This is to certify that the
dissertation entitled

INHERENT AND REGULATED MRNA STABILITY IN *A. THALIANA*

presented by

Rodrigo Antonio Gutiérrez

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Biochemistry and Molecular Biology

Major Professor's Signature

December 16, 2002

Date

DATE DUE	DATE DUE	DATE DUE

INHERENT AND REGULATED MRNA STABILITY IN *A. THALIANA*

By

Rodrigo Antonio Gutiérrez

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Graduate Program in Biochemistry and Molecular Biology

2003

ABSTRACT

INHERENT AND REGULATED MRNA STABILITY IN *A. THALIANA*

By

Rodrigo Antonio Gutiérrez

mRNA degradation provides a powerful means for controlling gene expression during growth, development and many physiological transitions in plants and other systems. Enormous advances have been made in the understanding of the mRNA decay process, particularly related to the control of rapid transcript turnover. However there is still limited knowledge regarding the nature and associations of genes with unstable transcripts from a genomic perspective, or the physiological significance of rapid mRNA turnover in intact organisms. To address these questions, cDNA microarray technology was applied to identify and characterize genes with unstable transcripts in *Arabidopsis thaliana* (*AtGUTs*). At least 1% of the 11,521 clones represented on Arabidopsis Functional Genomics Consortium microarrays correspond to transcripts that are rapidly degraded, with estimated half-lives of less than 60 min. Analysis of public microarray expression data for these genes indicates that mRNA instability is of high significance during plant responses to mechanical stimulation and is associated with specific genes controlled by the circadian clock. Control of mRNA stability has often been proposed as a component of circadian gene expression. However there is no direct evidence that the rate of mRNA turnover can be regulated by the circadian clock in plants or any other

system. In this dissertation it was shown that mRNA stability for two *AtGUTs*, *Ccr*-like and *SEN1*, changes throughout the day and that this change is commanded by the clock. Furthermore, it was found that *DST1* gene function was required for the normal diurnal oscillation of these genes. The evidence obtained indicates a previously unknown connection between expression of clock-controlled genes and the DST-mediated mRNA decay pathway. In contrast to the current understanding of rapid mRNA decay, little is known regarding the determinants for the long half-life of extremely stable transcripts in plants or other systems. One of the reasons that the study of stable mRNAs has lagged behind studies of unstable transcripts in plants, is that current methods are not well suited for the study of long-lived mRNAs. The significance in plants of the best known stability determinant, the pyrimidine-rich sequence from the human α-globin transcript, was evaluated. This and a related synthetic sequence were fused to reporter transcripts and expressed in tobacco cell cultures, maize cell cultures and in Arabidopsis plants. The results obtained indicate that these sequences are not recognized as stability elements in plants. To facilitate identification of plant stability elements, and to improve future studies of stable as well as unstable transcripts in plants, a novel regulated promoter system that allows for transcriptional-pulse type experiments in Arabidopsis was developed. This system makes use of the natural transcriptional regulatory properties of the *At-EXPL1* gene. The promoter of this gene is reproducibly and transiently induced producing a synchronized population of transcripts that can be monitored overtime. This tool should be useful for the study of specific aspects of the mRNA degradation process of unstable as well as stable transcripts in Arabidopsis

To Julia, mother of father, my inspiration and model of perseverance.

To my family, thank you all for your patience and support

A Julia, madre de padre, mi inspiración y modelo de perseverancia.

A mi familia,gracias por la paciencia y el apoyo.

ACKNOWLEDGMENTS

Many thanks are due at the end of this adventure. But because my memory is fragile and because I want to get rid of this manuscript "asap" I apologize up front for the names I will never print in these two pages.

First in my mind, I would like to thank my advisor Dr. Pam Green, for her superb guidance throughout my doctoral program at the MSU-DOE Plant Research Laboratory. For helping me discern what was most scientifically interesting, for giving me freedom to pursue all my ideas, and especially for the time spent to improve my presentation and writing skills. *"A gene codes for"* or a *"Gene encodes"* not a *"Gene encode for"*... just one of the many suggestions that are now part of my vocabulary (or certainly hope so) and English thinking process.

I want to thank all past and present members of the Green lab which I was fortunate to interact with: Dr. Yukako Chiba, Dr. Jay de Rocher, Dr. Scott Diehn, Linda Danhof, Dr. Michael Feldbrügge, Dr. Mark Johnson, Dr. Jim Kastenmayer, Preet Lidder, Dr. Nikki LeBrasseur, Dr. Gustavo MacIntosh, Dr. Miguel Pérez and Dr. Ambro van Hoof. For great discussions, comments, and for making the "Green lab" a stimulating and thought provoking environment for research. I want to especially acknowledge Dr. Scott Diehn for teaching me the basic RNA techniques when I started in the lab and his guidance during my rotation. I would also like to thank Dr. Mark Johnson, Dr. James Kastenmayer, and Dr. Ambro van Hoof for critical comments of the two manuscripts that were published during this dissertation. I want to thank Linda Danhof, for providing all the lab basics, keeping everything running smooth, and for excellent technical support. I

should also especially acknowledge the patience of Nikki and Preet, for bearing with the "drumming" that went on during my incubations, spins, precipitations, reading, writing, thinking, etc. in the back room. I would also like to thank the members of the Arabidopsis Functional Genomics Consortium for technical advice during my microarray experiments and especially Dr. Robert Schaffer, Dr. Jeff Landgraf and Dr. Ellen Wisman for great discussions.

I want to thank the members of my PhD guidance committee: Dr. Christoph Benning, Kenneth Keegstra, Michael Thomashow and Steven Triezenberg; for great comments and professional advice during my PhD studies.

I would like to thank Dr. Tom Newman for providing and sequencing EST clones. I would also like to thank our collaborators, Dr. Rob Ewing and Dr. Mike Cherry for assistance in the sequence analysis of *AtGUTs* by the oligomer counting method.

I also want to thank everybody in the Plant Research Laboratory, for sharing your knowledge and skills whenever I needed it. This is a great scientific community and I have benefited greatly from being part of it. I also want to thank the Plant Research Laboratory staff for making paperwork, ordering, mailing, meeting and course registrations, reimbursements, etc. so much easier.

I also want to thank many people in the Biochemistry and Molecular Biology Department, for also sharing knowledge and research advice whenever I needed it. And I want to thank the Biochemistry and Molecular Biology staff for facilitating my visa paperwork, keeping track of my PhD requirements etc.

I want to thank the Graduate School for the financial support that help me attend the course "Bioinformatics: Writing Software for Genome Research" at the Cold Spring Harbor Laboratory.

Last but definitely not least, I want to thank my beautiful wife Maite, for her tremendous support and patience during these years. Thanks for helping me go through the difficult times and for celebrating the successes. Thanks also to mom, dad, brothers... all my family in Chile, which despite disagreeing on the benefits of being so far away, lend me unconditional support throughout my PhD.

I would also like to acknowledge the funding agencies that made possible this research, grants from the DOE (DE-FG02-91ER20021), USDA (9801498), USDA (2000-01491), NSF (DBN987638), NSF (IBN9408052) and Michigan State University Research Excellence Fund to Dr. Pam J. Green.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AFGC : Arabidopsis Functional Genomics Consortium

AREs : Adenylate/uridylate-rich elements

At-EXPL1 : *Arabidopsis thaliana* expansin-like gene 1

AtGUT : *Arabidopsis thaliana* gene with unstable transcript

CCG : Clock-controlled gene

cDNA : Complementary DNA

CT : Circadian time

DNA : Deoxyribonucleic acid

DST : Downstream element

GTB2 : quality control parameter that indicates the fraction of pixels in one spot in a microarray slide that have intensity values 1.5 times the background.

MEME : Multiple Expectation Maximization for Motif Elicitation

mRNA : Messenger RNA

ORF : Open reading frame

RNA : Ribonucleic Acid

SMD : Stanford Microarray Database

UTR : Untranslated region.

ZT : Zeitgeber (time-giver) time.

CHAPTER 1

mRNA stability in plants and genomic approaches to study mRNA decay[I]

[I] Part of this chapter was published in "Gutiérrez, R.A., MacIntosh, G.C, and Green P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. *Trends Plant Sci* 4:429-438".

Normal growth and development as well as the ability to adjust to ever changing environmental conditions requires the carefully regulated expression of many genes. Although much of this regulation is exerted at the transcriptional level, post-transcriptional mechanisms also play a fundamental role. For some genes, post-transcriptional mechanisms constitute the predominant form of control in response to a given stimulus. In other cases, an extra level of modulation is provided by post-transcriptional control that increases the flexibility and speed of responses beyond what could be achieved through transcriptional regulation alone. The control of mRNA stability is one of the most prominent forms of post-transcriptional regulation in eukaryotic cells. The stability of a particular mRNA influences its steady-state levels and directly affects the rate of its induction or repression following a change in transcription. Thus, a thorough understanding of how mRNA stability is controlled is essential to elucidate how the abundance of endogenous mRNAs is governed and to optimize the accumulation of transgene mRNAs in plants for biotechnological applications.

As illustrated in Figure 1.1, the molecular components that control mRNA stability can be considered in three layers. Recent work in yeast indicates that eukaryotic cells contain RNA-degrading activities and protein cofactors that appear to constitute the general/basal mRNA decay machinery, responsible for the degradation of most stable and unstable mRNAs. Superimposed on this basal machinery are the sequence-specific controls that dictate the inherent stability of various mRNAs, the half-lives of which can vary over a wide range. For those transcripts whose stability changes in response to exogenous or endogenous stimuli, a third layer of control must be evoked. This last layer would transduce the signals elicited by various stimuli into changes in mRNA turnover.

Theoretical Hierarchy

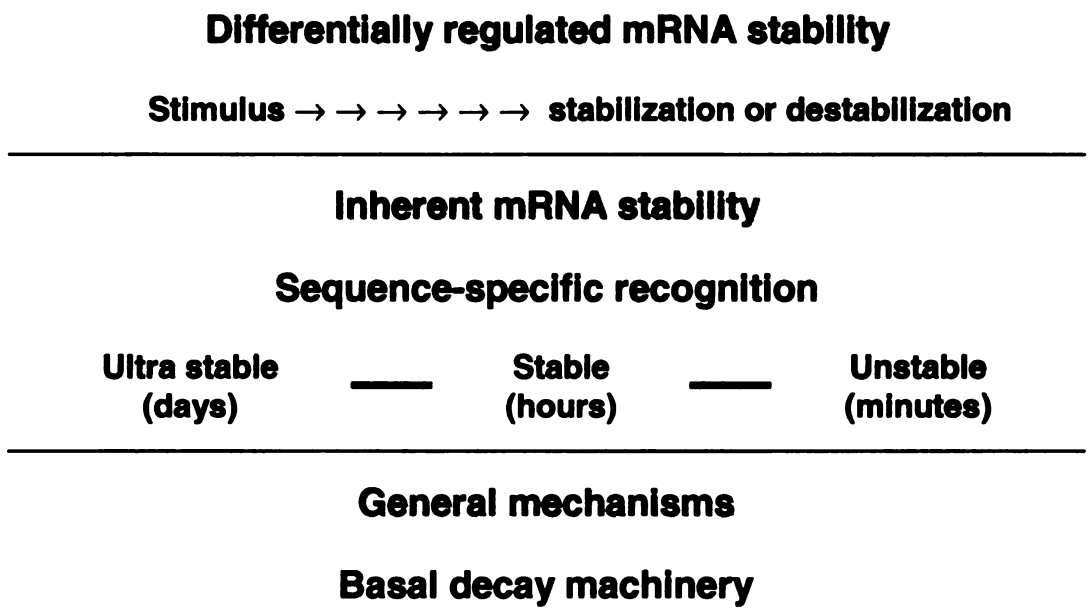


Figure 1.1. A conceptual framework of mRNA stability in eukaryotic cells. The molecular components that control mRNA stability can be considered in three inter-related layers. According to this framework, the underlying layer contains RNA-degrading activities and protein cofactors that constitute the general/basal mRNA decay machinery, responsible for the degradation of most mRNAs. Superimposed on this basal machinery are the sequence-specific components, represented by the second layer, that dictate the inherent stability of different mRNAs. mRNA half-lives (indicated in parenthesis) can vary over a wide range, with the average on the order of hours. The third layer of control would facilitate the transduction of signals into changes in mRNA turnover to adjust the stability of transcripts in response to exogenous or endogenous stimuli.

In this conceptual framework, investigation of all three layers is critical because of their individual importance and the probable inter-relationships among them. For example, differential control of the stability of a particular mRNA could be mediated by modulating the activity of a sequence-specific recognition factor, which interacts with the basal decay machinery. In this dissertation however, attention will be devoted to the two uppermost layers.

Most recent progress in our understanding of mRNA stability in plants has emerged from studies of sequence-specific recognition of transcripts for rapid and/or regulated decay. This chapter will highlight current knowledge for those nuclear encoded transcripts that have been studied in the most detail. Readers are referred to several previous reviews for more comprehensive presentations of the mRNA stability and post-transcriptional control literature in plants (Gallie, 1993; Abler and Green, 1996; Johnson *et al.*, 1998) and other eukaryotes (Ross, 1995; McCarthy, 1998; Mitchell and Tollervey, 2000; Tucker and Parker, 2000; Wilusz *et al.*, 2001).

Molecular determinants of mRNA stability

The decay rate of transcripts in plants seems to be similar to those observed in other multicellular eukaryotes. Half-lives range from less than an hour for unstable messages, to days or more for stable transcripts with the average being on the order of several hours (Johnson *et al.*, 1998; Taylor and Green, 1995). The decay rates of some transcripts can be rather dynamic, being modulated by the coordinated integration of internal and external stimuli. What then, are the molecular determinants that control the half-life of a particular transcript at any time in the cell? In recent years, research has mainly focused on the identification and characterization of structural features of the mRNA molecule, or *cis*-acting elements, that influence mRNA decay rates. These studies have shown that general structural elements found at the ends of virtually all mRNAs, as well as specific sequence elements located within a transcript, can all contribute to the overall stability.

In addition to their role as translational enhancers, the 7-methyl-G cap at the 5' end and the polyadenylate (poly(A)) tail at the 3' end have been shown to increase mRNA stability in transient assays (Gallie, 1998). By electroporating capped or uncapped mRNAs, and mRNAs with or without poly(A) tails into tobacco protoplasts, it was found that the 5' cap stabilizes reporter transcripts by two- to four-fold and the poly(A) tail by two- to three-fold (Gallie, 1991). Although it is yet unclear how the cap and poly(A) tail protect a transcript from degradation, an appealing model is that the physical interaction between the cap and poly(A) tail, via their associated factors (*e.g.* poly(A) binding protein, eIF4G, eIF4E, eIF4B) would sequester the ends of the mRNA protecting them from the action of nucleases (Gallie, 1998).

Within the body of the mRNA, specific sequence motifs that are only present in a subset of transcripts can act constitutively to establish the inherent instability (or stability) of a particular transcript or they can modulate the stability of an mRNA in response to certain physiological, developmental or environmental cues. Major examples of both classes of stability determinant are discussed in the following sections. Although they are presented separately, it should be noted that the division is organizational rather than biological. Some sequences that appear to affect mRNA decay rates constitutively may be later found to be regulated under special conditions. Conversely, regulatory sequences may also contribute to inherent stability in the absence of stimuli. In any event, the characterization of these sequences is already leading to mechanistic insights as to how they are recognized in the cell and how that recognition may be controlled.

A. *SAUR 15A* DST element

(GGAgactgac**ATAGATT**ggaggagacAt**TTtGTA**taata)$_2$

B. *SAUR-AC1* 3'UTR

AGTACTATACTACAACATTTCCATATTTTTTTTAGATTGTTAGCTAATTT

CCCCTGGAGATAATTGTAAATTGTTTCAATGAGAGGAATATACAATA

CATAGATCGTAATTGATCAATGCGTATTTGCATGTT

Figure 1.2. The DST sequence element. (A) The prototype DST element derived from the soybean *SAUR 15A* gene is shown as DNA sequence. Highly conserved residues across different species are shaded and invariant residues underlined. Mutational analysis identified important residues (bold) for DST function (see text for detail). (B) The *SAUR-AC1* 3'UTR shown as DNA sequence includes one DST element (shaded) and several ATAGAT-like (underlined) and GTA-like (upper bar) subdomains that may contribute to its instability function.

Sequence elements that control inherent mRNA stability

The DST element. The DST or downstream element was originally identified as a conserved region in the 3' untranslated region (UTR) of the unstable small auxin-up RNA (*SAUR*) transcripts (McClure and Guilfoyle, 1989). It consists of three highly conserved subdomains separated by two variable regions (Figure 1.2*a*). When a synthetic dimer of the soybean *SAUR-15A* DST sequence was placed in the 3'UTR of a reporter transcript, its turnover was significantly faster than that of a spacer or no-insert control in BY-2 cells (Newman *et al.*, 1993). Subsequent mutational analysis indicated that two conserved subdomains, designated ATAGAT and GTA regions after the invariant nucleotides they contain, are critical for DST function. Five- and six-base substitutions in

the ATAGAT and GTA regions respectively, resulted in slower turnover rates in BY-2 cells and higher reporter transcript accumulation in transgenic tobacco plants (Sullivan and Green, 1996). Two base substitution mutations within these two subdomains indicated that the first four bases of the ATAGAT subdomain are critical for instability function in tobacco cell culture. Interestingly, a 2-base substitution in the GTA subdomain inactivated DST function in transgenic tobacco leaves but not in cell culture. This finding suggests that the DST element may be differentially recognized in different cell types (Sullivan and Green, 1996).

Detailed studies of *SAUR* gene expression in *Arabidopsis thaliana* have been carried out on the *SAUR-AC1* gene. By examining the expression of chimeric genes it was shown that the promoter region of the gene is responsible for auxin induction and that sequences downstream of the promoter limit mRNA accumulation in an auxin-independent manner (Gil and Green, 1996). Measurements of the half-lives of the transcripts encoded by chimeric genes showed that the 3'UTR acts as a potent mRNA instability determinant (Gil and Green, 1996) (Figure 1.2*b*). Interestingly, the *SAUR-AC1* 3'UTR contains one canonical DST element and several ATAGAT-like and GTA-like subdomains that may contribute to instability of the mRNA (Figure 1.2*b*). This is intriguing since in previous work two copies of the prototype DST element from *SAUR-15A* were needed to cause instability of a reporter transcript (Newman *et al.*, 1993). Further studies will be necessary to investigate the contribution of particular sequences to the instability of *SAUR-AC1* mRNA, and the importance of context for DST element function.

The novel structure of DST sequences suggests that they may mediate mRNA decay through a pathway that is unique to plants. In an effort to address this issue, reporter

transcripts with and without DST sequences were expressed in NIH3T3 fibroblasts (Feldbrügge *et al.*, 2002). The presence of the DST tetramer accelerated deadenylation and decay rate of the reporter mRNA in the mammalian cells as compared to control transcripts lacking DST sequences. However a tetramer soybean DST element mutated in the ATAGAT domain, which is inactive in tobacco cells, was equally active as the wild-type version in fibroblasts (Feldbrügge *et al.*, 2002). Also contrary to what expected, two reporter transcripts containing different versions of the Arabidopsis DST sequence element decayed no faster than a control transcript lacking DST sequences. Together these results indicate that recognition of the DST sequence element in mammalian cells follow different rules as compared to plants cells.

To understand the molecular mechanisms underlying DST function, a genetic strategy was devised to isolate mutants defective in DST-mediated mRNA degradation (Johnson *et al.*, 2000). Two independent mutants were isolated, *dst1* and *dst2*, that showed elevated mRNA levels of a hygromycin phosphotransferase and a β-glucuronidase mRNA each containing four copies of the DST element (HPH-DSTx4 and GUS-DSTx4 respectively) (Johnson *et al.*, 2000). The *dst1* and *dst2* mutants also exhibited elevated mRNA levels of the endogenous *SAUR-AC1* gene (Johnson *et al.*, 2000). In addition, decay of HPH-DSTx4, GUS-DSTx4 and *SAUR-AC1* mRNAs was slower in the *dst* mutants as compared to parental. Because no morphological or developmental defects were apparent, DNA microarray analysis was used to investigate the molecular phenotypes of the *dst1* mutant (Pérez-Amador *et al.*, 2001). Eighteen of the approximately 7800 genes represented on the Arabidopsis Functional Genomics Consortium (AFGC) DNA microarray exhibited increased mRNA levels when

comparing *dst1* mutant and parental plants. In addition, seven genes with decreased levels in *dst1* as compared to the parental plants were also identified. Seven of these twenty five genes contained DST-like sequences in their 3' UTR indicating they might be primary targets of the DST-mediated decay pathway (Pérez-Amador *et al.*, 2001). Surprisingly, eight out of the twenty five genes were regulated by the circadian clock suggesting a connection between circadian rhythms and the *dst1* mutation (Pérez-Amador *et al.*, 2001). This number is higher than what expected based on current estimates of the total number of clock-controlled genes in Arabidopsis. DNA microarray experiments have shown that 2 to 6% of Arabidopsis mRNAs can oscillate (Harmer *et al.*, 2000; Schaffer *et al.*, 2001). The relationship between this sequence-specific mRNA decay pathway and circadian control of gene expression in Arabidopsis is the subject of the Chapter 3 of this dissertation. Efforts to clone the *dst1* gene are currently underway (Lidder & Green unpublished results).

