNA was sequenced twice from both its 5'-end and its 3'-end, the sequences obtained did not overlap, thus the entire sequence is not known. The sequences listed here are the consensus of the two sequencing runs. A nucleotide sequence comparison of pOA24 actin sequences from the 5'-end of the clone to other actin sequences from Genbank was performed. The results of these comparisons support the conclusion that pOA24 is an oat actin clone. Restriction mapping, determination of the protein sequence, and sequence comparisons were performed using the sequence analysis software package from The Genetics Computer Group Program Manual for the GCG Package, Version 7, April 1991, Madison, Wisconsin.

Nucleotide sequence, restriction map, protein sequence of the 5'-end of the pOA24 cDNA clone

```
                                    E     H
                          BM        c   SCa
                   F      Rca     B o BMcve                M   B
                   o      see     c R gwriI                n   s
                   k      afI     c I loFJI                l   r
                   I      III     I I IIIII                I   I
                                  /       // /
GAGACCTTCAACACTCCTGCTATGTACGTCGCCATCCAGGCCGTCCTCTCGCTGTATGCC
---------+---------+---------+---------+---------+---------+60
CTCTGGAAGTTGTGAGGACGATACATGCAGCGGTAGGTCCGGCAGGAGAGCGACATACGG
E  T  F  N  T  P  A  M  Y  V  A  I  Q  A  V  L  S  L  Y  A

                              H          B      C
          R                   iT         Bs     Hv        B    B
          s                   nf         cm     pi        c    c
          a                   fi         cF     hJ        g    c
          I                   II         II     II        I    I
                               /
AGTGGTCGTACCACAGGTATCGTGCTGGATTCTGGTGATGGTGTCAGCCACACTGTCCCC
---------+---------+---------+---------+---------+---------+120
TCACCAGCATGGTGTCCATAGCACGACCTAAGACCACTACCACAGTCGGTGTGACAGGGG
S  G  R  T  T  G  I  V  L  D  S  G  D  G  V  S  H  T  V  P
```


P
f
1 B N S
1 s l BE C a
1 F p aBB T ssM v u D
0 o 2 Icc a mpn i 3 p
8 k 4 Igc q A31 J A n
I I I III I III I I I
//
ATCTACGAAGGATATGCTCTGCCCCATGCCATCCTCCGTCTCGACTTGGCTGGTCGTGAT
+180
TAGATGCTTCCTATACGAGACGGGGTACGGTAGGAGGCAGAGCTGAACCGACCAGCACTA
I Y E G Y A L P H A I L R L D L A G R D
N S M B
l Ba M a s B CB
RA a suDM b e H p s EEvsF
cl I t3pn o I p 2 a aaiia
aw I YAnl I I h 4 J egJEu
II I IIII I I I I IIIII
/ / / /
CTCACTGATTACCTCATGAAGATCCTGACAGAGCGTGGTTACTCATTCACCACCTCGGCC
+242
GAGTGACTAATGGAGTACTTCTAGGACTGTCTCGCACCAATGAGTAAGTGGTGGAGCCGG
L T D Y L M K I L T E R G Y S F T T S A
T
H s N E
Ga B p l B C B BcS
deAsM M5 a s Av s soc
iIcrn nO I m li r aRr
IIiBl 19 I F uJ D JIF
IIIII II I I II I III
// // / / /
GAGCGGGAAATTGTGAGGGACATGAAAGGAGAAGCTGTCCTACATTGCCCTGGrmyTACk
+302
CTCGCCCTTTAACACTCCCTGTACTTTCCTCTTCGACAGGATGTAACGGGACCykrATGm
E R E I V R D M K G E A V L H C P G ? T
E
c S B
o c M s
R r n t
I F l X
I I I I
ACCAGGAAAATGGAGGAmTTyCA
+325
TGGTCCTTTTACCTCCTkAArGT
T R K M E ? F

Enzymes that do cut:

AciI	AluI	AlwI	BccI	BcefI	BcgI	BglI	BsaJI
BsiEI	BsmAI	BsmFI	Bsp24I	BsrI	BsrBI	BsrDI	BstXI
BstYI	CviJI	DpnI	EaeI	EagI	EcoRII	Esp3I	FauI
FokI	GdiII	HaeIII	HinfI	HphI	MaeII	MaeIII	MboII
MnlI	MwoI	NlaIII	Pfl1108I	RcaI	RsaI	Sau3AI	ScrFI
TaqI	TfiI	Tsp509I					

Enzymes that do not cut:

AatII	AccI	AflII	AflIII	AgeI	Alw21I	Alw44I	AlwNI
ApaI	ApaBI	ApoI	AscI	AvaI	AvaII	AvrII	BaeI
BamHI	BanI	BanII	BbsI	BbvI	Bce83I	BclI	BfaI
BglII	BpmI	Bpu10I	Bpu1102I	BsaI	BsaAI	BsaBI	BsaHI
BsaWI	BscGI	BseRI	BsgI	BsiI	BslI	BsmI	Bsp1286I
BspEI	BspGI	BspLU11I	BspMI	BsrFI	EsrGI	BssHII	Bst1107I
BstEII	Bsu36I	Cac8I	ClaI	CviRI	DdeI	DraI	DraIII
DrdI	DrdII	DsaI	EamI105I	EarI	EciI	Eco47III	Eco57I
EcoNI	EcoO109I	EcoRI	EcoRV	FseI	FspI	HaeI	HaeII
HgaI	HgiEII	HhaI	HincII	HindIII	HpaI	ItaI	KpnI
MluI	MmeI	MscI	MseI	MslI	MspI	MspA1I	MunI
NarI	NciI	NcoI	NdeI	NgoAIV	NheI	NlaIV	NotI
NruI	NsiI	NspI	NspV	PacI	PflMI	PleI	PmeI
PmlI	PshAI	Psp5II	Psp1406I	PstI	PvuI	PvuII	RleAI
RsrII	SacI	SacII	SalI	SapI	Sau96I	ScaI	SexAI
SfaNI	SfcI	SfiI	SgfI	SgrAI	SmaI	SnaBI	SpeI
SphI	SrfI	Sse8387I	SspI	StuI	StyI	SunI	SwaI
TaqII	TaqII	ThaI	Tsp45I	Tth111I	Tth111II	VspI	XbaI
XcmI	XhoI	XmnI					

Nucleotide sequence identity of 5'end of the pOA24 cDNA to other actin clones listed in genebank:

Organism	Plasmid or Cell Type	Accession Number	% Nucleotide Identity
Oryza sativa	RAc1 for actin	X16280	92.2% identity in 295 bp overlap
Oryza sativa	cell-type callus	D21122	70.3% identity in 155 bp overlap
Zea mays	Mac1 for actin	J01238	78.6% identity in 220 bp overlap
Nicotiana tobaccum	Tac9 for pollen specific actin	X69885	78.8% identity in 222bp overlap
Pisum sativum	actin	X67666	80.0% identity in 295 bp overlap
Sacchromyces carlsbergensis	pYacb-2,	L0025	68.8% identity in 295 bp overlap
Sacchromyces cerevisiae	pYA208	L00026	69.8% identity in 295 bp overlap
Glycine max	pSac3 (genomic clone)	J01297	75.7% identity in 251 bp overlap
Glycine max	pSac1	J01298	62.8% identity in 43 bp overlap