AUUUA-repeats. Adenylate/uridylate-rich elements (AREs) represent a common determinant of RNA stability in mammalian cells. Transcripts that contain AREs are selectively targeted for rapid decay (Chen and Shyu, 1995). AREs are approximately 50-150 nucleotides long, usually contain multiple copies of the AUUUA motif and a high content of uridine, and are located in the 3'UTR of mRNAs encoding a variety of proto-oncoproteins, cytokines and transcription factors (Chen and Shyu, 1995). Accordingly AUUUA sequences play important roles in the post-transcriptional regulation of gene expression during processes such as cell growth, differentiation, the immune response, etc. in mammalian systems. Due to the significance of AUUUA elements in mammals, a

synthetic AUUUA repeat was tested for the ability to act as instability determinant in plants. Reporter transcripts containing 11 repeats of the AUUUA motif in their 3'UTRs were degraded much more rapidly in stably transformed tobacco cells and accumulated to a lower level in transgenic tobacco plants than those of control constructs (Ohme-Takagi *et al.*, 1993) . The effect appeared to be AUUUA-specific because two other sequences with the same size and A+U content had no effect in parallel experiments. These results suggest that the mRNA decay pathway mediated by AUUUA repeats is conserved between animals and plants. However, the natural targets of the plant AUUUA-mediated decay pathway remain to be identified. Possible candidates include the *PvPRP1* transcript from *Phaseolus vulgaris*, *αAmy3* transcript from *Oryzae sativa* (discussed below) and three genes from Arabidopsis (discussed in Chapter 2). Recent experiments have shown that ARE-mediated decay is also present in the yeast *Saccharomyces cerevisiae* (Vasudevan and Peltz, 2001). Together these data suggest that ARE-mediated decay is conserved in eukaryotes, from yeast to mammals.

Nonsense codons. Premature nonsense codons decrease mRNA stability by activating nonsense-mediated decay pathways in several eukaryotic systems. The yeast nonsense-mediated decay pathway, discussed further below, is one of the best understood at the molecular and genetic levels. Nonsense mediated decay is presumably part of a mRNA surveillance system that rapidly removes abnormal mRNAs to prevent the formation of truncated or otherwise potentially detrimental polypeptides (Hilleren and Parker, 1999; Culbertson, 1999).

Initial evidence that nonsense-mediated mRNA decay occurred in plants came from studies conducted on natural alleles of the soybean Kunitz trypsin inhibitor (Jofuku *et al.*, 1989) and bean phytohemagglutinin A (PHA) genes (Voelker *et al.*, 1986). Cells containing alleles with early stop codons accumulate low levels of mRNA (Jofuku *et al.*, 1989; Voelker *et al.*, 1986) even when transcriptional rates were normal (Jofuku *et al.*, 1989). Nonsense mediated decay was also found responsible for the reduced mRNA accumulation of two mutant alleles of the *WAXY* gene in rice (Isshiki *et al.*, 2001). Generally nonsense codons affect mRNA abundance in a position-dependent manner, the closer to the initiation codon the greater the likelihood of affecting mRNA abundance. The effect of stop codons positioned at variable distance from the translation start codon of a reporter gene was directly addressed using the initially isolated PHA allele and other PHA alleles constructed *in vitro* (van Hoof and Green, 1996). By measuring mRNA decay rates in stably transformed tobacco cell lines it was demonstrated that transcripts with nonsense codons positioned 20, 40 and 60% of the way through the normal coding region yielded highly unstable mRNAs, whereas a transcript with a nonsense codon at 80% was as stable as wild type. These findings strongly support the idea that plants have a nonsense-mediated decay pathway similar to that found in other eukaryotes.

Differential control of mRNA stability

Light modulation. Light regulation at the post-transcriptional level has been well documented for the pea photosynthetic electron carrier *ferredoxin I* (*Fed-1*) gene (for a review see Dickey *et al.*, 1998). As with many other photosynthetic genes, *Fed-1* expression is induced by light, mRNA levels being five-fold higher in the light than in

darkness. When mRNA decay rates were measured, a two-fold higher mRNA half-life was observed for the transcript in light versus darkness in transgenic tobacco seedlings demonstrating that light regulation occurs through a change in mRNA stability (Petracek *et al.*, 1998). A sequence element was identified within the transcribed region that could confer light responsiveness to a reporter gene under the control of a constitutive promoter. This internal light regulatory element (iLRE), spans a portion of the 5' UTR and the first 20 codons of the coding region (Dickey *et al.*, 1998). The observations that *Fed-1* light-induced mRNA accumulation correlates with its polyribosomal association, prompted a model in which efficient translation of *Fed-1* in the light is associated with increased mRNA stability (Dickey *et al.*, 1998). This model was also initially argued based on the observation that nonsense mutations, which block ribosomal progression, abrogate *Fed-1* mRNA accumulation in response to light (Dickey et al., 1994; Dickey *et al.*, 1998). However as discussed above, nonsense mutations trigger rapid mRNA degradation through the nonsense mediated decay pathway in plants (van Hoof and Green, 1996). More recent studies have indeed found that normal degradation of *Fed-1* mRNA in the dark occurs through a pathway that is different from that mediated by the presence of nonsense mutations (Petracek et al., 2000). Further mutation analysis of the iLRE identified two regions that are critical for its function, a CATT repeat in the 5' UTR and the translation initiation region (Dickey *et al.*, 1998). Two different substitution mutations were made within the CATT repeat that blocked *Fed-1* mRNA accumulation, one of which affected ribosome loading (Dickey *et al.*, 1998). The simplest explanation of these studies is that *Fed-1* mRNA is stable in illuminated plants when associated with polyribosomes. In darkness, inefficient translation renders the transcript less stable

through a process involving the CATT repeat located in the 5' portion of the message (Dickey *et al.*, 1998). In an effort to identify *trans*-acting factors that mediate the regulation of *Fed-1* mRNA translation and stability, proteins that bind the iLRE were isolated (Ling *et al.*, 2000). Among several RNA binding activies that were found to associate with the iLRE, the heat shock protein HSP101 was identified. HSP101 was required to achieve high translation activity of iLRE containing reporter transcripts in yeast (Ling *et al.*, 2000). In addition, reporter transcripts containing iLRE were more efficiently translated in plant protoplasts expressing HSP101 than control transcripts (Ling *et al.*, 2000). This data suggest HSP101 might be important for the light-mediated translational regulation of *Fed-1*. Further studies should help determine whether the CATT region is a stability or instability determinant, as well as the exact mechanistic relationship between mRNA stability and translation.

Sucrose regulation. α-Amylases are endo-amylolytic enzymes, which catalyze the hydrolysis of α-1,4 linked glucose polymers and have an important role in degradation of starch in higher plants. The expression of the rice α-amylase gene family is coordinately induced by sucrose starvation and suppressed by sucrose availability, a process that depends on both transcriptional and post-transcriptional mechanisms (Sheu *et al.*, 1996). The sucrose-mediated effect on mRNA stability has been analyzed in detail for one of the most abundant α-amylase genes, *αAmy3*. When mRNA decay rates were measured, the half-life of *αAmy3* was about 1.5 h in the presence of sucrose and increased to 6 h in sucrose-starved cells (Sheu *et al.*, 1996). By examining the expression of chimeric genes in stably transformed rice cells it was shown that the *αAmy3* 3'UTR was sufficient and

probably the major determinant for controlling the stability of *αAmy3* mRNA in response to sucrose availability (Chan and Yu, 1998b; Chan and Yu, 1998a). Further analysis of the 3'UTR identified two subdomains, called I and III, that could each function as a sugar-dependent stability determinant (Chan and Yu, 1998b; Chan and Yu, 1998a). In addition, secondary structure analysis predicted extensive duplex formation in the *αAmy3* 3'UTR, and interestingly, conserved A/U rich regions were found in the loop of subdomains I and III (Chan and Yu, 1998b). Whether these A/U rich regions or the structural motifs that contain them are involved in modulation of mRNA stability in response to sucrose levels remains to be elucidated. Moreover, as in other cases of regulated mRNA stability, it is not yet clear whether a trans-acting factor slows down the turnover of the transcript in the presence of sucrose or speeds up the turnover in its absence. Treatment with the translation inhibitor cycloheximide enhanced the accumulation of *αAmy3* transcript in the presence or absence of sucrose (Sheu *et al.*, 1996). In contrast, cycloheximide did not significantly affect transcriptional rates of *αAmy3* regardless of whether or not the cells were provided with sucrose (unpublished results cited in (Sheu *et al.*, 1996)). These observations might suggest that labile proteins are involved in *αAmy3* mRNA decay. However cycloheximide may interfere with the normal decay of the message in other ways, *e.g.* translation of the message may be required for degradation to take place.

Methionine regulation. Cysthatione-γ-synthase (CGS) catalyzes the first committed step of the biosynthesis of the amino acid methionine (Met) and is thought to be the major site of regulation for the pathway in plants (Ravanel *et al.*, 1998). Regulation of CGS occurs

primarily at the level of gene expression and not through metabolic control of the enzyme activity (reviewed in (Ravanel *et al.*, 1998)). To elucidate the molecular mechanism underlying regulation of Met biosynthesis, Chiba *et al.* (1999) characterized a mutant that accumulates high levels of soluble Met (Inaba *et al.*, 1994). This *mto1-1* mutant also showed high levels of CGS mRNA, protein and enzyme activity as compared to wild-type (Chiba *et al.*, 1999). Wild-type plants respond to Met addition by destabilizing CGS mRNA. However degradation of CGS mRNA in the *mto1-1* mutant background was slower than in the wild-type and was not affected by the presence of Met (Chiba *et al.*, 1999). Sequence analysis of the *mto1-1* and four other alleles of the *mto1* locus revealed single base changes that altered the amino acid sequence in the N-terminus of the CGS protein (Chiba *et al.*, 1999). The region that contained these mutations (MTO1) was necessary and sufficient to confer Met responsiveness to reporter transcripts in transient expression experiments (Chiba *et al.*, 1999). In addition, silent mutations in the MTO1 region did not affect Met response indicating that the nucleotide sequence is not important for the regulation. Detailed analysis of the first exon of CGS identified the sequence (A)RRNCSNIGVAQ(I) (with uncertainty in the first and last position) as required for the feedback regulation mediated by Met (Ominato *et al.*, 2002). Interestingly, the decrease in CGS mRNA levels following addition of the amino acid correlated with accumulation of a mRNA decay intermediate truncated at the 5'end (Chiba *et al.*, 1999). Together, this evidence suggests a mechanism in which translation of the first exon of CGS in the presence of Met, including the (A)RRNCSNIGVAQ(I) sequence, would destabilize its own mRNA generating a 3' end fragment (Chiba *et al.*, 1999). A similar model has been proposed for autoregulation of the β-tubulin gene in

mammalian systems by unassembled β-tubulin subunits (Theodorakis and Cleveland, 1993).

Biotic stress. One of the best examples of modulation of mRNA stability in response to biotic stress (commonly a result of infection from bacteria, fungi or viruses) has been characterized in common bean cells. Fungal elicitor treatment of bean cells results in down-regulation of the PvPRP1 gene, which encodes a cell wall proline-rich protein32. Direct proof that the major control mechanism of this down-regulation is modulation of mRNA stability was provided by the observation that PvPRP1 mRNA half-life in the presence of the elicitor was shorter than in its absence. Moreover transcriptional rates remained constant regardless of the presence or absence of the elicitor (Zhang *et al.*, 1993). Subsequent studies identified a 50 kDa protein (PRP-BP) that can be specifically crosslinked to the 3' UTR of the *PvPRP1* transcript (Zhang and Mehdy, 1994). Using deletion analysis, the binding site for PRP-BP was mapped to a 27 nt, U-rich site that contains one copy of the AUUUA motif. It remains to be demonstrated that this binding site is important for the regulation of transcript stability. Nevertheless the observation that *PvPRP1* mRNA degradation, in response to fungal elicitor treatment, was preceded by increased PRP-BP binding activity in bean cells suggests that this protein and the *cis*-element it binds are involved in the regulation (Zhang and Mehdy, 1994). PRP-BP activity *in vitro* was increased by the reducing agents DTT or β-mercaptoethanol and reversibly eliminated with the –SH oxidizing agent diamide or the –SH alkylating agent N-methylmaleimide (Zhang and Mehdy, 1994). The defense response in bean and many other species is accompanied by production of active oxygen species and other redox

perturbations. Hence, these observations suggest that PRP-BP binding activity could be modulated by the redox changes that take place during the plant defense response (Mehdy and Brodl, 1998; Zhang and Mehdy, 1994).

Other stimuli. Hormones play an indisputable role in the regulation of a multitude of physiological and developmental processes in plants. Although it is clear that hormones can influence gene expression at both transcriptional and post-transcriptional levels, a detailed understanding of the molecular basis of hormone action, especially at the post-transcriptional level is lacking. One recent example of hormonal regulation of mRNA stability arose during a study of cytokinin effects on the soybean mRNA Cim135. The predicted Cim1 protein product is related to a group of proteins termed β-expansins, which are involved in cell wall expansion during the vegetative and/or reproductive phases of plant development. *Cim1* mRNA abundance increases 20-60-fold upon addition of cytokinin to cytokinin-starved soybean suspension cultures. When half-life of the *Cim1* mRNA was determined following actinomycin D treatment, cytokinin addition to cytokinin-starved soybean cells increased the mRNA half-life of *Cim1* about 4-fold (Downes and Crowell, 1998). Further experiments were aimed to characterize the role of protein phosphorylation/dephosphorylation in cytokinin-mediated induction of *Cim1*. It was observed that accumulation of *Cim1* message was stimulated by staurosporine (kinase inhibitor) in the absence of cytokinin and inhibited by okadaic acid (phosphatase inhibitor) in the presence of cytokinin. These results suggest a role for protein phosphatases in cytokinin regulation of *Cim1* mRNA abundance (Downes and Crowell, 1998).

In addition to the examples described above, a number of other mRNAs have been reported to show modulation of mRNA stability in response to biotic stress, abiotic stress (*e.g.* cold, heat, salinity), hormone treatments, etc. In the majority of these cases, conclusions have been drawn after finding a poor correlation between the rate of transcription and mRNA accumulation in response to the stimulus. However most of this research is still at a preliminary stage and the mechanisms through which this modulation is achieved have not yet been reported (for reviews see (Gallie, 1993; Johnson *et al.*, 1998; Mehdy and Brodl, 1998; Marcotte, 1998)).

Stable mRNAs in plants

Although it is clear that unstable mRNAs contain instability sequences, no discrete stabilizing determinant has been demonstrated to be responsible for the long half-life of an extremely stable transcript in plant systems. The search for mRNA stabilization sequences has lagged behind those of destabilizing elements in eukaryotes in general, but at least one example has been well characterized in mammals. Studies aimed at understanding the mechanism for the selective stabilization of the α-globin message during erythroid cell development, identified a pyrimidine-rich sequence in the 3'UTR that was responsible for the long half-life of this transcript (reviewed in (Russell *et al.*, 1997)). This finding negates a previous idea that all mRNAs are stable by default rather than by the presence of stabilizing sequences. Further, it makes it likely that stabilizing sequences exist in other systems as well. Specific mRNA sequences could, for example, contribute to the stability of seed storage protein mRNAs (Marcotte, 1998), as in the case of wild cultivars of oat (Johnson *et al.*, 1999), perhaps by influencing their

compartmentalization. Studies conducted in these and other plant systems may provide insights into the mechanisms of mRNA stabilization. The identification of the sequence elements, and *trans*-acting factors they interact with, as well as understanding the mechanisms of their function could provide tools to improve transgene expression in crop plants, and would certainly contribute to a more complete understanding of mRNA metabolism.

Genomic approaches for the study of mRNA decay

While enormous advances have been made in understanding the mRNA decay process and the determinants of mRNA stability, basic questions remain unanswered. For example, how many unstable transcripts are there in the cell? What is the physiological significance of rapid mRNA turnover? Further, most of what we now know about the *cis*- and *trans*-acting factors involved in mRNA decay derives from studies on a relatively small group of model genes. Therefore the relevance of these studies for the whole cell or intact organism is unclear.

In an effort to address these questions several groups have developed genome-scale approaches to study mRNA degradation in *E. coli* (Bernstein *et al.*, 2002; Selinger *et al.*, 2002), *S. cerevisiae* (Wang *et al.*, 2002) and *A. thaliana* (Gutiérrez *et al.*, 2002), discussed in Chapter 2 of this dissertation). Experimentally all these studies combine time courses performed after general transcription is arrested with the highly parallel power of microarray technology. Thus it is possible to monitor genome-wide mRNA disappearance over time. The experimentally measured decline in mRNA levels can then be used to estimate the stability of each of the transcripts represented on the array.

New insights into mRNA stability derived from microarray studies

The first indication that this approach held promise came from a study carried out in Richard Young's laboratory in 1998 (Holstege *et al.*, 1998). Holstege *et al.* (1998) used oligonucleotide arrays to analyze global mRNA levels in yeast carrying mutations in components of the yeast transcription initiation machinery. In one of their experiments, inactivation of the thermo-sensitive RNA polymerase II in the *rpb1-1* mutant background (Nonet *et al.*, 1987) allowed them to estimate half-lives for more than 5000 yeast mRNAs (http://web.wi.mit.edu/young/expression/) (Holstege *et al.*, 1998).

More recently, Bernstein *et al.* (2002) used spotted DNA microarrays to study mRNA degradation in *Escherichia coli*. Rifampicin was used to prevent initiation of new transcripts in cells growing at 30 °C in LB and M9 + 0.2% glucose media. Overall, the mRNA half-life distributions were similar in the two growth conditions, despite a 3-fold slower generation time in the M9 + glucose media. mRNA half-lives ranged from 1-2 min for the most unstable to 10 min or more for the most stable with approximately 80% of all mRNAs exhibiting half-lives between 3 and 8 min. An exponential decay model was used to fit the time-series data and only those with a regression coefficient greater than 0.7 were considered for further studies. This and other filtering criteria allowed half-life measurements for 3835 transcripts with a mean of 5.7 min in M9 + glucose and 2267 transcripts with a mean of 5.2 min in LB media. Several aspects of the transcripts measured were analyzed. Interestingly and contrary to what was anticipated, mRNA stability was found to be a poor predictor of mRNA abundance (Bernstein *et al.*, 2002). Although there were cases in which stable mRNAs did accumulate to high levels, no

positive correlation between transcript stability and abundance was observed. In an effort to understand the determinants of mRNA stability in *E. coli*, structural features were also inspected. The enzyme RNaseE catalyzes the rate-limiting endonucleolytic cleavage that initiates decay of many mRNAs in *E. coli* (Coburn and Mackie, 1999). However the density of putative sites of cleavage by RNaseE was not predictive of mRNA stability. Secondary structures are known to slow the action of mRNases in *E. coli* (Bouvet and Belasco, 1992; Grunberg-Manago, 1999), but the free energy of folding, G/C content, or length of 5' or 3' UTR sequences could not be correlated with mRNA half-lives. Further, no correlation could be detected between half-lives and open reading frame (ORF) length, operon length or codon usage. Interestingly, despite the fact that stability did not correlate with obvious molecular characteristics it seemed to be similar in transcripts that encode related metabolic functions. For example, mRNAs encoding amino acid synthesis or macromolecule synthesis/modification functions showed shorter half-lives than average, whereas those belonging to the cell-envelope maintenance or recycling of small molecules category showed half-lives longer than average.

mRNA decay rates appear to be related to physiological function in a second study in *E. coli* (Selinger *et al.*, 2002). Oligonucleotide arrays containing on average one 25-mer probe every 30 bp throughout the entire bacterial genome were used to measure stability of transcripts corresponding to 1036 ORFs and 329 operons. The drug rifampicin was used to shut-off transcription in bacteria cultures grown in LB at 37 °C. In accordance to the assumed first order kinetic of mRNA degradation, the intensity of most mRNAs studied decreased exponentially over time with an average mRNA half-life of 6.8 min. In this study, translation and post-translational modification functions were

under-represented in the group of labile mRNAs. In contrast, transcripts that encode putative enzymes were significantly over-represented among short lived mRNAs. Furthermore, genes involved in energy metabolism were over-represented among transcripts with half-lives between 10 and 20 min (Selinger *et al.*, 2002). The high resolution of the oligonucleotide microarrays used in this study allowed mechanistic aspects of mRNA degradation in *E. coli* to be examined. Disappearance of the 5'UTR, 3'UTR and three equally spaced internal regions was monitored over time in operons with 2 ORFs or more. Consistent with current models of mRNA degradation in bacteria (Coburn and Mackie, 1999), 5' ends of operons degraded on average more quickly than the rest of the transcript with stability increasing in the 3' direction. Interestingly, hierarchical clustering of the decay patterns for 149 operons indicated that this pattern predominates but it is not the only one. Alternatives to the 5' to 3' model of bacterial mRNA degradation have been postulated (Coburn and Mackie, 1999). This approach should greatly aid in evaluating the significance of the different pathways of mRNA degradation and identifying the cellular targets in bacteria.

The past year was also fruitful for global mRNA stability studies in eukaryotic systems. Wang *et al.* (2002) reported the use of spotted DNA microarrays to investigate mRNA decay rates in the yeast *Saccharomyces cerevisiae*. In this study, mRNA synthesis was halted by thermal inactivation of the temperature sensitive RNA polymerase II (*rpb1-1*) (Nonet *et al.*, 1987). Similar to bacteria, exponential decay was a good model to explain mRNA disappearance in yeast and allowed half-lives of 4687 yeast transcripts to be determined. The half-life measurements ranged from ~3 to 90 min and showed a global mean value of 23 min. Several features of the mRNAs measured were investigated,

but no simple correlation was observed between mRNA half-lives and ORF size, codon bias, ribosome density or mRNA abundance. However consistent with the data in bacteria, coordination of mRNA decay rates and gene function was apparent. The mRNAs encoding components of cellular complexes such as the nucleosome core, the 20S proteasome core, the ribosome or the trehalose phosphate synthase complex, showed strikingly similar turnover rates. This observation was extended to 95 other complexes analyzed emphasizing the remarkable coordination of mRNA decay in yeast. Coordination of the decay of yeast mRNAs was also observed in more broadly related physiological functions. For example, transcripts encoding enzymes that participate in the central systems of energy metabolism (glycolysis / gluconeogenesis, the tricarboxylic acid cycle and the glyoxylate cycle) were among those that live the longest. In contrast, transcripts encoding the proteins of the mating pheromone signal transduction pathway turned over relatively rapidly.

Lam *et al.* (2001) used a specialized lymphocyte array (spotted DNA microarray) to estimate mRNA stabilities in lymphoid cell cultures. The initial objective of this study was to determine the mode of action of the anti-cancer drug flavopiridol. It was found that flavopiridol inhibited general transcription in the cell cultures, presumably by inhibiting the transcription elongation factor P-TEFb (Price, 2000) in a similar fashion as 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB). The effect of flavopiridol on mRNA levels was comparable to that obtained with DRB or the DNA interchalating agent actinomycin D. Thus Lam *et al.* (2001) used flavopiridol to stop transcription and determine the mRNA half-lives of 2794 mammalian mRNAs. Although the gene sample analyzed was biased towards lymphocyte-related functions, some of the conclusions

reached in this study echoed those of other systems. The great majority of the well-measured transcripts decreased in abundance with first order kinetics after transcription inhibition. In addition, association between mRNA stability and gene function was also observed in the mammalian cell cultures studied. Transcripts encoding apoptosis regulators often decayed rapidly, as did mRNAs coding for several key cell-cycle regulators. AREs are among the best characterized instability sequences in eukaryotes and are often found in labile mRNAs encoding proto-oncoproteins, cytokines and transcription factors (Chen and Shyu, 1995). The relationship between the number of transcripts containing AREs and mRNA stability was investigated. The number of ARE-containing mRNAs increased as the mRNA stability decreased. However, the great majority of unstable transcripts lack AREs and 10% of stable mRNAs contained ARE sequences. Hence AREs are not predictive of rapid mRNA turnover. As in other systems, additional unknown transcript features should contribute to the individual mRNA stabilities observed.

mRNA decay studies with microarray technology have also been conducted in *Arabidopsis thaliana* (Gutiérrez *et al.*, 2002). What we have learned in Arabidopsis, to date the only effort in an intact multicellular eukaryote, is the foundation of this doctoral dissertation and will be discussed in depth in Chapter 2.

Common themes and new trends in mRNA decay

The coupling of global transcriptional shut-off assays with DNA microarray analysis has been a successful approach for monitoring mRNA decay on a global basis. The few

studies reported to date support basic notions of mRNA degradation. It is generally assumed that mRNA degradation, like radioactive decay, is a stochastic process. Therefore the change in mRNA concentration at any time is a first-order process, that depends only on the amount of mRNA present at the time (Brawerman, 1993; Ross, 1995; Caponigro and Parker, 1996). Based on the data obtained in bacteria, yeast and mammalian cell cultures, it now appears that first order decay kinetics is indeed a good approximation to model mRNA disappearance of most cellular transcripts after transcription ceases. Detailed analysis of individual time-series should help identify the exceptions to this rule. Characterization of the features of transcripts with unusual decay kinetics should provide additional insight into the mRNA degradation process.

The studies in yeast and bacteria are in agreement with the main pathways of mRNA decay. By comparing global decay rates of oligo(dT) and random primer labeled mRNA samples, Wang *et al.* (2002) showed that 3' ends are more labile on average than the body of mRNAs. This evidence supports the two major pathways of mRNA degradation in yeast (Caponigro and Parker, 1996). In addition, Selinger *et al.* (2002) showed that disappearance of most operons with 2 ORFs or more proceeded in a 5' to 3' direction consistent with the major model of mRNA degradation in bacteria (Coburn and Mackie, 1999). These results indicate that microarray studies should be useful to address mechanistic aspects of mRNA degradation. Such an application would be particularly attractive for less characterized organisms. Perhaps sub-genic resolution affymetrix experiments as those described by Selinger *et al.* (2002) could be used to identify transcripts with stable decay intermediates. Analysis of the decay intermediates should be instrumental in dissecting the steps in the corresponding decay pathways.

The genome-wide studies described have also challenged previous notions about mRNA stability. The view that mRNA abundance is directly correlated with mRNA stability has not been supported in the studies in bacteria, yeast or plants. Direct measurements of mRNA abundance in bacteria and indirect estimates based on fluorescent intensity in yeast and plants (Chapter 2) indicate that mRNA stability is a poor predictor of mRNA abundance. Because mRNA levels are determined primarily by the balance between the rate of synthesis and degradation (Hargrove *et al.*, 1991), this data implies that transcription plays a predominant role in determining mRNA steady-state levels. This data also suggest that the regulation of mRNA half-life may have an alternative biological significance. For example as illustrated in Chapter 2 for touch-controlled genes, a role for rapid mRNA turnover might be to allow rapid and transient changes in transcript abundance in response to environmental cues. Alternatively, and as illustrated in Chapter 3, regulation of mRNA stability might be essential to achieve precise expression patterns that are not possible through transcriptional regulation alone.

An interesting genome-wide property that emerged from these studies is the coordination of transcript stability based on functional and physiological associations. Overall, long-lived mRNAs seem to be involved in central metabolic functions whereas those involved in regulatory systems turn over relatively rapidly. At least in yeast, this association goes beyond broad physiological relationships and seems important in ensuring proper expression of the components of stoichiometric complexes such as the ribosome.

Future prospects

Microarray technology has already proved to be a valuable tool to study post-transcriptional regulation of gene expression in various model organisms. But the use of this approach for the study of mRNA degradation and post-transcriptional processes in general is in its infancy. New applications for this approach can be easily foreseen. For example, determining mRNA turnover rates over different developmental, environmental or other treatments should help us evaluate the extent and significance of this mechanism of regulation. In addition, the role of new candidate regulators of the mRNA degradation process as well as components of the decay machinery could be readily addressed by comparing mRNA turnover rates in KO mutants and wild-type. Because mRNAs will decay with slower rates in the relevant KO mutants, this analysis should help categorize transcripts on the basis of their decay strategy.

The exciting corollary of these studies is that much remains to be learned about mechanisms of mRNA degradation in biological systems, for example regarding the structural features that determine the stability of individual transcripts. It is likely that the next years will see more applications of this tool to address the questions posed. Detailed knowledge of this level of regulation is necessary to better comprehend the complex gene expression program in plants and other systems.

References

Abler,M.L. and Green,P.J. (1996). Control of mRNA stability in higher plants. Plant Mol. Biol. *32*, 63-78.

Bernstein,J.A., Khodursky,A.B., Lin,P.H., Lin-Chao,S., and Cohen,S.N. (2002). Global analysis of mRNA decay and abundance in Escherichia coli at single-gene resolution using two-color fluorescent DNA microarrays. Proc. Natl. Acad. Sci. USA *99*, 9697-9702.

Bouvet,P. and Belasco,J.G. (1992). Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in E. coli. Nature *360*, 488-491.

Brawerman,G. (1993). mRNA degradation in eukaryotic cells:an overview. In Control of Messenger RNA Stability, J.Belasco and G.Brawerman, eds. Academic Press, Inc. San Diego, CA), pp. 149-160.

Caponigro,G. and Parker,R. (1996). Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol. Rev. *60*, 233-249.

Chan,M.T. and Yu,S.M. (1998a). The 3 ' untranslated region of a rice alpha-amylase gene functions as a sugar-dependent mRNA stability determinant. Proc. Natl. Acad. Sci. USA *95*, 6543-6547.

Chan,M.T. and Yu,S.M. (1998b). The 3 ' untranslated region of a rice alpha-amylase gene mediates sugar-dependent abundance of mRNA. Plant J. *15*, 685-695.

Chen,C.Y.A. and Shyu,A.B. (1995). AU-rich elements - Characterization and importance in messenger-RNA degradation. T. Biochem. Sci. *20*, 465-470.

Chiba,Y., Ishikawa,M., Kijima,F., Tyson,R.H., Kim,J., Yamamoto,A., Nambara,E., Leustek,T., Wallsgrove,R.M., and Naito,S. (1999). Evidence for autoregulation of cystathionine gamma-synthase mRNA stability in Arabidopsis. Science *286*, 1371-1374.

Coburn,G.A. and Mackie,G.A. (1999). Degradation of mRNA in Escherichia coli: An old problem with some new twists. Prog. Nucleic Acid Res. Mol. Biol.Vol. 62 *62*, 55-108.

Culbertson,M.R. (1999). RNA surveillance - unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet. *15*, 74-80.

Dickey,L.F., Nguyen,T.T., Allen, G.C. and Thompson,W.F. (1994). Light modulation of Federroxin mRNA abundance requires an open reading frame. Plant Cell *6*, 1171-1176.

Dickey,L.F., Petracek,M.E., Nguyen,T.T., Hansen,E.R., and Thompson,W.F. (1998). Light regulation of Fed-1 mRNA requires an element in the 5 ' untranslated region and correlates with differential polyribosome association. Plant Cell *10*, 475-484.

Downes,B.P. and Crowell,D.N. (1998). Cytokinin regulates the expression of a soybean beta-expansin gene by a post-transcriptional mechanism. Plant Mol. Biol. *37*, 437-444.

Feldbrügge M., Arizti, P., Sullivan, M.L., Zamore, P.D., Belasco, J.G. and Green, P.J. (2002). Comparative analysis of the plant mRNA-destabilizing element, DST, in mammalian and tobacco cells. Plant Mol. Biol.*49*, 215-223.

Gallie,D.R. (1991). The cap and poly(A) tail function synergistically to regulate messenger-RNA translational efficiency. Genes Dev. *5*, 2108-2116.

Gallie,D.R. (1993). Posttranscriptional regulation of gene-expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. *44*, 77-105.

Gallie,D.R. (1998). A tale of two termini: A functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. Gene *216*, 1-11.

Gil,P. and Green,P.J. (1996). Multiple regions of the *Arabidopsis SAUR-AC1* gene control trascript abundance: the 3' untranslated region functions as an mRNA instability determinant. EMBO J. *15*, 1678-1686.

Grunberg-Manago,M. (1999). Messenger RNA stability and its role in control of gene expression in bacteria and phages. Annu. Rev. Genet. *33*, 193-227.

Gutiérrez,R.A., Ewing,R.M., Cherry,J.M., and Green,P.J. (2002). Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: rapid decay is associated with a group of touch- and specific clock-controlled genes. Proc. Natl. Acad. Sci. U. S. A *99*, 11513-11518.

Hargrove,J.L., Hulsey,M.G., and Beale,E.G. (1991). The kinetics of mammalian gene-expression. Bioessays *13*, 667-674.

Harmer,S.L., Hogenesch,J.B., Straume,M., Chang,H.S., Han,B., Zhu,T., Wang,X., Kreps,J.A., and Kay,S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science 290 , 2110-2113.

Hilleren,P. and Parker,R. (1999). Mechanisms of mRNA surveillance in eukaryotes. Annu. Rev. Genet. *33*, 229-260.

Holstege,F.C.P., Jennings,E.G., Wyrick,J.J., Lee,T.I., Hengartner,C.J., Green,M.R., Golub,T.R., Lander,E.S., and Young,R.A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Inaba,K., Fujiwara,T., Hayashi,H., Chino,M., Komeda,Y., and Naito,S. (1994). Isolation of an Arabidopsis thaliana Mutant, mto1, That Overaccumulates Soluble Methionine (Temporal and Spatial Patterns of Soluble Methionine Accumulation). Plant Physiol. *104*, 881-887.

Isshiki,M., Yamamoto,K., Satoh,H., and Shimamoto,K. (2001). Nonsense-mediated decay of mutant waxy mRNA in rice. Plant Physiol. *125*, 1388-1395.

Jofuku,K.D., Schipper,R.D., and Goldberg,R.B. (1989). A frameshift mutation prevents kunitz trypsin-inhibitor messenger-RNA accumulation in soybean embryos. Plant Cell *1*, 427-435.

Johnson,M.A., Baker,E.J., Colbert,J.T., and Green,P.J. (1998). Determinants of mRNA stability in plants. In A look beyond transcription: Mechanisms determining mRNA stability and translation in plants, J.Bailey-Serres and D.R.Gallie, eds. (Rockville: American Society of Plant Physiologists Press), pp. 40-53.

Johnson,M.A., Pérez-Amador,M.A., Lidder,P., and Green,P.J. (2000). Mutants of Arabidopsis defective in a sequence-specific mRNA degradation pathway. Proc. Natl. Acad. Sci. U. S. A *97*, 13991-13996.

Johnson,R.R., Chaverra,M.E., Cranston,H.J., Pleban,T., and Dyer,W.E. (1999). Degradation of oat mRNAs during seed development. Plant Mol. Biol. *39*, 823-833.

Lam,L.T., Pickeral,O.K., Peng,A.C., Rosenwald,A., Hurt,E.M., Giltnane,J.M., Averett,L.M., Zhao,H., Davis,R.E., Sathyamoorthy,M., Wahl,L.M., Harris,E.D., Mikovits,J.A., Monks,A.P., Hollingshead,M.G., Sausville,E.A., and Staudt,L.M. (2001). Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. Genome Biol. *2*, 1-11.

Ling,J., Wells,D.R., Tanguay,R.L., Dickey,L.F., Thompson,W.F., and Gallie,D.R. (2000). Heat shock protein HSP101 binds to the Fed-1 internal light regulatory element and mediates its high translational activity. Plant Cell *12*, 1213-1227.

Marcotte,W.R. (1998). Developmental regulation of translation and mRNA stability. In A look beyond transcription: Mechanisms determining mRNA stability and translation in plants, J.Bailey-Serres and D.R.Gallie, eds. American Society of Plant Physiologists), pp. 64-67.

McCarthy,J.E.G. (1998). Posttranscriptional control of gene expression in yeast. Microbiol. Rev. *62*, 1492-1553.

McClure,B.A. and Guilfoyle,T. (1989). Rapid redistribution of auxin-regulated RNAs during gravitropism. Science *243*, 91-93.

Mehdy,M.C. and Brodl,M.R. (1998). The role of stress in regulating mRNA stability. In A look beyond transcription: Mechanisms determining mRNA stability and translation in plants, J.Bailey-Serres and D.R.Gallie, eds. American Society of Plant Physiologists), pp. 54-63.

Mitchell,P. and Tollervey,D. (2000). mRNA stability in eukaryotes. Curr. Opin. Genet. Dev. *10*, 193-198.

Newman,T.C., Ohme-Takagi,M., Taylor,C.B., and Green,P.J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell *5*, 701-714.

Nonet,M., Scafe,C., Sexton,J., and Young,R. (1987). Eukaryotic RNA-polymerase conditional mutant that rapidly ceases messenger-RNA synthesis. Mol. Cell. Biol. *7*, 1602-1611.

Ohme-Takagi,M., Taylor,C.B., Newman,T.C., and Green,P.J. (1993). The effect of sequences with high AU content on mRNA stability in tobacco. Proc. Natl. Acad. Sci. USA *90*, 11811-11815.

Ominato,K., Akita,H., Suzuki,A., Kijima,F., Yoshino,T., Yoshino,M., Chiba,Y., Onouchi,H., and Naito,S. (2002). Identification of a short highly conserved amino acid sequence as the functional region required for Posttranscriptional autoregulation of the cystathionine gamma-synthase gene in Arabidopsis. J. Biol. Chem. *277*, 36380-36386.

Pérez-Amador,M.A., Lidder,P., Johnson,M.A., Landgraf,J., Wisman,E., and Green,P.J. (2001). New molecular phenotypes in the dst mutants of Arabidopsis revealed by DNA microarray analysis. Plant Cell *13*, 2703-2717.

Petracek,M.E., Dickey,L.F., Nguyen,T.T., Gatz,C., Sowinski,D.A., Allen,G.C., and Thompson,W.F. (1998). Ferredoxin-1 mRNA is destabilized by changes in photosynthetic electron transport. Proc. Natl. Acad. Sci. USA *95*, 9009-9013.

Price,D.H. (2000). P-TEFb, a Cyclin-Dependent Kinase Controlling Elongation by RNA Polymerase II. Mol. Cell. Biol. *20*, 2629-2634.

Ravanel,S., Gakiere,B., Job,D., and Douce,R. (1998). The specific features of methionine biosynthesis and metabolism in plants. Proc. Natl. Acad. Sci. U. S. A *95*, 7805-7812.

Ross,J. (1995). Messenger-RNA stability in mammalian-cells. Microbiol. Rev. *59*, 423-450.

Russell,J.E., Morales,J., and Liebhaber,S.A. (1997). The role of mRNA stability in the control of globin gene expression. Prog. Nucleic Acid Res. Mol. Biol. Vol 57 *57*, 249-287.

Schaffer,R., Landgraf,J., Accerbi,M., Simon,V., V, Larson,M., and Wisman,E. (2001). Microarray Analysis of Diurnal and Circadian-Regulated Genes in Arabidopsis. Plant Cell 13, 113-123.

Selinger, D. W., Saxena, R. M., Cheung, K. J., Church, G. M., and Rosenow, C. Global RNA half-life analysis in Escherichia coli reveals positional patterns of transcript degradation. Genome Res. 2002. In Press

Sheu,J.J., Yu,T.S., Tong,W.F., and Yu,S.M. (1996). Carbohydrate starvation stimulates differential expression of rice alpha-amylase genes that is modulated through complicated transcriptional and posttranscriptional processes. J. Biol. Chem. *271*, 26998-27004.

Sullivan,M.L. and Green,P.J. (1996). Mutational analysis of the DST element in tobacco cells and transgenic plants: Identification of residues critical for mRNA instability. RNA *2*, 308-315.

Taylor,C.B. and Green,P.J. (1995). Identification and characterization of genes with unstable transcripts (*GUT*s) in tobacco. Plant Mol. Biol. *28*, 27-38.

Theodorakis,N.G. and Cleveland,D.W. (1993). Translationally coupled degradation of tubulin mRNA. In Control of Messenger RNA Stablilty, J.Belasco and G.Brawerman, eds. Academic Press, Inc. San Diego, CA), pp. 219-238.

Tucker,M. and Parker,R. (2000). Mechanisms and control of mRNA decapping in Saccharomyces cerevisiae. Annu. Rev.Biochem. *69*, 571-595.

van Hoof,A. and Green,P.J. (1996). Premature nonsense codons decrease the stability of phytohemagglutinin mRNA in a position-dependent manner. Plant J. *10*, 415-424.

Vasudevan, S. and Peltz, S.W. (2001). Regulated ARE-mediated mRNA decay in Saccharomyces cerevisiae. Mol. Cell 7, 1191-1200.

Voelker,T.A., Staswick,P., and Chrispeels,M.J. (1986). Molecular analysis of 2 phytohemagglutinin genes and their expression in *Phaseolus-vulgaris* cv pinto, a lectin-deficient cultivar of the bean. EMBO J. *5*, 3075-3082.

Wang,Y.L., Liu,C.L., Storey,J.D., Tibshirani,R.J., Herschlag,D., and Brown,P.O. (2002). Precision and functional specificity in mRNA decay. Proc. Natl. Acad. Sci. USA *99*, 5860-5865.

Wilusz,C.J., Wormington,M., and Peltz,S.W. (2001). The cap-to-tail guide to mRNA turnover. Nature Rev. Mol. Cell Biol. *2*, 237-246.

Zhang,S.Q. and Mehdy,M.C. (1994). Binding of a 50-kD protein to a U-rich sequence in an messenger-RNA encoding a proline-rich protein that is destabilized by fungal elicitor. Plant Cell *6*, 135-145.

Zhang,S.Q., Sheng,J.S., Liu,Y.D., and Mehdy,M.C. (1993). Fugal elicitor-induced bean proline-rich protein messenger-RNA down-regulation is due to destabilization that is transcription and translation dependent. Plant Cell *5*, 1089-1099.

CHAPTER 2

Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: Rapid decay is associated with a group of touch- and specific clock-controlled genes[II]

[II] The original form of this chapter was published in "Gutiérrez , R.A., Ewing R.M., Cherry, J.M., and Green, P.J. (2002). Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: rapid decay is associated with a group of touch- and specific clock-controlled genes. *Proc. Natl. Acad. Sci. U.S.A.* 99: 11513-11518".

Introduction

Regulation of the stability of mRNAs is an important process in the control of gene expression. This point is perhaps most evident in the wide range of half-lives that is typically observed for nuclear encoded transcripts. In plants, similar to what reported in mammalian systems, half-lives of mRNAs span several orders of magnitude. Unstable messages have half-lives of less than 60 minutes, very stable ones on the order of days, with the average being on the order of several hours (Siflow and Key, 1979; Hargrove *et al.*, 1991).

Most research has emphasized the study of unstable mRNAs. These transcripts have attracted attention because they often code for regulatory functions that are important for growth and development. For example, mRNAs known to be highly unstable include those for the transcription factors *c-myc* and *c-fos* in mammalian cells (Greenberg and Belasco, 1993) and the mating-type transcripts in yeast (Peltz and Jacobson, 1992). In plants, transcripts that fall into this category include the mRNAs for photo-labile phytochrome (Seeley *et al.*, 1992) and several auxin-inducible transcripts (McClure and Guilfoyle, 1989; Koshiba *et al.*, 1995). The instability of these mRNAs facilitates fast changes in mRNA levels that result in transient and tightly controlled gene expression (Treisman, 1985).