Nucleotide sequence, restriction map of the 3'-end of the pOA24 cDNA clone

```
              T
              s                      H
              p       NB             i         C      C
M   M         5    B  ls           SAnT        v      vB
n   n         0    a  ae           acca        i      is
l   l         9    n  IR           lcIq        J      Jr
I   I         I    I  VI           IIII        I      II
                                     /
AACGACGGAGGAGGTAATTGGCACCTGTTTCAAAAGTCGACCTGATAGCCACTGGCTAAA
---------+---------+---------+---------+---------+---------+60
TTGCTGCCTCCTCCATTAACCGTGGACAAAGTTTTCAGCTGGACTATCGGTGACCGATTT

                                                       M
                                                       s
                                                       e
                                                       I
CAATGATAAGTTTTTTTCTGACATTATAGATAACGATGACCACGAACAGTGAACATTAAA
---------+---------+---------+---------+---------+---------+120
GTTACTATTCAAAAAAAGACTGTAATATCTATTGCTACTGGTGCTTGTCACTTGTAATTT
```

```
                    T
                    s                S          S
          C         p              ARa  B       a
          v       ND5      A       vsuA s       u D
          i       sro      c       ar9c r       3 p
          J       ea9      c       II6I B       A n
          I       III      I       IIII I       I I
                                     //
ACCTGTGTTATACAAGCCAAGTAAGTTTAAATTAGTCTACGGACCGCTCCGACGATCTCT
---------+---------+---------+---------+---------+---------+180
TGGACACAATATGTTCGGTTCATTCAAATTTAATCAGATGCCTGGCGAGGCTGCTAGAGA

                                  T
                                  t          T
                                  h          s
                                  1          p
          M               R       A 1        5
          m               s       c 1        0
          e               a       c I        9
          I               I       I I        I
TTTTATGCCTACCACAAGACCAAACAGTACACAGTAGACATAGAACAATTTACTTATTAT
---------+---------+---------+---------+---------+---------+240
AAAATACGGATGGTGTTCTGGTTTGTCATGTGTCATCTGTATCTTGTTAAATGAATAATA

         T
         S
         P
        A5
        P0
        O9
        II
         /
AGTTCACAGAATTTTTTTTTTTT
---------+---------+----264
TCAAGTGTCTTAAAAAAAAAAAA
```

Enzymes that do cut:

AccI	AciI	ApoI	AvaII	BanI	BseRI	BsrI	BsBI
CviJi	DpnI	DraI	HincII	MmeI	MnlI	MseI	NlaIV
RsaI	RsrII	SalI	Sau96I	Sau3AI	TaqI	Tsp509I	Tth111I

Enzymes that do not cut:

AatII	AflII	AflIII	AgeI	AluI	AlwI	Alw21I	Alw44I
AlwNI	ApaI	ApaBI	AscI	AvaI	AvrII	BaeI	BamHI
BanII	BbsI	BbvI	BccI	Bce831	BcefI	BcgI	BclI
BfaI	BgII	BglII	BpmI	Bpu10I	Bpu1102I	BsaI	BasAI
BsaBI	BsaHI	BsaJI	BsaWI	BscGI	BsgI	BsiI	BsiEI
BslI	BsmI	BsmAI	BsmFI	Bsp24I	Bsp1286I	BspEI	BspGI
BspLU11I	BspMI	BsrDI	BsrFI	BsrGI	BssHII	Bst1107I	BstEII
BstXI	BstYI	Bsu36I	Cac8I	ClaI	CviRI	DdeI	DraIII
DrdI	DrdII	DsaI	EaeI	EagI	Eam1105I	EarI	EciI
Eco47III	Eco57I	EcoNI	Eco0109I	EcoRI	EcoRII	EcoRV	Esp31
FauI	FokI	FseI	FspI	GdiII	HaeI	HaeII	HaeIII
HgaI	HgiEII	HhaI	HindIII	HinfI	HpaI	HphI	ItaI
KpnI	MaeII	MaeIII	MboII	MluI	MscI	MslI	MspI
MspA1I	MunI	MwoI	NarI	NciI	NcoI	NdeI	NgoAIV
NheI	NlaIII	NotI	NruI	NsiI	NspI	NspV	PacI
Pfl1108I	PlfMI	PleI	PmeI	PmlI	PhAI	Psp5I	Psp1406I
PstI	PvuI	PvuII	RcaI	RleAI	SacI	SacII	SapI
ScaI	ScrFI	SexAI	SfaNI	SfcI	SfiI	SgfI	SgrAI
SmaI	SnaBI	SpeI	SphI	SrfI	Sse8387I	SspI	StuI
StyI	SunI	SwaI	TaqII	TfiI	ThaI	Tsp45I	Tth111I
VspI	Xbai	XcmI	Xho	XmnI			