Previous work on unstable transcripts has concentrated on the identification of sequence elements and *trans*-acting factors that regulate the stability of individual or small groups of transcripts. In eukaryotic cells, transcripts destabilized by multiple overlapping of AUUUA sequences or other AU-rich elements (AREs) located in 3'

untranslated regions (UTRs), have been a major focus (Shaw and Kamen, 1986; Ohme-Takagi *et al.*, 1993; Chen and Shyu, 1995; Vasudevan and Peltz, 2001). Several proto-oncogene, cytokine, and transcription factor mRNAs involved in growth and differentiation are recognized for rapid decay via AREs (Shaw and Kamen, 1986; Ohme-Takagi *et al.*, 1993; Chen and Shyu, 1995; Vasudevan and Peltz, 2001). In plants, one of the best characterized instability sequences is the DST or downstream element (McClure *et al.*, 1989; Newman *et al.*, 1993). This instability determinant is found in the 3'UTR of the small auxin up RNA (*SAUR*) genes. DST elements have a complex structure (Sullivan and Green, 1996) and the recognition requirements appear to be unique to plants (Feldbrügge *et al.*, 2002). Other sequences that cause instability have also been described (Ross, 1995; Caponigro and Parker, 1996; Gutiérrez *et al.*, 1999). Nevertheless, the number of structural features identified to date that target transcripts for rapid decay is relatively modest, and many more are likely to be discovered (Taylor and Green, 1995).

Although study of individual transcripts is a viable avenue to address this problem, genomic-scale analysis is necessary to evaluate the nature of unstable transcripts within an organism and the regulatory associations they share. Genomic approaches using DNA microarrays have emphasized the study of mRNA levels and how are those levels affected under different conditions (Brown and Botstein, 1999; Schaffer *et al.*, 2000), but they have been rarely applied to the study of post-transcriptional processes. The first indication that this approach held promise came from data presented in a web site (http://web.wi.mit.edu/young/expression/) referred to in Holstege *et al.* (Holstege *et al.*, 1998) that estimated stabilities of yeast mRNAs. More recently, Lam *et al.* (Lam *et al.*, 2001) used a specialized lymphocyte array to estimate mRNA stabilities

in lymphoid cell cultures. Both investigations suggested that global analysis of mRNA stability in intact multi-cellular organisms should be feasible and more revealing.

In this study, we examined mRNA degradation in intact Arabidopsis plants using cDNA arrays containing more than 11,000 clones. Similar to the situation in other organisms, the identity and percentage of unstable transcripts in plants had not been evaluated on this scale. Our analysis indicated that at least 1% of the transcripts represented on our arrays decayed with half-lives of less than 60 minutes. Further, we identified specific functional and regulatory associations among groups of unstable mRNAs that provide insight into the biological significance of rapid mRNA decay mechanisms.

Materials and Methods

Half-life measurements and preparation of RNA samples.

Half-lives were determined as described by Seeley *et al.* (Seeley *et al.*, 1992) with the following modifications. *Arabidopsis thaliana* ecotype Columbia were grown on plates containing 1x Murashige and Skoog salts, 1x Gamborg's vitamins and 1% sucrose for two weeks at 22 °C and 16/8h light/dark cycles. The plants were then transferred to a flask with incubation buffer (Seeley *et al.*, 1992). After a 30 min incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and quickly frozen in liquid nitrogen. Total RNA was isolated and analyzed by northern blot using standard techniques. Cordycepin was used to inhibit transcription in these studies because its use is routine in plants (Seeley *et al.*, 1992; Johnson *et al.*, 2000) in contrast to other inhibitors such as alpha-amanitin, and is more effective in leaf tissue than Actinomycin D (Johnson *et al.*, 2000) presumably due to poor penetration.

Hybridization of cDNA Microarrays.

The 11,521 element cDNA microarray, print name MSU-2_03-00, prepared by the AFGC was used in all experiments (Schaffer *et al.*, 2001). 100 μg of total RNA corresponding to time 0 and 120 min after cordycepin treatment was labeled during first strand cDNA synthesis with Cy3- and Cy5-labeled dUTP, respectively, as previously described (Schaffer *et al.*, 2001). Three independent cordycepin treatments (biological

replicas) were performed and RNA samples were isolated. Each pair of samples from the 0 and 120 time points was used in two microarray hybridizations, the second with reverse labeling relative to the first (technical replicas). Measurement of the fluorescence corresponding to hybridization intensities was performed with the ScanArray 4000 Microarray Acquisition System (Packard BioChip Technologies. Billerica, MA). We used the ScanAlyze v2.44 software (http://rana.lbl.gov/EisenSoftware.htm) to extract the information of the images generated. The raw data for these experiments is available from the Stanford Microarray Database (http://genome-www.stanford.edu/microarray/) (Sherlock *et al.*, 2001), ExptID: 11374, 11333, 11339, 11323, 11375 and 11342.

Microarray data analysis.

Stringent quality control measures were applied to define the working data set. Spots with abnormal shapes or high local background were discarded manually. Spots with channel intensity values smaller than the mean plus two standard deviations of each slide background or with GTB2 values (GTB2 indicates the fraction of pixels in the spot that have intensity values 1.5 times the background) smaller than 0.65 in more than two channels were discarded because of low signal. The quality of the hybridization was also evaluated by visual inspection of the gradients, using the most sensitive setting of the "Array Color Plot" tool implemented in the Stanford Microarray Database (SMD). Slides that showed gradients in more than 25% of the array surface and/or that had R-squared values > 0.15 (indicating a strong dependence on spatial location) were not used for data analysis. The percent of the array surface that exhibited gradients was also used to order the slides from worst to best or best to worst in Figure 2.1*b*.

The z-score method in log space with a 90% trimmed data set was used for global normalization of the data (Schaffer *et al.*, 2001). The difference in mRNA levels between the time points considered (0 and 120 min) can be used to estimate the rates of decay using the equation: ln (Normalized Ratio) = $-k_{decay}t$, with the half-life being: $t_{1/2} = 0.693 / k_{decay}$, because mRNA degradation generally obeys first-order kinetics (Lam *et al.*, 2001). Statistical analysis of the ratios was performed using the *t*-test as described in the text.

Sequence and gene expression analysis

Sequences of 59 clones representing *Arabidopsis thaliana* genes for unstable transcripts (*AtGUTs*) were determined and found to be consistent with sequences deposited in Genbank. For EST identification, the BLASTN program was used to search the completed Arabidopsis genome sequence downloaded from The Institute for Genomic Research (TIGR). Functional categories were obtained from the Munich Information Center for Protein Sequences. For analysis of gene expression data across multiple experiments, the Cluster and Treeview software were used (Eisen *et al.*, 1998) (http://rana.lbl.gov/EisenSoftware.htm). Visual images were generated with the Treeview software using the output generated by the hierarchical clustering program of the Cluster software. The uncentered correlation similarity metric was used to perform average linkage clustering.

Sequences of *AtGUTs* (5'UTR, coding sequence, 3'UTR) were obtained from the TIGR Arabidopsis genome. Because UTRs are not an annotated feature of the Arabidopsis genome, the 3'UTR sequences of AtGUTs were assembled by extracting the average length of an Arabidopsis 3'UTR, that is 150 bp downstream of the annotated stop

codon. Similarly, 5'UTR sequences were assembled by extracting 75 bp upstream of the annotated start codon. Average 3UTR and 5'UTR sizes were estimated from the 5,000 full lenghtcDNAs released by CERES (also available from TIGR).

The oligomer counting method was used in an effort to identify candidate instability determinants in *AtGUTs* (van Helden *et al.*, 1998). This is a rigorous and exhaustive method that is based on the detection of over-represented oligonucleotides in the input set of sequences as compared to a control set. Frequencies of overlapping (1 bp window) oligonucleotides (up to 6 nt in length) were determined in the 3'UTR sequences of *AtGUTs* and also in the 3'UTR sequences of a control set of genes as described by van Helden *et al.*, (van Helden *et al.*, 1998). The control set was derived from 4064 clones corresponding to stable transcripts on the array. To assess significance, 1000 random samples of the same size as the test set were taken from the control sequences and oligonucleotide frequencies were determined in these samples. The criteria for significance were as follow: (1) Oligonucleotide was at least 2-fold more abundant in unstable than in whole control set; (2) Oligonucleotide had frequencies > 2 stdev above mean frequency in the 1000 random samples from control set; (3) Oligonucleotide was present in >10% of test sequences.

Additional MEME (Multiple Expectation Maximization for Motif Elicitation) searches (Bailey and Elkan, 1994) were carried out as described at http://meme.sdsc.edu. MEME is a computational tool for discovering short sequence patterns (motif) that occur repeatedly in a group of related DNA or protein sequences. MEME output describes each motif it finds by the probability of each possible nucleotide (if using DNA or RNA sequences) at each position in the motif. MEME indicates the location in the input

sequences and provides a *p*-value to evaluate the significance of each motif. Various combinations of the MEME parameters were tested: motif distribution, 1-3; number of motifs, 3-5; motif width, 5-50. Programs written in the Practical Extraction and Report Language (Perl) were used for sequence extraction and manipulation.

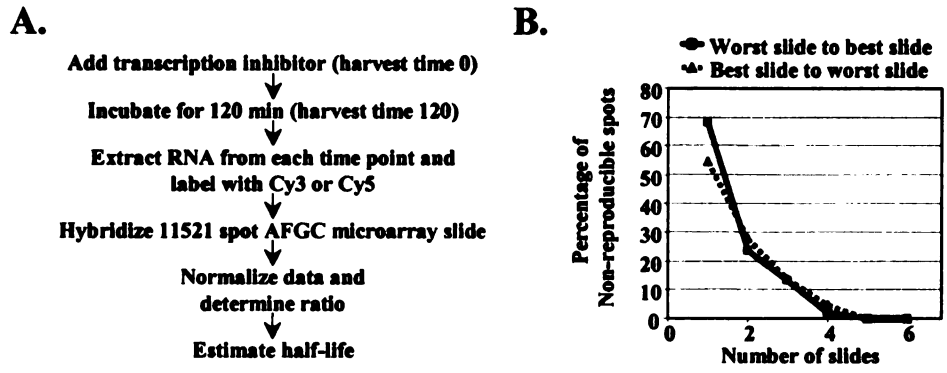


Figure 2.1. Strategy for monitoring mRNA stability using cDNA microarrays.
(A) RNA samples corresponding to 0 and 120 min after the addition of the transcriptional inhibitor cordycepin were labeled with Cy3 and Cy5 respectively and used to hybridize 11K microarray slides. Each pair of RNA samples were reverse labeled for a separate microarray hybridization. These hybridizations were performed with samples from three independent cordycepin treatments for a final data set of six slides. Half-life values were then estimated from the normalized ratios. (B) Non-reproducible spots decrease as a function of the number of slides, nearly leveling out when the data from four slides is combined. The quality of the slides, best to worst or worst to best based on the extent of visible gradients (see *Materials and Methods*), does not significantly affect the reproducibility of the data when two or more slides are considered, although the curves are slightly steeper with better slides.

Results and Analysis

Monitoring mRNA stability using cDNA microarrays.

mRNA decay rates, expressed as half-life values, are typically measured by monitoring the disappearance of a transcript by northern blot after transcription of the corresponding gene has been halted. We combined this simple experimental strategy with the highly parallel power of DNA microarray analysis (Schena *et al.*, 1995) as outlined in Figure 2.1*a*. Total RNA samples corresponding to 0 and 120 min time points after transcriptional inhibition with cordycepin were isolated. 100 μg of total RNA from each of these samples was used to synthesize cDNA probes by incorporating Cy3- or Cy5-labeled dUTP during oligo(dT)-primed reverse transcription. The probes were combined and used for hybridization of the 11,521 elements cDNA microarray (11K microarray) prepared on glass slides by the AFGC. We performed three biological replica experiments, each with a reverse-labeling technical replicate. The purpose of these repetitions was to increase the likelihood of detecting significant differences in mRNA levels, while decreasing the likelihood of false positives, which might be common on microarray studies with one or two slides (Lee *et al.*, 2000). Quite reasonably, the number of non-reproducible normalized intensity ratios ≥2 decreased as a function of the number of slides, nearly leveling out below 5% when 4 slides were considered (Figure 2.1*b*). Based on these data, and to be rigorous, we defined our working data set as all those clones with reproducible normalized intensity ratios ≥2 in 5 of the 6 slides.

At least 1% of clones on the 11K Arabidopsis microarrays correspond to unstable messages

To identify and characterize the most unstable transcripts from our working data set, we focused our attention on the transcripts that were most diminished after treatment with cordycepin for 120 minutes. Clones whose median normalized intensity ratios were ≥ 4 (0 versus 120 min) and that met several quality control criteria (see *Materials and Methods*) were used for further analysis. In this study, expressed sequence tags (EST) that overlap with the same annotated open reading frame were considered as representing the same gene. This is a reasonable assumption because the expression patterns of groups of ESTs that match the same open reading frame were well correlated across multiple experiments (data not shown). Based on this criterion, the selected clones corresponded to 100 genes that were termed *Arabidopsis thaliana* genes with unstable transcripts (*AtGUTs*), because the calculated half-lives of the encoded transcripts were 60 min or less (Table 2.1). Similarly, we identified 225 genes with moderately unstable mRNAs, whose estimated half-lives ranged from 60 to 120 min. The great majority of the transcripts in Arabidopsis appeared to decay with rates greater than two hours, consistent with the idea that most messages in plants are relatively stable (Siflow and Key, 1979).

The 11K microarray used in these studies represents an estimated 7800 unique genes (Schaffer *et al.*, 2001) so the 100 *AtGUTs* we identified correspond to about 1%. This number likely represents an underestimate of unstable Arabidopsis transcripts especially when extrapolated to the whole genome for several reasons. First, unstable mRNAs with low steady state levels may be underrepresented in the EST collections used for the 11K microarray. Second, some unstable transcripts likely fall below the

Table 2.1. Arabidopsis thaliana genes with unstable transcripts (*AtGUTs*)

Representative EST CloneID	Accession	GI	t1/2 median	*P* value	BLASTN score	BLASTN *E* value	Locus	Pub locus	Com name	Major MIPS role
162J18T7	R29894	936605	29.6	1.9E-27	605	e-172	T28M21.16	At2g40000	Putative nematode-resistance	11
G7E10T7	N96265	2748596	30.0	1.1E-28	396	e-110	T6K21_190	AT4g18010	Putative protein	1
201F23T7	H76698	1053949	31.8	4.1E-24	482	e-135	K18I23_25	AT5g05440	Putative protein	4
191D5T7	R90660	958200	32.7	2.1E-26	585	e-166	MMB12_13	AT3g19680	Unknown protein	99
110D19XP	AA585854	2393266	33.7	8.1E-23	167	1.00E-40	F28P10_210	AT3g54810	Similar to GATA transcription factor 3	4
108O17XP	AA395006	2048203	34.3	5.2E-23	157	8.00E-38	F16L2_180	AT3g45970	Putative protein	30
249B1T7	AA597982	2413405	34.7	3.8E-21	80	2.00E-14	C225EPL23M	At5g03430	Putative protein	1
105B6T7	T22424	2597035	37.3	2.9E-17	418	e-116	F1L3_1	At1g17420	Lipoxygenase	1
184G24T7	H37631	907130	37.7	4.1E-19	137	1.00E-31	MBK5_27	AT5g63790	Putative protein	4
222C9T7	N38276	1159418	38.5	1.3E-17	591	e-168	T10D17_50	AT3g44260	CCR4-associated factor 1-like	4
E5E10T7	AA042089	2414049	38.7	1.3E-17	355	3.00E-97	T14P8_2	AT4g02380	Similar to several small proteins (~100 aa) that are induced by heat, auxin, ethylene, and wounding.	11
97A6T7	T21700	2757211	39.6	5.9E-19	567	e-161	F7G19_6	At1g09070	Unknown protein	1

38B3T7	T04337	315497	39.6	1.1E-18	307	4.00E-83	F14M13.17	At2g22430	Homeodomain transcription factor	4
125A2XP	AI099804	3449543	39.6	1.6E-15	416	e-116	MXC7_6	AT3g23030	Auxin-inducible gene (IAA2)	99
122I20XP	AA395351	2048562	40.0	8.0E-14	404	e-112	K9I9_4	AT5g67480	Putative protein	11
165P2T7	R64779	937312	40.1	8.2E-20	618	e-176	F15E12_6	At1g66160	Unknown protein	10
142G24T7	T76090	935125	40.5	1.4E-18	535	e-152	K7L4_1	AT3g15210	Ethylene responsive element binding factor 4	4
148N24T7	T76263	2763629	41.0	4.4E-25	502	e-141	F22K18_230	AT4g24570	Putative mitochondrial uncoupling	7
41H3T7	T13839	2759901	41.2	2.3E-19	323	3.00E-88	MJK13_11	AT3g15450	Unknown protein	1
F2C7T7	N96483	2747965	41.3	7.4E-22	198	5.00E-50	F22D22.10	At2g32150	Putative hydrolase	25
171E9XP	AA651102	2749348	41.3	2.1E-15	462	e-129	F22D1_50	AT5g20880	Putative protein	29
144L3XP	AA404905	2062895	41.9	1.9E-14	712	0	F13D13.3	At1g57990	Unknown protein	99
113D5T7	T42394	2757718	42.0	2.2E-16	82	4.00E-15	dl4785w	AT4g17500	Ethylene responsive element binding factor 1-like	4
203O8T7	AA712865	2722782	42.2	5.5E-16	258	2.00E-68	F9L11_25	At1g32920	Unknown protein	99
184O7T7	H37666	907165	42.2	2.3E-15	454	e-127	T32G9_32	At1g35140	Phosphate-induced (phi-1) protein,	98
F2B9T7	N96457	2747956	42.2	4.6E-16	359	2.00E-98	MSJ1_10	AT5g64260	phi-1-like protein	99

162M13T7	R29917	936624	42.4	4.2E-15	***** No match against *Arabidopsis thaliana* coding sequences *****					
86D5T7	T20525	501966	42.7	2.1E-15	393	e-109	F22O6_220	AT3g52400	Syntaxin-like protein synt4	8
H10A4T7	AA042412	2446142	42.8	8.9E-17	626	e-179	F3C3_8	At1g32130	Unknown protein	99
216H23T7	N38405	1159547	42.9	4.2E-15	668	0	F28L22_2	At1g37130	Nitrate reductase 2	67
121G2T7	T43596	2758442	42.9	2.5E-17	603	e-172	T7M7.3	At2g40140	Putative CCCH-type zinc finger protein	4
F4B4T7	N95988	2748060	43.0	1.5E-13	507	e-143	F2J7_21	At1g25550	Hypothetical protein	10
188B23T7	R90032	2733297	43.0	5.4E-15	184	5.00E-46	F9K21_220	AT3g45640	Mitogen-activated protein kinase 3	10
209O17T7	N37328	1158470	43.3	3.4E-15	755	0	T6J4_3	At1g13260	DNA-binding protein RAV1	4
E12D8T7	AA042669	2581635	43.7	6.7E-16	460	e-129	T4O12_13	At1g75900	Anter-specific proline-rich -like	1
305A4T7	AA394319	2047544	43.8	4.8E-15	408	e-113	T8M16_210	AT3g56880	Putative protein	99
180E15T7	H36869	906368	43.9	3.9E-16	64	1.00E-09	T5P19_10	AT3g56360	Putative protein	99
172F17T7	H36431	905930	44.6	1.1E-14	452	e-126	F10N7_170	AT4g32020	Putative protein	99
220O22T7	N38209	2747827	46.2	1.5E-14	385	e-106	F6F22.17	At2g19800	Unknown protein	98
89I20T7	T20915	2756834	46.4	4.4E-11	182	2.00E-45	F28K19_29	At1g78080	AP2 domain containing protein,	99

98J7XP	AI100650	3450611	46.7	5.5E-13	266	1.00E-70	F22L4_7	At1g01550	Hypothetical protein	99
181F2XP	AA651342	2749585	47.1	6.8E-13	285	2.00E-76	T17F15_170	AT3g47960	Putative peptide transporter	7
223F20T7	N64958	1216584	47.2	1.2E-16	757	0	T32G6.16	At2g41640	Unknown protein	99
189I19XP	AA605453	2445981	47.3	2.2E-12	442	e-123	T1D16.17	At2g26190	Unknown protein	99
103P14T7	T21879	2596568	47.5	9.3E-14	521	e-147	T19N18_70	AT5g04340	Putative c2h2 zinc finger	4
159F8T7	R30283	936952	47.6	1.2E-13	434	e-121	F3L17_120	AT4g31550	WRKY family transcription factor	4
211N1T7	N37850	1158992	47.7	4.6E-12	624	e-178	T17F3_8	At1g69890	Hypothetical protein	99
50A9BT7	T14209	931155	48.4	2.6E-20	666	0	T29M8_5	At1g19180	Hypothetical protein	4
187O13T7	R90490	958030	48.4	3.1E-10	622	e-178	F25P17.13	At2g24570	Putative WRKY-type DNA binding	4
116J23T7	T43171	2597733	48.6	1.8E-10	315	2.00E-85	T18B16_200	AT4g19230	Cytochrome P450	11
172F13T7	H36428	905927	49.2	1.7E-11	646	0	F24K9_8	AT3g11410	Protein phosphatase 2C (PP2C)	10
H10A12T7	AA042408	2446138	49.9	7.3E-12	135	5.00E-31	F27B13_190	AT4g29950	Putative protein	99
164H19T7	R30505	937156	49.9	1.0E-09	529	e-150	F8G22_8	At1g47210	Cyclin, putative	3
168C16T7	R64886	2764185	50.3	9.4E-13	***** No match against *Arabidopsis thaliana* coding sequences *****					

H1H12T7	W43539	2748861	50.5	1.2E-09	165	5.00E-40	T29H11_120	AT3g48360	Putative protein	99
147B19T7	AA720100	2733710	50.8	1.2E-05	393	e-109	F17I23_220	AT4g30440	Nucleotide sugar epimerase-like	1
88K20XP	AI100427	3450388	50.8	8.8E-11	511	e-144	dl3920c	AT4g15760	Hypothetical protein	1
215C17T7	AA597715	2413138	50.9	1.2E-11	561	e-159	k11j9_110	AT5g61590	Ethylene responsive element binding factor 5-like	4
122F7XP	AA395343	2048554	51.1	1.9E-11	618	e-176	F27L4.1	At2g23810	Similar to senescence-associated	14
303H9T7	AA395910	2047526	51.4	7.3E-12	329	1.00E-89	T12C14_250	AT3g62550	Putative protein	10
195P14T7	H76142	1053393	51.6	3.5E-11	365	e-100	MBK20_1	AT5g07580	Transcription factor-like protein	4
92O12T7	T20793	502234	52.2	6.8E-11	605	e-172	T22I11_4	At1g21130	O-methyltransferase, putative	1
149C14T7	AA720131	2733741	52.5	1.2E-10	252	2.00E-66	MRG7_8	AT5g18120	Putative protein	6
154C6T7	T76510	935493	52.6	1.1E-10	274	6.00E-73	T26I12_120	AT3g55240	Putative protein	99
192C22T7	R90276	957816	52.8	2.9E-17	155	4.00E-37	F28A23_90	AT4g34150	Putative protein	1
76D8T7	T21456	2756999	52.9	4.2E-11	595	e-169	T7B11_13	AT4g01870	Predicted protein of unknown	99
156A6XP	AA394587	2047868	53.0	2.3E-17	280	1.00E-74	MEE6_15	AT5g41080	Putative protein	1
215B3T7	N37995	2747798	53.2	1.4E-09	220	9.00E-57	MDA7_25	AT5g56190	WD-repeat protein-like	40

195F24T7	H76116	2733395	53.2	1.8E-09	484	e-136	F16B3_18	AT3g02550	Unknown protein	99
159D24XP	AI099625	3449937	53.9	6.6E-10	373	e-102	F16J13_110	AT4g12040	Similar to zinc finger protein ZNF216-*M. musculus*	4
169B10T7	R64946	937450	54.4	3.2E-09	***** No match against *Arabidopsis thaliana* coding sequences *****					
182G6XP	AA651380	2749622	54.6	9.7E-13	141	9.00E-33	K19B1_18	AT5g62570	Putative protein	10
117G15XP	AA395200	2048397	54.7	5.2E-09	414	e-115	T2E22_105	AT3g12630	Unknown protein	99
88J8T7	T20600	502041	55.0	2.4E-09	569	e-162	F3L17_70	AT4g31500	Cytochrome P450 monooxygenase	11
197N9T7	AA597420	2412843	55.2	1.5E-10	456	e-128	F25A4.8	At1g74950	Unknown protein	99
E6E1T7	AA042331	2414114	55.6	7.4E-11	599	e-170	MLJ15_14	AT3g26740	Light regulated protein, putative	99
K3A4T7	AA728492	2747449	56.4	5.3E-13	605	e-172	T4C15.7	At2g35260	Unknown protein	99
189J17T7	AA712424	2722341	56.6	6.1E-10	410	e-114	F13F21_6	At1g49500	Unknown protein	99
171P12T7	AA720344	2733954	56.7	9.9E-10	127	8.00E-29	dl4650c	AT4g17230	Scarecrow-like 13 (SCL13)	4
181L15T7	H37452	906951	56.8	1.9E-09	466	e-130	F10A5.2	At1g75800	Thaumatin-like protein, putative, 3	10
92I17T7	AA067525	1565874	56.9	1.6E-06	513	e-145	K22F20_10	AT5g37770	Calmodulin-related protein 2	10
245O10T7	AA597849	2413272	57.1	2.2E-06	517	e-146	T10D10_8	At1g72450	Unknown protein	99

44G9T7	T13991	930995	57.1	1.8E-10	373	e-103	F16P2.17	At2g29450	Glutathione S-transferase	1
165M23T7	AA720308	2733918	57.2	6.8E-09	234	3.00E-61	F6N18_4	At1g32640	Putative prot. kinase; bHLH protein	4
F2H12T7	AA713007	2722924	57.4	1.3E-11	317	6.00E-86	F13H10.4	At2g41410	Calmodulin-like protein	10
63A8T7	T41667	931306	57.5	2.8E-09	466	e-130	T5I7.3_	At2g39730	Rubisco activase	3
K3H8TP	AA728680	2750052	57.6	2.6E-09	676	0	T8H10_120	AT3g57520	Imbibition protein homolog	1
F2E8T7	N96502	1268253	58.0	5.8E-09	***** No match against *Arabidopsis thaliana* coding sequences *****					
147A21T7	AA720093	2733703	58.1	9.2E-10	254	3.00E-67	M4I22_90	AT4g27280	Putative protein	10
169P15T7	R65359	937831	58.1	1.7E-10	650	0	F18G18_90	AT5g25350	Leucine-rich repeats containing	10
149C22T7	AA720132	2733742	58.7	2.4E-08	385	e-106	F23A5_22	At1g80920	J8-like protein	11
128L4XP	AA394474	2047685	59.0	3.2E-10	***** No match against *Arabidopsis thaliana* coding sequences *****					
ATHATL2A	L76926	3873407	59.2	1.8E-09	2777	0	MGL6_17	AT3g16720	Putative RING zinc finger protein	4
117A20XP	AA395188	2576770	59.4	8.8E-08	206	1.00E-52	T2H3_13	AT4g02200	Drought-induced-19-like 1	11
186F13T7	R89921	957461	59.8	1.0E-09	440	e-123	T24G5_90	AT5g19190	Putative protein	99
166F15XP	AA651001	2749248	59.9	2.8E-07	581	e-165	f2o15_210	AT5g59550	Similar to COP1-interacting protein CIP8	4

121G24T7	R87001	2758441	60.0	7.1E-10	589	e-168	k11j9_120	AT5g61600	Ethylene responsive element binding factor4-like	4
126B3T7	T44869	949125	60.5	2.4E-08	660	0	T22I11_7	At1g21100	O-methyltransferase, putative	1
109G12T7	T41881	932826	60.5	2.5E-09	414	e-115	F10N7_140	AT4g32060	Putative protein	10
198L20T7	H76584	1053835	60.6	7.0E-10	454	e-127	T10O22.27	At1g18300	Unknown protein	11

detection limits of the microarray technique or might not meet our stringent reproducibility requirements. However, the channel intensity distribution for *AtGUTs* resembled that of the whole array, suggesting the *AtGUTs* identified were not strongly biased to either high or low expression levels on the 11K microarray. The identification of highly expressed *AtGUTs* was an added bonus from this analysis because these transcripts should greatly facilitate future studies of steps in their degradation. Finally, multiple members of closely related gene families may be missed because the 11K microarray was not designed to resolve gene family members. Thus, on the basis of our work, it seems valid to estimate, that at least 1% of the genes of Arabidopsis correspond to unstable transcripts.

We used conventional northern blot analysis of cordycepin time courses with several time points to confirm the 11K microarray data. Four randomly selected transcripts with half-lives of less than 60 min showed comparable turnover rates in full cordycepin time courses (Figure 2.2*a*). Two representative examples are shown in Figure 2.2*b-c*. In addition, we assessed the statistical significance of the ratio values for the genes of interest. Using the *t*-test and the conservative Bonferroni method to adjust *p* values (Samuels, 1989) all selected *AtGUTs* showed significantly different ratios from the mean of the population at $\alpha < 0.0001$ (Table 2.1). Several genes with moderately unstable messages according to the microarray studies were at least moderately unstable in northern blots (data not shown). Four stable transcripts according to microarray data were also found stable by northern blot analysis further validating our results. Two representative examples of these stable transcripts are shown in Figure 2.2*b-c*.

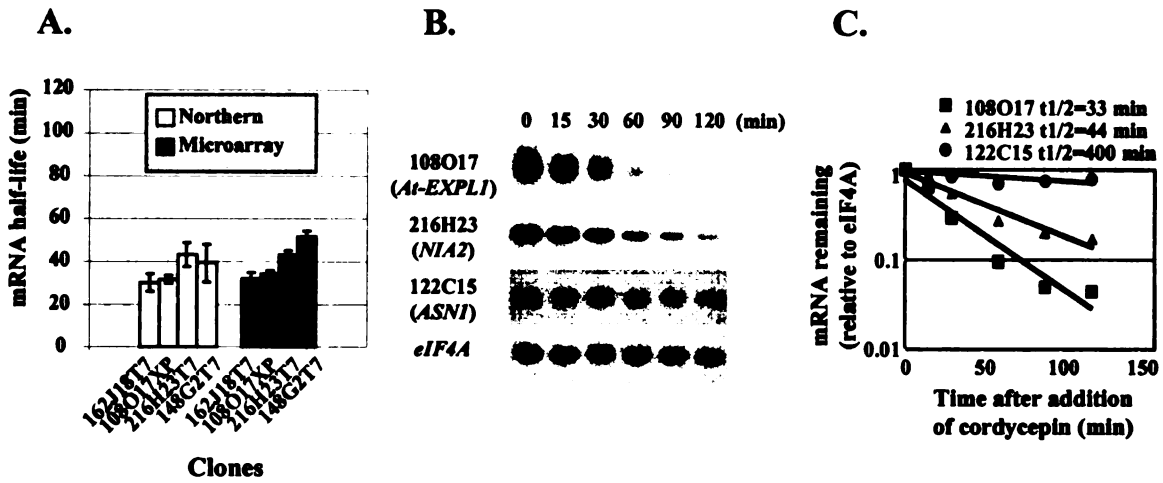


Figure 2.2. Confirmation of the instability of transcripts identified by microarray analysis. (A) Half-life values determined by northern blot are comparable to estimates from microarray analysis for four randomly selected unstable transcripts. Northern blot values are representative of at least three independent cordycepin time courses. (B) Representative northern blot analysis of cordycepin time courses for two randomly selected unstable and two stable transcripts. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (C) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for *eIF4A* does not change significantly during the time courses and was used as a reference for equal loading.

General structural features of genes with unstable and stable transcripts are similar.

The identification of the *AtGUTs* allowed us to evaluate them for structural properties that might play a role in determining their instability. We compared the sequences of the 100 *AtGUTs* against genes that encode stable transcripts under our conditions. *AtGUTs* were evenly distributed throughout the Arabidopsis genome and showed no significant differences in nucleotide composition, number of introns, size of the coding sequence and codon usage as compared to genes with stable mRNAs.

We did not expect to find a simple sequence that would be present in the 3'UTR of all or most *AtGUTs* because previous observations suggest that many instability

sequences exist (Ross, 1995; Caponigro and Parker, 1996; Gutiérrez *et al.*, 1999). Consistent with this prediction, neither an oligonucleotide frequency approach (van Helden *et al.*, 1998) nor a probabilistic approach using the MEME software (Bailey and Elkan, 1994) was indicative of a simple sequence common to all or most *AtGUTs* compared to controls (see *Materials and Methods* for more details) . However a few *AtGUTs* have potential ARE-like instability sequences (Greenberg and Belasco, 1993; Ohme-Takagi *et al.*, 1993; Chen and Shyu, 1995) typified by repeats of the AUUUA motif: a putative nematode-resistance gene (At2g4000) which encodes the most unstable transcript in our conditions and two genes of unknown function (At1g72450 and At2g41640). Though the functional significance of these sequences remains to be determined, these transcripts are potential targets for the AUUUA-mediated decay pathway in Arabidopsis. Similarly two *AtGUTs,* the senescence-associated gene *SEN1* (At4g35770) and a putative light regulated gene similar to the *ccr* gene from *Citrus paradisi* (At3g26740), have DST-like elements in their 3'UTRs. Interestingly, the expression of these two transcripts is altered in *dst1*, a mutant deficient in DST-mediated decay (Pérez-Amador *et al.*, 2001). Therefore, these transcripts are potential primary targets of the DST-mediated decay pathway in Arabidopsis (Pérez-Amador *et al.*, 2001).

***AtGUTs* are predicted to play a role in a broad range of cellular processes but most prominently in transcription.**

To explore the potential cellular roles of *AtGUTs*, we analyzed how they were distributed among the functional categories assigned by the Munich Information Center for Protein Sequences (MIPS; Figure 2.3). More than half of the *AtGUTs* could be

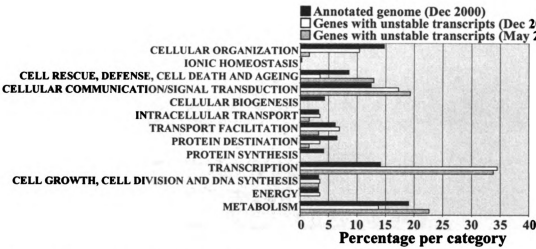


Figure 2.3. Instability is associated with a broad range of plant processes. Genes with unstable transcripts were classified according to the scheme of MIPS. *AtGUTs* are predicted to participate in a broad range of cellular processes with transcriptional functions over-represented compared to what is expected based on the whole genome annotation. To allow comparison, *AtGUTs* were classified based on the information released for the annotation of the whole *A. thaliana* genome sequence in Dec. 2000. The most updated annotation for the *AtGUTs* is also included (May 2002), although a whole genome annotation based on this updated information is not yet available.

assigned to a MIPS category (Table 2.1) with the remainder lacking similarity to known proteins. The distribution of predicted functions for *AtGUTs* suggests they participate in a broad range of plant processes and in roughly the same proportion as the whole complement of Arabidopsis genes. Interestingly, enrichment was observed for transcriptional functions. *AtGUTs* encode transcriptional functions more than twice the expected frequency based on the whole Arabidopsis genome annotation (The Arabidopsis Genome Initiative, 2000). BLAST search analysis (Altschul *et al.*, 1990) indicated that 14 of the 21 *AtGUTs* that belong to this transcriptional class were not found in the sequenced genome of *H. sapiens*, *Mus musculus*, *Rattus norvegicus*, *C. elegans*, *D. melanogaster*, *S. cerevisiae*, *Synechocystis*, *Eubacteria* and *Archebacteria* (BLASTX program, p-value < 0.01) (Table 2.2). This is in line with the detailed analysis of Arabidopsis transcription factors performed by Riechmann *et al.* (37) that indicated that 45% of those annotated on

Table 2.2. Arabidopsis genes with unstable messages that belong to the MIPS transcriptional category (04) as of May 2002. It should be noted that this category includes transcription as well as other aspects of RNA metabolism.

Locus	Description
At1g13260	DNA-binding protein RAV1[1,2]
At1g19180	Hypothetical protein[1,2]
At1g32640	Putative protein kinase; bHLH protein
At2g22430	Homeodomain transcription factor (ATHB-6)
At2g24570	Putative WRKY-type DNA binding protein[1,2]
At2g40140	Putative CCCH-type zinc finger protein
At3g15210	Ethylene responsive element binding factor 4[1,2]
At3g16720	Putative RING zinc finger protein
At3g44260	CCR4-associated factor 1-like protein
At3g54810	Similar to GATA transcription factor 3[1]
At4g12040	Similar to zinc finger protein ZNF216-*M. musculus*
At4g17230	Scarecrow-like 13 (SCL13)[1,2]
At4g17500	Ethylene responsive element binding factor1-like[1,2]
At4g31550	WRKY family transcription factor[1,2]
At5g04340	Putative c2h2 zinc finger transcription factor[1]
At5g05440	Putative protein[1]
At5g07580	Transcription factor-like protein[1,2]
At5g59550	Similar to COP1-interacting protein CIP8[1]
At5g61590	Ethylene responsive element binding factor 5-like[1,2]
At5g61600	Ethylene responsive element binding factor4-like[2]
At5g63790	Putative protein[1]

1. Plant specific genes as determined by BLAST search analysis (June 2000).
2. Plant specific genes according to the classification by Reichman *et al.* (2001) (37).

the genome are from families specific to plants, reflecting the independent evolution of many plant transcription factors. It is possible that plants might also have evolved mechanisms for regulating the stability of these transcripts that are distinct from those of other eukaryotes. Plant specific mechanisms might not be exclusive to transcriptional functions but could also extend to other *AtGUTs* which are unique to plants.

Rapid mRNA degradation is associated with Arabidopsis responses to mechanical stimulation and circadian rhythms.

To begin understanding the physiological implications of instability in Arabidopsis, we examined expression of the identified *AtGUTs* for patterns of regulation. Hierarchical cluster analysis was performed (see *Materials and Methods*) using the publicly available microarray data for the *AtGUTs*, deposited in the Stanford Microarray Database (Sherlock *et al.*, 2001) by the AFGC. At the time of this study, 112 slides corresponding to 47 different experiments carried out under various treatments, environmental conditions, developmental stages or in different genotypes were available.

Two main clusters of genes were observed. The largest contained 32 genes, the majority of which were induced by mechanical stimulation (touch; see Table 2.3 for SMD experiment identifiers) (Figure 2.4). Several of the genes in this cluster also appeared repressed in an auxin treatment and induced in the histone deacetylase mutant *axe1-4* relative to wild type Arabidopsis plants (Murfett *et al.*, 2001). In addition, most showed organ-preferential expression with low levels in flowers and high levels in roots compared to a reference sample prepared from a mixture of plant organs (Figure 2.4 and Table 2.3). The identification of a touch-induced cluster of *AtGUTs* is consistent with touch responses being fast (Braam and Davis, 1990), and instability being critical when rapid changes in mRNA steady state levels are to be achieved. In fact, the touch gene transcripts initially characterized were detected within minutes of treatment and disappeared very rapidly thereafter, consistent with rapid turnover (Braam and Davis, 1990). Interestingly, 32 (34%) of the 95 genes induced by the touch treatment in SMD encode unstable transcripts (Table 2.3). In contrast, only 0.8% of the genes repressed

Table 2.3. Comparison of gene expression data for all genes in the AFGC 11K microarray and *AtGUTs* in selected treatments

Treatment	SMD Expt ID	Direction of change	Differentially regulated genes (greater or equal to 2)		*AtGUTs*
			Number	% of all genes in array	(n = 100)
Touch treatment vs. untreated control	3714, 3715	Increased	95	1	32
		Decreased	1	0.01	0
Auxin treatment vs. untreated control	3743, 3749	Increased	1	0.01	0
		Decreased	20	0.3	7
axr1-4 mutant vs. wild type	6654, 6655	Increased	78	1	27
		Decreased	0	0	0
Diurnal*	10178, 2344, 10179, 10180, 2362, 2359, 2365, 10177, 2361, 2363, 3242, 2368, 10186	na	740	10	29
Circadian*		na	188	2	9
Circadian†		na	453	6	9
Far red light vs. untreated control	8266, 8130	Increased	30	0.4	4
		Decreased	56	0.7	1
Flowers vs. leaves	2370, 2371	Increased	707	9	2
		Decreased	680	9	42
Flowers vs. reference	7200, 7201	Increased	248	3	2
		Decreased	552	7	38
Roots vs. reference	7203, 7205	Increased	437	6	18
		Decreased	1117	14	11

Genes differentially regulated in the AFGC 11K microarray as well as the number of AtGUTs that exhibited regulation in the same treatments are presented. Data was examined from a total of 112 slides, representing 47 different treatments, publically available from SMD. Because data processing steps might be different, the numbers indicated in this table might differ from what published by the authors of each experiment. To identify the differentially regulated genes, the following conservative quality control criteria were used: (i) Sum of raw channel intensities was ≥1000 (ii) Channel intensity values could not be saturated in more than 1 channel per slide. (iii) GTB2 parameter was ≥0.5 in at least one channel per slide. (iv) Flag = 0 in all slides. After spot filtering, slides were normalized. Normalized expression data for clones printed several times on the AFGC array were averaged. We calculated the median of the normalized expression data for all those EST clones that represent the same gene (see text for details). Genes differentially expressed were those that showed 2-fold or greater difference among the treatment in all replicas available.

na, not applicable.
*Based on data by Schaffer *et al.* (Plant Cell (2001) 13, 113-123).
†Based on data by Harmer *et al.* (Science (2000) 290, 2110-2113). These experiments were performed with the Affymetrix Arabidopsis Genome Array (~8200 genes).

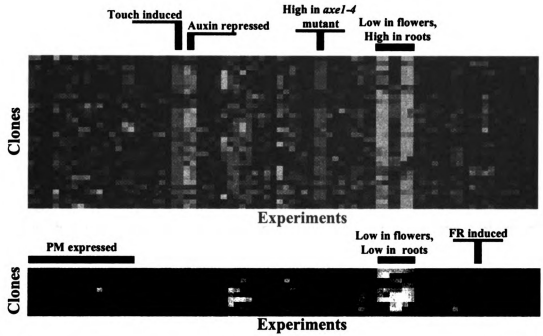


Figure 2.4. Cluster analysis indicates that a set of AtGUTs is induced by mechanical stimulation (touch) and another is controlled in a diurnal fashion. Hierarchical cluster analysis of expression data for the *AtGUTs* across multiple microarray experiments was performed with Cluster and Treeview software (28). Expression characteristics shared by genes in each cluster are indicated (PM = post meridiem; FR= far red light). Each row represents a gene and each column represents an experiment. Small labels on top of the clusters are SMD experiment identifiers. Small labels on right side of clusters indicate *Arabidopsis thaliana* loci or EST clone identifiers.

under high CO_2 conditions (experiments 7561 and 7562) and 5% of the genes induced after 1.5h and 3h H_2O_2/NO_2 co-treatment (experiments 7523 and 9371) did the same. The number of genes differentially expressed in the CO_2 and H_2O_2/NO_2 experiments is in the same range as the overall number of *AtGUTs* identified. These results indicate that not all physiological responses have an equal instability component. Moreover, our data suggest that mRNA instability is an important regulatory component of the touch response.

It is relevant to note that we were unable to confirm the auxin regulation shown by genes inside the touch cluster. Indole acetic acid (IAA) treatment of two weeks old Arabidopsis plants did not affect the expression of three different genes in the touch

cluster over a 24 hours time course (data not shown). These three genes showed a transient induction irrespective of the presence or absence of IAA in the solution sprayed. However, IAA treatment strongly induced the expression of the small auxin up RNA gene *SAUR-AC1* (McClure *et al.*, 1989) compared to the control (data not shown). Our data suggest that, at least in the conditions tested, the genes that belong to the touch cluster are not regulated by the phytohormone auxin but are induced by spraying alone. Presumably the genes in the touch cluster are more sensitive to experimental variation especially if there is mechanical stimulation involved.

A second smaller cluster contained 6 genes whose expression was regulated diurnally (Figure 2.4). In addition, some of these genes were induced by a far-red light treatment and showed organ-preferential expression with low levels in flowers and roots (Figure 2.4). Interestingly, some of these genes (At5g67480, At3g62550, At3g26740, At1g80920) have been also shown to be controlled by the circadian clock with a peak in mRNA abundance in the afternoon (Schaffer *et al.*, 2001). Further, 12 other *AtGUTs* (At3g15450, At1g37130, At1g75900, At4g31500, At2g39730, At2g32150, At1g13260, At3g55240, At2g35260, At1g49500, At2g29450, At4g32060) have been reported as clock-controlled genes (Table 2.3) (Schaffer *et al.*, 2001; Harmer *et al.*, 2000). At similar transcriptional rates, different mRNA stabilities would translate into circadian mRNA profiles with distinct phases and amplitudes (So and Rosbash, 1997). Therefore, instability might be essential for the mRNA oscillatory patterns observed for specific *AtGUTs* that are regulated by the clock. Post-transcriptional regulation of mRNA stability has been shown to play a role in the expression of the Drosophila clock gene *per* (So and Rosbash, 1997). There is also evidence that transcription makes a small contribution to

the observed circadian expression pattern of the Arabidopsis *NIA2* gene (Pilgrim *et al.*, 1993). Hence, modulation of mRNA stability could also contribute to the clock-regulated expression of specific *AtGUTs*. An interesting common feature of the genes in the touch and diurnal cluster was their low expression level in flowers (Figure 2.4). In fact 40% of the *AtGUTs* identified showed similar diminished expression suggesting rapid mRNA turnover might not be as prominent in flowers as in other organs. Perhaps reproductive tissues do not require rapid response control as much as vegetative tissues do, but instead favor the more economical long-lived mRNAs which are common in specialized cells (Weiss and Liebhaber, 1994).

Because genes that showed similar patterns of expression might share regulatory mechanisms, the 3'UTR sequences of *AtGUTs* that belong to the touch and light regulated clusters were analyzed for the presence of common sequence elements as described previously. Neither the oligonucleotide frequency approach nor the MEME software provided candidate sequence motifs overrepresented among these subsets of *AtGUTs*. Signals that control mRNA metabolism are often composite sequence elements, *e.g.* mRNA localization signals (Bashirullah *et al.*, 1998), whose key motifs have been difficult to identify. Alternatively, a sequence conserved in a small number of *AtGUTs* might not be detected by our approach.

Perhaps the most intriguing question for future studies prompted by our work relates to the *AtGUTs* regulated by touch, and light. Are these *AtGUTs* "constitutively" unstable, or is their mRNA stability regulated in response to light, touch, or other signals? The approach we describe has the potential to address this and other new exciting questions about post-transcriptional processes in plants. By comparing global turnover

rates under different conditions, post-transcriptional regulatory networks might be unraveled. This large-scale approach for analysis of post-transcriptional control mechanisms should help uncover the extent and significance of this level of regulation in plants in response to a variety of stimuli.

References

Altschul,S.F., Gish,W., Miller,W., Myers,E.W., and Lipman,D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Bailey, T. L. and Elkan, C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology , 28-36. 1994. AAAI Press.

Bashirullah,A., Cooperstock,R.L., and Lipshitz,H.D. (1998). RNA localization in development. Annu. Rev. Biochem. *67*, 335-394.

Braam,J. and Davis,R.W. (1990). Rain-induced, wound-induced, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell *60*, 357-364.

Brown,P.O. and Botstein,D. (1999). Exploring the new world of the genome with DNA microarrays. Nature Genet. *21*, 33-37.

Caponigro,G. and Parker,R. (1996). Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol. Rev. *60*, 233-249.

Chen,C.Y.A. and Shyu,A.B. (1995). AU-rich elements - Characterization and importance in messenger-RNA degradation. Trends Biochem. Sci. *20*, 465-470.

Eisen,M.B., Spellman,P.T., Brown,P.O., and Botstein,D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA *95*, 14863-14868.

Feldbrügge,M., Arizti,P., Sullivan,M.L., Zamore,P.D., Belasco,J.G., and Green,P.J. (2002). Comparative analysis of the plant mRNA-destabilizing element, DST, in mammalian and tobacco cells. Plant Mol. Biol. *49*, 215-223.

Greenberg,M.E. and Belasco,J.G. (1993). Control of the Decay of Labile Protooncogene and Cytokine mRNAs. In Control of Messenger RNA Stability, J.Belasco and G.Brawerman, eds. Academic Press, Inc. San Diego, CA), pp. 199-218.

Gutiérrez,R.A., MacIntosh,G.C., and Green,P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. *4*, 429-438.

Hargrove,J.L., Hulsey,M.G., and Beale,E.G. (1991). The kinetics of mammalian gene-expression. Bioessays *13*, 667-674.

Harmer,S.L., Hogenesch,J.B., Straume,M., Chang,H.S., Han,B., Zhu,T., Wang,X., Kreps,J.A., and Kay,S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science *290* , 2110-2113.

Holstege,F.C.P., Jennings,E.G., Wyrick,J.J., Lee,T.I., Hengartner,C.J., Green,M.R., Golub,T.R., Lander,E.S., and Young,R.A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell *95*, 717-728.

Johnson,M.A., Pérez-Amador,M.A., Lidder,P., and Green,P.J. (2000). Mutants of Arabidopsis defective in a sequence-specific mRNA degradation pathway. Proc. Natl. Acad. Sci. U. S. A *97*, 13991-13996.

Koshiba,T., Ballas,N., Wong,L.M., and Theologis,A. (1995). Transcriptional regulation of *Ps-IAA4/5* and *Ps-IAA6* early gene-expression by indoleacetic-acid and protein-synthesis inhibitors in pea (*Pisum-sativum*). J. Mol. Biol. *253*, 396-413.

Lam,L.T., Pickeral,O.K., Peng,A.C., Rosenwald,A., Hurt,E.M., Giltnane,J.M., Averett,L.M., Zhao,H., Davis,R.E., Sathyamoorthy,M., Wahl,L.M., Harris,E.D., Mikovits,J.A., Monks,A.P., Hollingshead,M.G., Sausville,E.A., and Staudt,L.M. (2001). Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. Genome Biol. *2*, 1-11.

Lee,M.L.T., Kuo,F.C., Whitmore,G.A., and Sklar,J. (2000). Importance of replication in microarray gene expression studies: Statistical methods and evidence from repetitive cDNA hybridizations. Proc. Natl. Acad. Sci. USA *97*, 9834-9839.

McClure,B.A. and Guilfoyle,T. (1989). Rapid redistribution of auxin-regulated RNAs during gravitropism. Science *243*, 91-93.

McClure,B.A., Hagen,G., Brown,C.S., Gee,M.A., and Guilfoyle,T.J. (1989). Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. Plant Cell *1*, 229-239.

Murfett,J., Wang,X.J., Hagen,G., and Guilfoyle,T.J. (2001). Identification of arabidopsis histone deacetylase HDA6 mutants that affect transgene expression. Plant Cell *13*, 1047-1061.

Newman,T.C., Ohme-Takagi,M., Taylor,C.B., and Green,P.J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell *5*, 701-714.

Ohme-Takagi,M., Taylor,C.B., Newman,T.C., and Green,P.J. (1993). The effect of sequences with high AU content on mRNA stability in tobacco. Proc. Natl. Acad. Sci. USA *90*, 11811-11815.

Peltz,S.W. and Jacobson,A. (1992). mRNA stability: in trans-it. Curr. Opin. Cell Biol. *4*, 979-983.

Pérez-Amador,M.A., Lidder,P., Johnson,M.A., Landgraf,J., Wisman,E., and Green,P.J. (2001). New molecular phenotypes in the dst mutants of Arabidopsis revealed by DNA microarray analysis. Plant Cell *13*, 2703-2717.

Pilgrim,M.L., Caspar,T., Quail,P.H., and McClung,C.R. (1993). Circadian and light-regulated expression of nitrate reductase in Arabidopsis. Plant Mol. Biol. *23*, 349-364.

Ross,J. (1995). Messenger-RNA stability in mammalian-cells. Microbiol. Rev. *59*, 423-450.

Samuels,M.L. (1989). Statistics for Life Science., M.L.Samuels, ed. (San Francisco: Dellen), pp. 504-505.

Schaffer,R., Landgraf,J., Accerbi,M., Simon,V., V, Larson,M., and Wisman,E. (2001). Microarray Analysis of Diurnal and Circadian-Regulated Genes in Arabidopsis. Plant Cell *13*, 113-123.

Schaffer,R., Landgraf,J., Pérez-Amador,M., and Wisman,E. (2000). Monitoring genome-wide expression in plants. Curr. Opin. Biotech. *11*, 162-167.

Schena,M., Shalon,D., Davis,R.W., and Brown,P.O. (1995). Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray. Science *270*, 467-470.

Seeley,K.A., Byrne,D.H., and Colbert,J.T. (1992). Red Light-Independent Instability of Oat Phytochrome mRNA in Vivo. Plant Cell *4*, 29-38.

Shaw,G. and Kamen,R. (1986). A conserved AU sequence from the 3' untranslated region of the GM-CSF messenger-RNA mediates selective messenger-RNA degradation. Cell *46*, 659-667.

Sherlock,G., Hernandez-Boussard,T., Kasarskis,A., Binkley,G., Matese,J.C., Dwight,S.S., Kaloper,M., Weng,S., Jin,H., Ball,C.A., Eisen,M.B., Spellman,P.T., Brown,P.O., Botstein,D., and Cherry,J.M. (2001). The Stanford Microarray Database. Nucl. Acids Res. *29*, 152-155.

Siflow,C.D. and Key,J.L. (1979). Stability of polysome-associated polyadenylated RNA from soybean suspension culture cells. Biochemistry *18*, 1013-1018.

So,W.V. and Rosbash,M. (1997). Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. EMBO J. *16* , 7146-7155.

Sullivan,M.L. and Green,P.J. (1996). Mutational analysis of the DST element in tobacco cells and transgenic plants: Identification of residues critical for mRNA instability. RNA *2*, 308-315.

Taylor,C.B. and Green,P.J. (1995). Identification and characterization of genes with unstable transcripts (*GUT*s) in tobacco. Plant Mol. Biol. *28*, 27-38.

The Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature *408*, 796-815.

Treisman,R. (1985). Transient accumulation of *c-fos* RNA following serum stimulation requires a conserved 5' element and *c-fos* 3' sequences. Cell *42*, 889-902.

van Helden,J., Andre,B., and Collado-Vides,J. (1998). Extracting regulatory sites from the upstream region of yeast genes by computational analysis of oligonucleotide frequencies. J. Mol. Biol. *281*, 827-842.

Vasudevan,S. and Peltz,S.W. (2001). Regulated ARE-mediated mRNA decay in Saccharomyces cerevisiae. Mol. Cell *7*, 1191-1200.

Weiss,I.M. and Liebhaber,S.A. (1994). Erythroid cell-specific determinants of alpha-globin messenger-RNA stability. Mol. Cell. Biol. *14*, 8123-8132.

CHAPTER 3

Circadian rhythms and control of mRNA decay: oscillation of the Arabidopsis *Ccr-like* and *SEN1* transcripts is dependent on normal DST-mediated mRNA degradation[III].

[III] This chapter is part of a bigger project developed in collaboration with Ms. Preet Lidder (PhD candidate. MSU-DOE Plant Research Laboratory). For completeness, in this Chapter, I discuss experiments carried out by Lidder P. hereinafter cited as "Lidder, P. and Green, P.J. unpublished results".

Introduction

Plants, like many other organisms, have internal clocks that command biological rhythms with a period close to 24 hr. These rhythms provide selective advantages because they allow anticipation of the daily changes in environmental conditions (Green *et al.*, 2002; Ouyang *et al.*, 1998). Examples of processes that can exhibit circadian rhythms in plants are leaf movement, hypocotyl elongation, stomatal opening and floral induction (reviewed in (McClung, 2001)). At the molecular level, DNA microarray experiments have shown that 2 to 6% of Arabidopsis mRNAs can oscillate (Schaffer *et al.*, 2001; Harmer *et al.*, 2000). The underlying "master" oscillator that controls these rhythms is believed to include the late elongated hypocotil or *LHY* (Schaffer *et al.*, 1998), the circadian clock-associated protein 1 or *CCA1* (Wang and Tobin, 1998) and the timing of CAB expression 1 or *TOC1* (Millar *et al.*, 1995; Strayer *et al.*, 2000) genes. Similar to what is observed in other systems (Glossop *et al.*, 1999), LHY, CCA1 and TOC1 proteins would form a regulatory loop in which TOC1 positively regulates *LHY* and *CCA1* gene expression, and LHY and CCA1 proteins in turn repress *TOC1* gene expression (Alabadi *et al.*, 2001). This loop would output cyclic positive or negative expression commands to the downstream targets.

Transcriptional regulation is a well established mechanism for the circadian expression of *LHY*, *CCA1*, *TOC1* and other clock-controlled genes (CCGs). Recently, a novel sequence element termed the evening element was identified in the promoter region of 31 Arabidopsis circadian genes, 30 of which peaked at the end of the subjective day (Harmer *et al.*, 2000). This element was shown to be important for cycling of the *CCR2* and *TOC1* promoter activities (Harmer *et al.*, 2000; Alabadi *et al.*, 2001) and has been

proposed as the binding site for LHY and CCA1 transcription factors (Alabadi *et al.*, 2001).

In addition to the cycling in transcriptional activity, it is also clear that post-transcriptional mechanisms that affect mRNA accumulation can play a role in CCG expression. The best characterized example corresponds to the *per* gene (reviewed in (Stanewsky, 2002)), one of the components of the pacemaker in *Drosophila* (Panda *et al.*, 2002). The mRNA of the *Drosophila* clock gene *per* can oscillate in the absence of its natural promoter region (So and Rosbash, 1997). Furthermore, the 5' upstream portion of the gene is not sufficient to confer wild-type like oscillation of a luciferase reporter transcript (Stanewsky *et al.*, 1997). Regulatory elements located in both promoter and transcribed regions of *per* gene are necessary to replicate wild-type cycling (So and Rosbash, 1997; Stanewsky *et al.*, 1997). The functional significance of this post-transcriptional level of regulation has been well documented (Stanewsky, 2002). For example, a *per*-transgene completely devoid of its promoter sequences but including parts of the first intron is capable of restoring rhythmic behavior in per^{01} mutant flies solely through post-transcriptional cycling of its mRNA (Frisch *et al.*, 1994; So and Rosbash, 1997). In addition, disruption of the 3' untranslated region of the *per* gene affects circadian behavioral rhythms in Drosophila (Chen *et al.*, 1998). Hence post-transcriptional mechanisms are not only important for normal circadian gene expression but also for proper gene function.

Post-transcriptional mechanisms have also been invoked to explain circadian oscillation of a plant gene. Run-on experiments have shown that transcription makes a small contribution to the observed circadian expression pattern of the Arabidopsis *NIA2*

gene (Pilgrim *et al.*, 1993). Interestingly, overexpression of the Arabidopsis RNA binding protein *At*GRP7/*CCR2* can negatively affect accumulation of its own mRNA and that of the CCG *At*GRP8 (Heintzen *et al.*, 1997). Effectors of mRNA cycling at the post-transcriptional level are likely to include clock-controlled RNA binding proteins.

Although no examples of changes in mRNA stability regulated by the circadian clock have been reported, recent evidence suggests a more prominent role of control of mRNA stability in CCG expression in Arabidopsis. Genes with altered expression in *dst1*, a mutant deficient in DST-mediated mRNA decay, were biased towards CCGs (Pérez-Amador *et al.*, 2001). In addition, 16 out of 100 genes with unstable transcripts recently identified are known clock-controlled genes (Gutiérrez *et al.*, 2002). This is a higher than expected proportion of CCGs with labile mRNAs based on current estimates of total CCGs in Arabidopsis (Schaffer *et al.*, 2001; Harmer *et al.*, 2000). Together these data suggest rapid mRNA turnover, perhaps at specific times of the day, and the DST-mediated rapid mRNA decay, might be important for the specific circadian oscillation of CCGs in Arabidopsis.

To explore the relationship between mRNA stability and circadian gene expression we analyzed the stability of selected CCGs with unstable transcripts in Arabidopsis plants at two times during the day. We found two CCGs that exhibit circadian regulation of mRNA stability. In addition, analysis of mRNA levels throughout the day in wild-type and *dst1* mutant lines for these genes uncovered a previously unknown connection between diurnal mRNA oscillations and the sequence specific mRNA degradation mediated by the DST element.

Materials and Methods

Arabidopsis strains and growth conditions

Arabidopsis thaliana ecotype Columbia, and *dst1* mutant and 1519 parental lines (Johnson *et al.*, 2000) were grown on agar plates containing 1x Murashige and Skoog salts, 1x Gamborg's vitamins and 1% sucrose for two weeks at 22 °C and 16h light and 8h dark cycles unless indicated otherwise. Lighting was provided by fluorescent light bulbs.

Half-life measurements and preparation of RNA samples.

Half-lives were determined as described by Seeley *et al.* (Seeley *et al.*, 1992) with the following modifications. Two-week old Arabidopsis plants were transferred to a flask with incubation buffer (Seeley *et al.*, 1992). After a 30 min incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and quickly frozen in liquid nitrogen. Total RNA was isolated and analyzed by northern blot using standard techniques.

Results

Stability of *Ccr*-like and *SEN1* mRNAs changes during the day.

Previously, it was reported that some Arabidopsis genes that encode highly unstable mRNAs are also regulated by the circadian clock (Gutiérrez *et al.*, 2002). To determine whether regulation of mRNA stability contributes to the expression of these genes we measured mRNA decay rates at two times during the day. Two-week old plants grown on 16 h light / 8 h darkness cycles (16/8 LD) were used for cordycepin time courses one hour after dawn (zeitgeber time 1 or ZT1) as detailed in the *Materials and Methods*. Similar experiments were performed 8 h after dawn (ZT8). As shown in Figure 3.1*a-c*, the putative light regulated gene similar to the *ccr* gene from *Citrus paradisi* (*Ccr*-like; At3g26740), exhibited mRNA stability changes during the day. The transcript for this gene was significantly more stable in the morning, ZT1, as compared to the afternoon (ZT8) (Figure 3.1*c*). Similarly, turnover of the senescence associated gene 1 (*SEN1*; At4g35770) mRNA was also differentially regulated during the day (Figure 3.2*a-c*). The transcript was also more stable in the morning as compared to the afternoon. These data indicate regulation of mRNA stability is important for the diurnal oscillation of *Ccr*-like and *SEN1*, two Arabidopsis genes that are controlled by the circadian clock (Schaffer *et al.*, 2001; Harmer *et al.*, 2000). In contrast, no difference in mRNA turnover rates was observed for the clock controlled *NIA2* gene that encodes nitrate reductase 2 (At1g37130, Figure 3.3*a-c*) and for the light regulated *ATHB6* gene which encodes a homeodomain transcription factor (At2g22430, Figure 3.3*c*). The mRNA for these genes decayed at similar rates in the morning and in the afternoon in the conditions tested.

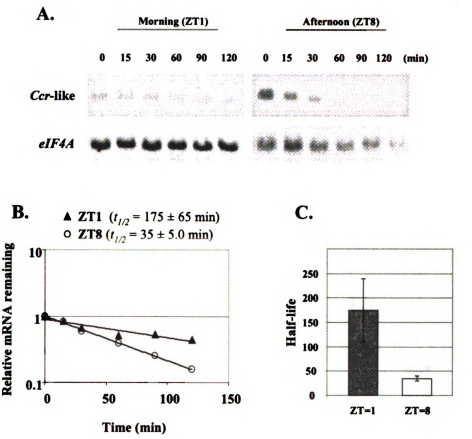


Figure 3.1. Ccr-like mRNA stability is regulated during the day. (A) Representative northern blot analysis of cordycepin time courses performed in the morning, 1 hour after dawn (zeitgeber time1 or ZT1), and in the afternoon, 8 hours after dawn (ZT8) for *Ccr*-like and the loading control *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for *eIF4A* does not change significantly during the time courses and was used as a reference for equal loading. (C) Half-life values determined by northern blot indicate *Ccr*-like mRNA is more stable in the morning than in the afternoon. Values are representative of three independent cordycepin time courses.

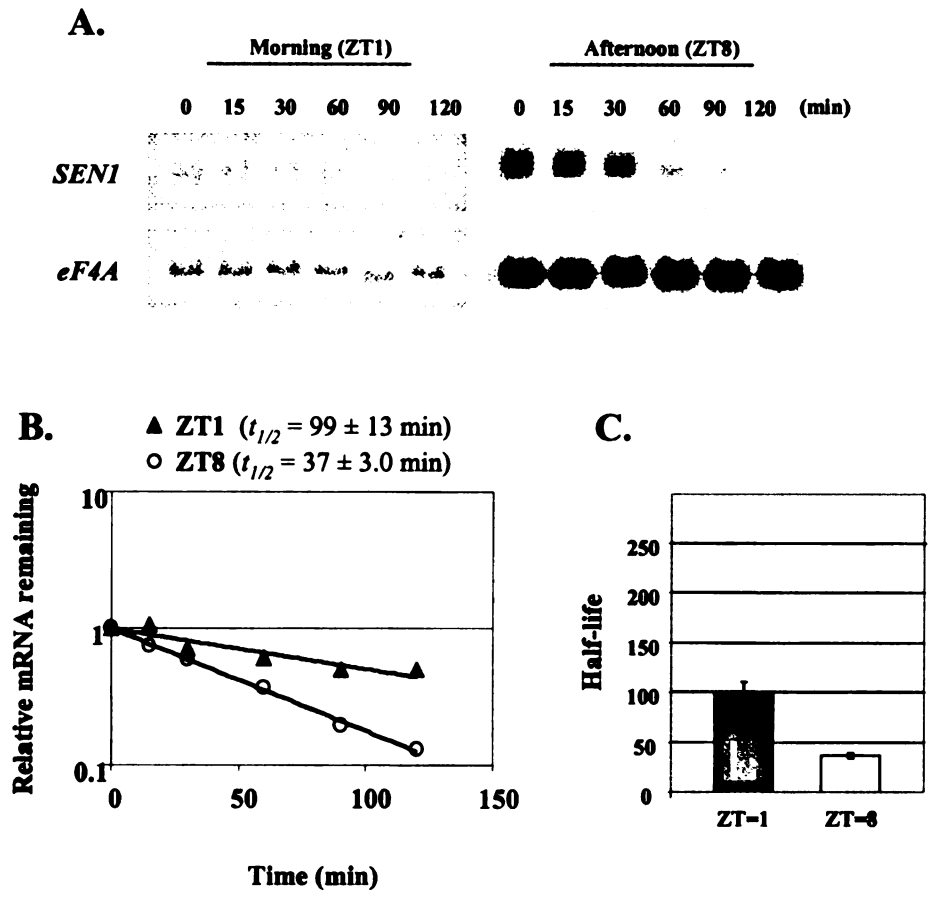


Figure 3.2. SEN1 mRNA stability is regulated during the day. (A) Representative northern blot analysis of cordycepin time courses performed in the morning, 1 hour after dawn (ZT1), and in the afternoon, 8 hours after dawn (ZT8), for *SEN1* and *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for the stable *eIF4A* mRNA was used as a reference for equal loading. (C) Half-life values indicate *SEN1* mRNA is more stable in the morning than in the afternoon. Values are representative of three independent cordycepin time courses.

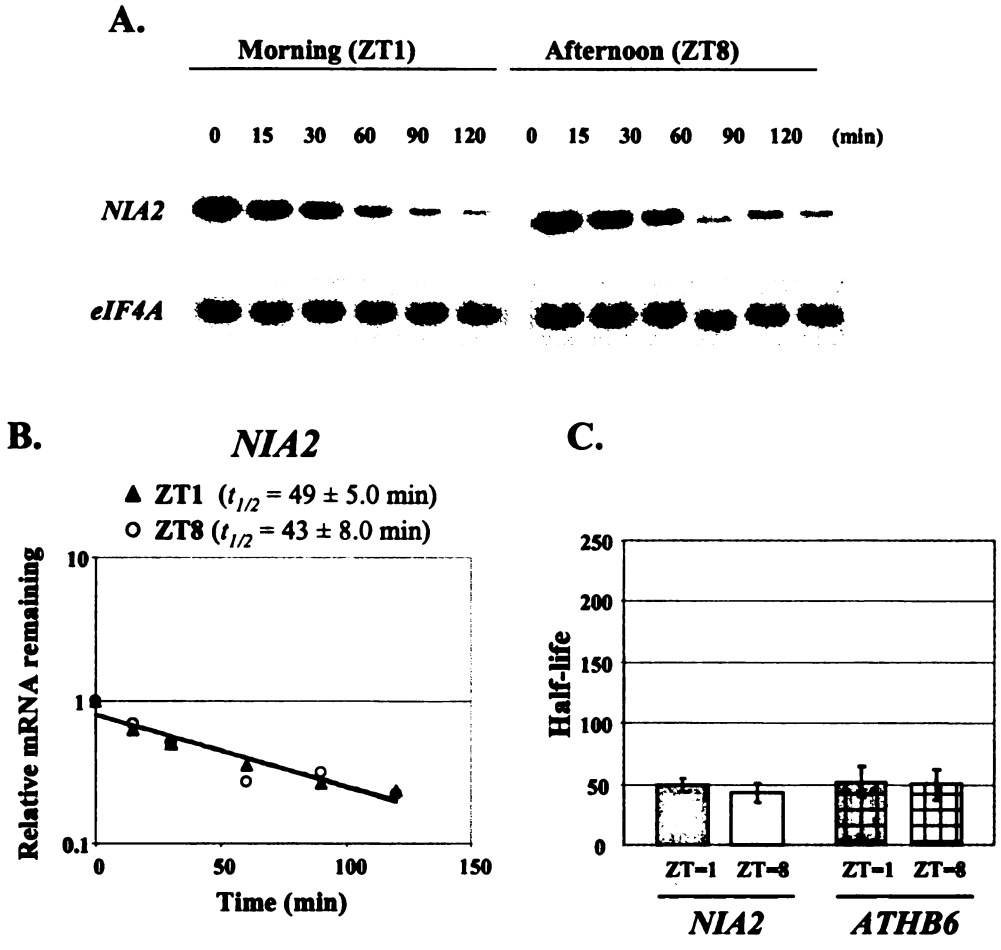


Figure 3.3. NIA2 mRNA stability is comparable in the morning and in the afternoon. (A) Representative northern blot analysis of cordycepin time courses performed in the morning, 1 hour after dawn (ZT1), and in the afternoon, 8 hours after dawn (ZT8), for *NIA2* and *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points in a cordycepin time course. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for *eIF4A* was used as a reference for equal loading. (C) Half-life values determined by northern blot indicate *NIA2* and *ATHB6* mRNAs decay at similar rates in the morning and in the afternoon. Values are representative of three independent cordycepin time courses.

The well characterized clock gene *LHY* was used to control for timing of the experiments. *LHY* mRNA has a circadian expression pattern with a peak around dawn and with very low levels throughout most of the day (Schaffer *et al.*, 1998). As expected, *LHY* was easily detectable in the morning and was near background levels in the afternoon (Figure 3.4*a*). *LHY* mRNA was relatively stable in the morning ($t_{1/2}$ > 130 min. Figure 3.4*b*), but low levels precluded determination of its half-life in the afternoon experiments. Together these experiments indicate the half-life changes observed are not the result of differences in the global cellular mRNA turnover rates in the morning and afternoon. They also indicate that regulation at the level of mRNA stability is not a general property but rather specific to some clock-controlled genes.

Ccr-like and *SEN1* mRNA stability changes are dictated by the circadian clock.

Two possible mechanisms could explain the changes in mRNA stability observed during the day for *Ccr*-like and *SEN1* genes. Signaling pathways activated by the changes in light patterns during the normal day cycle could be responsible as previously observed for the pea *FED1* (Elliott *et al.*, 1989) and other genes (Silverthorne and Tobin, 1990; Vorst *et al.*, 1993). Alternatively, the circadian clock could promote the change. To discriminate between these two possibilities, mRNA half-lives were determined under free running conditions. Arabidopsis plants were grown for 12 days in 16/8 LD cycles. In the morning of the 12th day they were transferred to continuous light conditions. Half-lives were then measured 1 (circadian time 1 or CT1) and 8 (CT8) hours after the subjective dawn of the 14th day. As shown in Figures 3.5 and 3.6, both *Ccr*-like and

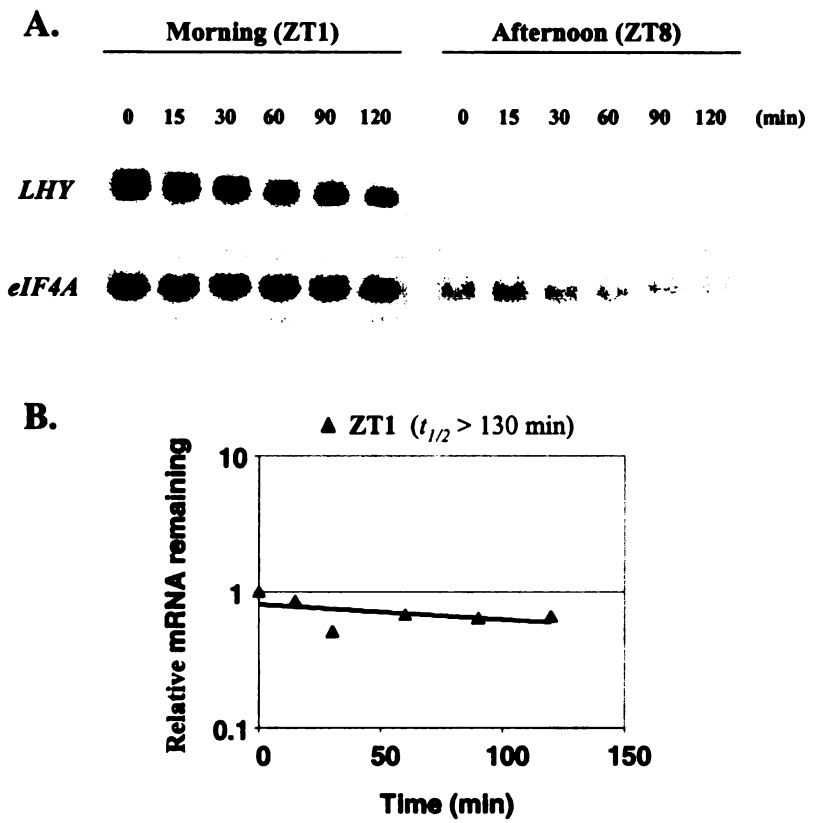


Figure 3.4. LHY mRNA is highly expressed in the morning and decreases to background levels in the afternoon. (A) Representative northern blot analysis of cordycepin time courses performed in the morning, 1 hour after dawn (ZT1), and in the afternoon, 8 hours after dawn (ZT8), for *LHY* and *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for *eIF4A* does not change significantly during the time courses and was used as a reference for equal loading. Values are representative of three independent cordycepin time courses.

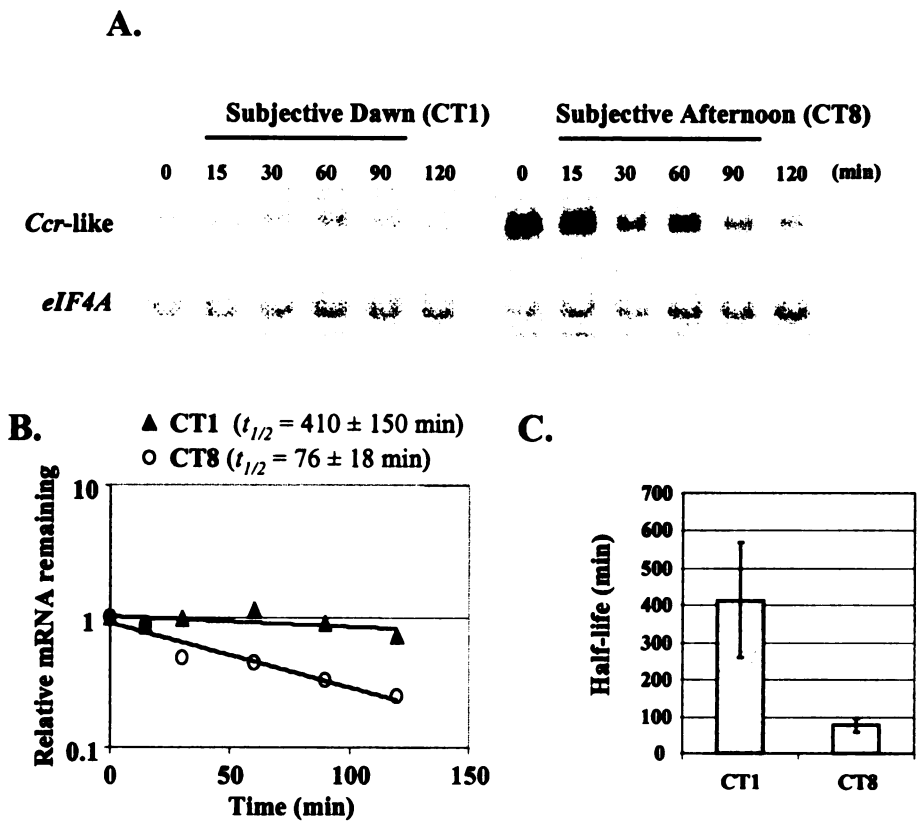


Figure 3.5. Ccr-like mRNA stability is regulated by the Arabidopsis circadian clock. (A) Representative northern blot analysis of cordycepin time courses performed in the subjective morning, 1 hour after subjective dawn (circadian time 1 or CT1), and in the subjective afternoon, 8 hours after subjective dawn (CT8) for *Ccr*-like and *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points in a cordycepin time course. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The stable *eIF4A* transcript was used as a reference for equal loading. (C) Half-life values indicate *Ccr*-like mRNA is more stable in the subjective morning than in the subjective afternoon. Values are representative of three independent cordycepin time courses.

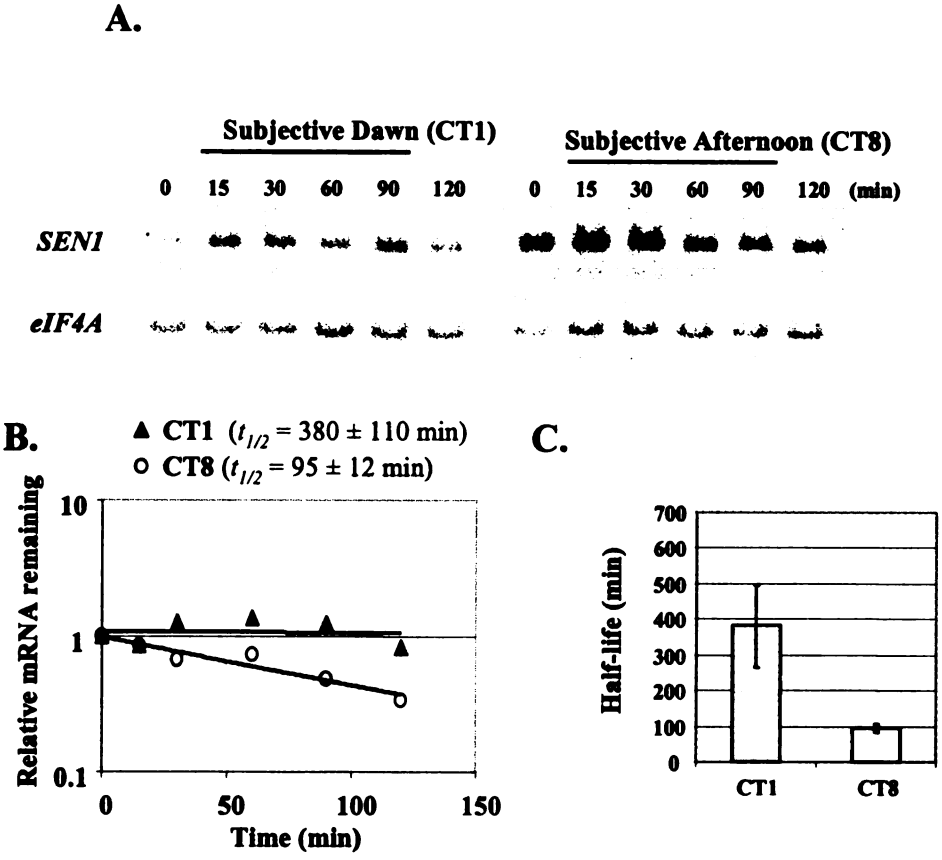


Figure 3.6. SEN1 mRNA stability is regulated by the Arabidopsis circadian clock. (A) Representative northern blot analysis of cordycepin time courses performed in the subjective morning, 1 hour after subjective dawn (CT1), and in the subjective afternoon, 8 hours after subjective dawn (CT8), for *SEN1* and *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for *eIF4A* does not change significantly during the time courses and was used as a reference for equal loading. (C) Half-life values indicate *SEN1* mRNA is more stable in the subjective morning than in the subjective afternoon. Values are representative of three independent cordycepin time courses.

SEN1 mRNA stability was regulated under continuous light conditions as seen previously in the day/night cycles. The transcripts were significantly more stable in the subjective morning (CT1) as compared to the subjective afternoon (CT8). In addition, *Ccr*-like and *SEN1* mRNAs were more stable in continuous light as compared to the equivalent times of the day under regular 16/8 LD cycles. In contrast to *SEN1* and *Ccr*-like mRNAs, the stability of the *NIA2* transcript was not affected by the time of the day and was comparable at CT1 and CT8 (Figure 3.7). *LHY* expression was readily detectable at CT1 and close to background levels at CT8, consistent with the planned timing of the experiments (Figure 3.7*a*). As before, *LHY* mRNA was relatively stable in the subjective morning ($t_{1/2}$ > 180 min, Figure 3.7*b*). This data indicates regulation of *Ccr-like* and *SEN1* mRNA stability is controlled by the Arabidopsis circadian clock.

***DST1* function is involved in the normal oscillatory expression of *Ccr*-like and *SEN1* genes.**

Ccr-like and *SEN1* genes contain DST-like sequences in the 3'UTR and showed altered expression levels in *dst1* mutant as compared to wild-type (Pérez-Amador *et al.*, 2001). In addition both genes encode unstable mRNAs in the afternoon (Figure 3.1 and 3.2). These features suggest they are targets of the DST-mediated decay pathway. To test whether *DST1* function is necessary for the normal diurnal expression of *Ccr*-like and *SEN1*, mRNA levels were examined throughout the day in *dst1* mutant and 1519 parental lines. Two-week old Arabidopsis plants grown on 16/8 LD cycles were harvested every two hours after dawn. Total RNA was isolated and mRNA levels were examined by

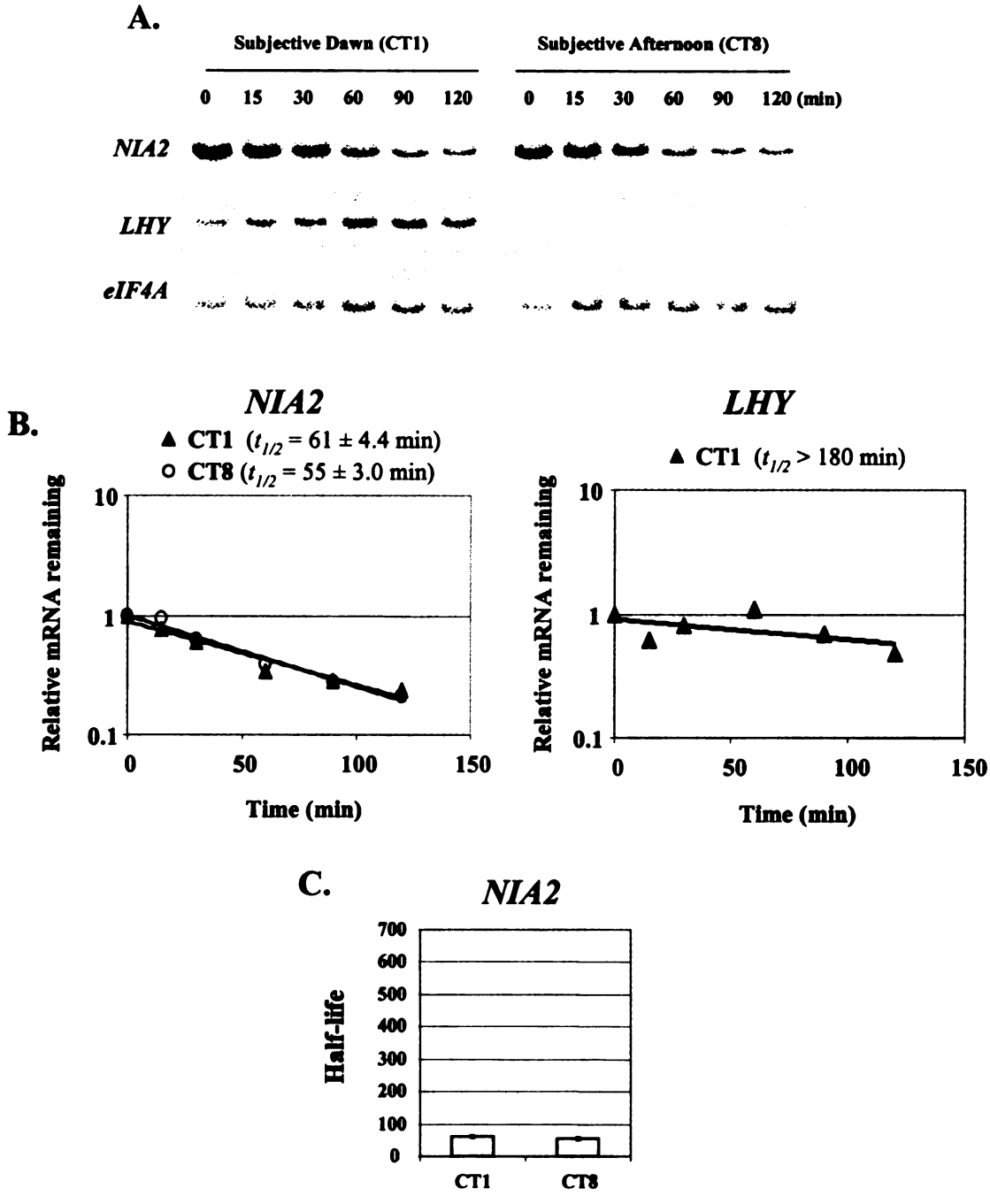


Figure 3.7. LHY mRNA is highly expressed in the subjective morning and decreases to background levels in the subjective afternoon. NIA2 mRNA stability is comparable in the two conditions. (A) Representative northern blot analysis of cordycepin time courses performed in the morning, 1 hour after dawn (ZT1), and in the afternoon, 8 hours after dawn (ZT8), for *NIA2*, *LHY* and *eIF4A* mRNA. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The stable *eIF4A* mRNA was used as a reference for equal loading. (C) Half-life values indicate *NIA2* mRNA decays at similar rates in the subjective morning and in the subjective afternoon. Values are representative of three independent cordycepin time courses.

northern blotting. As shown in Figure 3.8*a-b*, *Ccr*-like peaked late during the day in the parental 1519 line. In contrast, in the *dst1* mutant *Ccr*-like mRNA started to accumulate later and peaked at least 2 hours later than in the 1519 line (Figure 3.8*a-b*). In addition to the delay in the phase, the amplitude of *Ccr*-like mRNA oscillation was also reduced as compared to the parental (Figure 3.8*b*).

The impact of the *dst1* mutation on *SEN1* mRNA oscillation was less dramatic but nevertheless significant. We considered the curves of mRNA levels throughout the day to be significantly different between the two lines when the error bars of three consecutive time points or more did not overlap. *SEN1* gene was greatly induced during the dark period as reported previously (Oh *et al.*, 1996; Schaffer *et al.*, 2001) (Figure 3.9*a-b*).. Transcriptional control is thought to be the main mechanism responsible for this dark induction (Chung *et al.*, 1997) and as shown in Figure 3.9*a-b* was mostly unaffected by the *dst1* mutation. In contrast to the dark-induced mRNA levels, the clock-controlled accumulation of *SEN1* mRNA in the afternoon (Harmer *et al.*, 2000) was abolished in the *dst1* mutant (Figure 3.9*a-b*). These data indicate *DST1* function is required for the normal oscillation of *Ccr*-like and *SEN1* mRNAs.

The effect of the *dst1* mutation is specific to a subset of DST1 targets

To further understand the impact of the *dst1* mutation on the diurnal oscillation of targets of the DST-mediated decay pathway, we examined mRNA levels throughout the day for the *SAUR-AC1* gene (Johnson *et al.*, 2000; Pérez-Amador *et al.*, 2001). This gene was first shown to be controlled by the circadian clock in the microarray experiments of Harmer et al. (2000). *SAUR-AC1* transcript levels were monitored throughout the day by

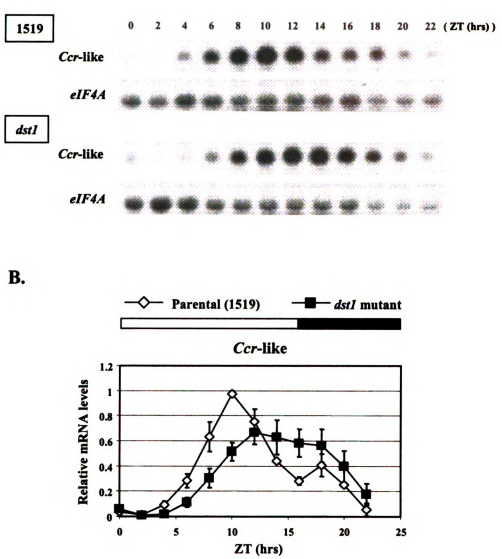


Figure 3.8. Diurnal oscillation of Ccr-like mRNA is altered in the dst1 mutant. (A) Representative northern blot analysis of time courses performed throughout an entire day for *Ccr-like* mRNA and *eIF4A* mRNA in *dst1* mutant and parental 1519 plants. Samples consisted of 10 µg of total RNA isolated from the indicated times of the day after dawn (ZT=0). (B) Quantitation of mRNA levels. Data from three independent experiments was used for the time points ZT=0 to ZT=16. Data from two independent experiments was used for the latest time points (ZT=18, ZT=20 and ZT=22). All values are relative to the highest mRNA accumulation in either of the two genetic backgrounds. The signal for *eIF4A* was used as a reference for equal loading. The rectangle above the graph illustrates the 16h day (white segment) and 8h night (grey segment) period used in these experiments.

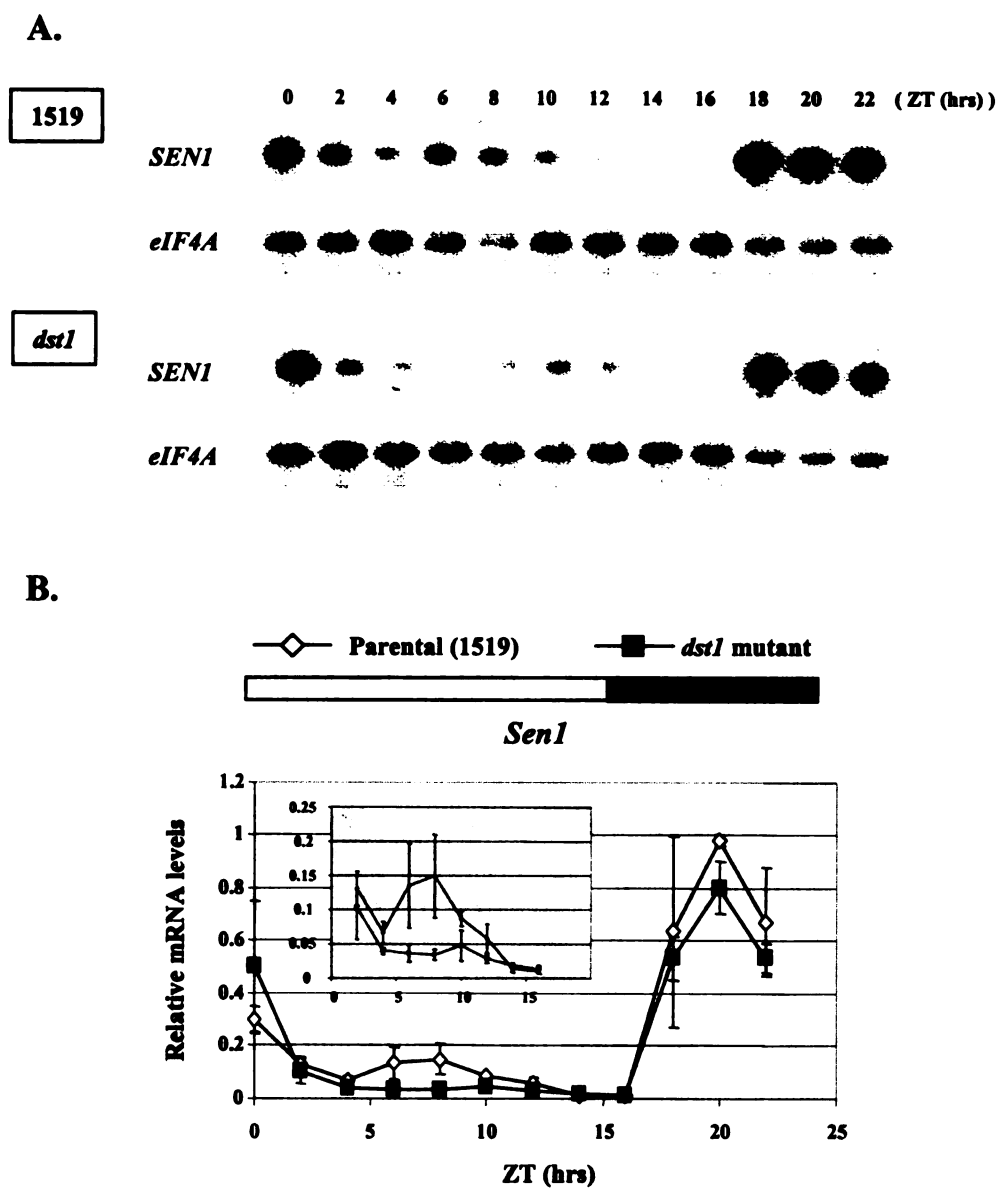


Figure 3.9. Diurnal oscillation of SEN1 mRNA is altered in the dst1 mutant. (A) Representative northern blot analysis of time courses performed throughout an entire day for *SEN1* mRNA and *eIF4A* mRNA in *dst1* mutant and parental 1519 plants. Samples consisted of 10 µg of total RNA isolated from the indicated times of the day after dawn (ZT=0). (B) Quantitation of mRNA levels. Data from three independent experiments was used for the time points ZT=0 to ZT=16. Data from two independent experiments was used for the latest time points (ZT=18, ZT=20 and ZT=22). All values are relative to the highest mRNA accumulation in either of the two genetic backgrounds. The signal for *eIF4A* was used as a reference for equal loading. Inset shows a magnification of *SEN1* mRNA levels between ZT=2 and ZT=16. Y- and X-axis in the inset are "Relative mRNA levels" and "ZT (hrs)" respectively. The rectangle above the graph illustrates the 16h day (white segment) and 8h night (grey segment) period used in these experiments.

northern blotting as described before. As shown in Figure 3.10*a*, *SAUR-AC1* oscillation was similar in mutant and parental plants. These data suggest the *dst1* mutation does not disturb the diurnal expression patterns of all DST1 targets, but is associated with a subset of them.

Diurnal expression of other CCGs is not compromised in the *dst1* mutant.

As an initial effort to evaluate the impact of the *dst1* mutation on general CCG expression, the diurnal oscillation of additional CCGs was investigated in mutant and parental lines. Two-week old *dst1* and 1519 parental plants grown on 16/8 LD cycles were harvested every two hours after dawn. Total RNA was isolated and transcript levels were examined for *At*GRP7/*CCR2*, a well characterized CCG that oscillates with opposite phase to *LHY* and *CCA1* and that is thought to be a slave oscillator downstream of the master clock (Heintzen *et al.*, 1997). As shown in Figure 3.10*b*, *At*GRP7/*CCR2* mRNA oscillation was nearly indistinguishable in *dst1* mutant and parental lines. Oscillation of *LHY* and *CCA1* expression, two genes thought to be components of the central clock, was also examined. As shown in Figure 3.10*c-d*, *LHY* and *CCA1* mRNA oscillation was similar in the *dst1* mutant and in the parental 1519 line and was consistent with what previously described (Wang and Tobin, 1998; Schaffer *et al.*, 1998). Interestingly, albeit similar in amplitude and phase, both *LHY* and *CCA1* mRNAs appeared to start accumulating later in the *dst1* mutant as compared to the parental. These results indicate that *dst1* mutant affect diurnal expression of selected Arabidopsis CCGs.

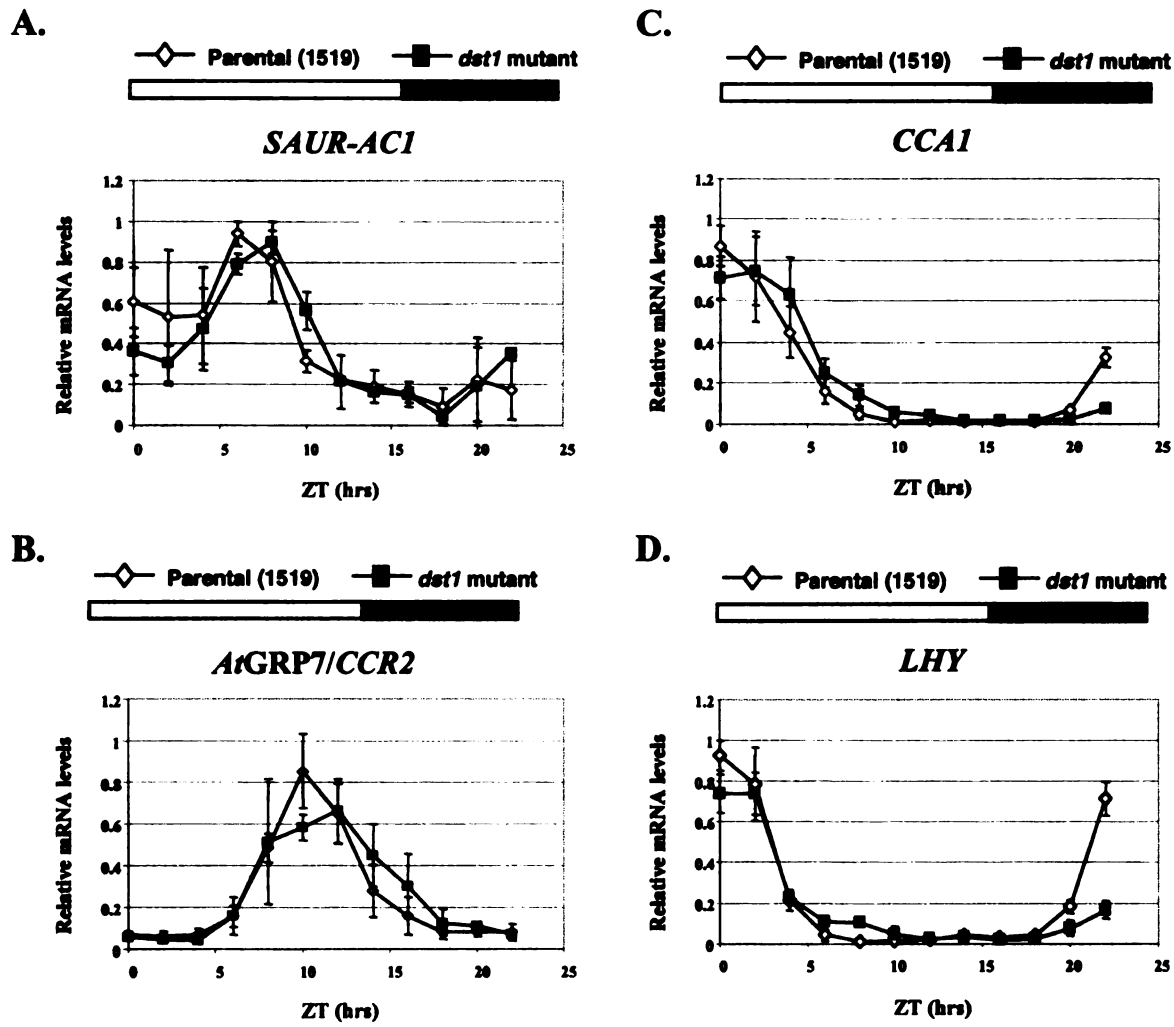


Figure 3.10. Diurnal oscillation of SAUR-AC1, AtGRP7/CCR2, CCA1 and LHY in dst1 mutant and 1519 parental plants. Transcript levels throughout an entire day for (A) *SAUR-AC1*, data represented corresponds to two independent experiments. (B) *At*GRP7/*CCR2*, (C) *CCA1*, and (D) *LHY*. mRNA levels were determined by northern blot analysis of time course experiments as described earlier (*e.g.* legend to Figure 3.9).

Regulation of *SEN1* mRNA stability is defective in the *dst1* mutant

To determine whether altered mRNA degradation in the *dst1* mutant plays a role in the abnormal diurnal expression of *SEN1* mRNA, half-life measurements were carried out at ZT1 and ZT8 in both *dst1* and 1519 plants. Preliminary results are presented in Figure 3.11 with permission from the author (Preet, L. and Green, P.J. unpublished results). Consistent with previous experiments (Figure 3.2), *SEN1* mRNA was rapidly degraded in the afternoon (ZT8) and stabilized in the morning (ZT1) in parental plants (Figure 3.11*a*). Interestingly, the opposite was true for the *dst1* mutant (Figure 3.11*b*). *SEN1* mRNA decayed faster in the morning (ZT1) than in the afternoon (ZT8). Moreover, *SEN1* mRNA decay measured in the morning in *dst1* plants was comparable to the rate of decay measured in the afternoon in 1519 plants (Figure 3.11*c*). This data suggests normal *DST1* function is required for proper timing of the degradation of *SEN1* mRNA. This data further suggests that the aberrant oscillation of *SEN1* mRNA in *dst1* might be caused at least partly by a defective regulation of its mRNA stability.

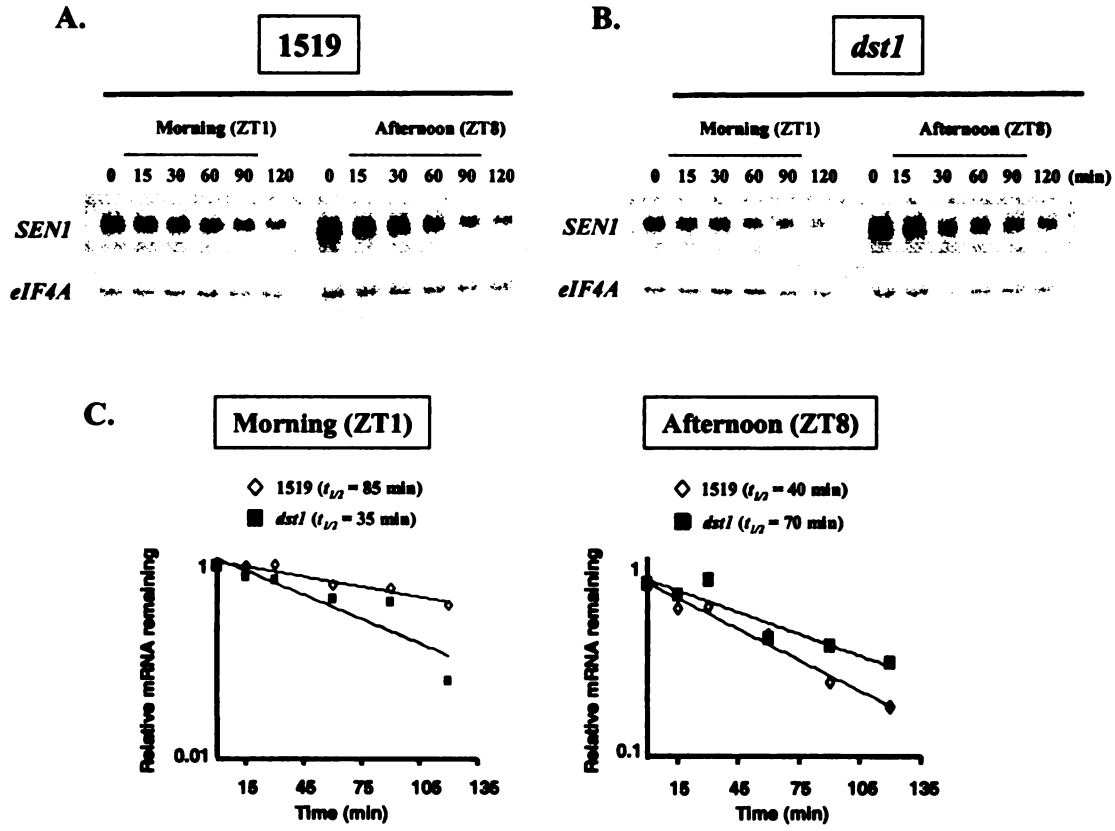


Figure 3.11. Regulation of SEN1 mRNA stability is altered in the dst1 mutant. (A) Northern blot analysis of cordycepin time course performed in 1519 plants, 1 hour after dawn (ZT1) and 8 hours after dawn (ZT8), for *SEN1* and *eIF4A* mRNAs. (B) Northern blot analysis of cordycepin time course performed in *dst1* plants, 1 hour after dawn (ZT1) and 8 hours after dawn (ZT8), for *SEN1* and *eIF4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (C) Quantitation of the decrease in mRNA abundance and half-life estimation. The signal for *eIF4A* does not change significantly during the time courses and was used as a reference for equal loading.

Discussion

It is clear that transcriptional control plays an important role in the circadian expression of many genes. Indeed, a transcriptional regulatory loop lays at the heart of the master oscillator in plants and other systems ((Alabadi *et al.*, 2001); reviewed in (Panda *et al.*, 2002)). However, transcription alone is not sufficient to explain the oscillation in mRNA levels of every clock gene (Pilgrim *et al.*, 1993; So and Rosbash, 1997). In fact, both transcriptional and post-transcriptional control of mRNA accumulation are necessary for normal *per* function and circadian behavior in flies (So and Rosbash, 1997; Stanewsky *et al.*, 1997; Chen *et al.*, 1998). Although it is now clear that post-transcriptional mechanisms can contribute significantly to the expression of clock genes, to date there is no direct evidence that the stability of *per* transcript, or that of any other gene, can be controlled by the circadian clock.

We have shown that *Ccr*-like and *SEN1* mRNA decay is regulated as a function of the time of the day. Both *Ccr*-like and *SEN1* mRNAs were stable in the morning at ZT1 and highly unstable in the afternoon at ZT8 in Arabidopsis plants grown in 16/8 LD cycles. This change in mRNA turnover rates prevailed in free running conditions indicating that it is controlled by the circadian clock. *Ccr*-like and *SEN1* mRNAs were more stable in the subjective morning (CT1) as compared to the subjective afternoon (CT8) two days after plants entrained in 16/8 LD cycles were transferred to continuous light. In contrast to *Ccr*-like and *SEN1*, the *NIA2* and *ATHB6* mRNAs decayed at similar rates at ZT1 and ZT8. Curiously, previous run-on experiments in 5 weeks plants grown on soil indicated that *NIA2* transcription was relatively constant throughout the day (Pilgrim *et al.*, 1993). These results suggested that mRNA stability changes are

responsible for the approximately 2-fold circadian oscillation of the *NIA2* mRNA (Pilgrim *et al.*, 1993). However, our results suggest that under the conditions studied, regulation of mRNA stability does not contribute to diurnal oscillation of the *NIA2* transcript. Developmental regulation could account for the discrepancy. Alternatively, the presence of sucrose in our experiments could override the circadian regulation. Sucrose in the growth media have been shown to induce nitrate reductase activity irrespective of the light conditions (Crawford *et al.*, 1992). Together these results indicate that the changes observed are not the result of a global change in mRNA turnover rates. Instead regulation of mRNA stability is a property of selected clock-controlled genes. This data further suggests that post-transcriptional control of mRNA stability is important for the circadian expression of *Ccr*-like and *SEN1* genes. Circadian rhythms allow anticipation of the daily changes in environmental conditions, thus providing an adaptive advantage (Ouyang *et al.*, 1998). Consistent with this anticipatory nature of clock-controlled processes, increased mRNA stability preceded the circadian mRNA accumulation phase and rapid turnover was followed by the decrease in transcript levels.

Intriguingly, *Ccr*-like and *SEN1* mRNAs were more stable in continuous light as compared to the equivalent times of the day under regular 16/8 LD cycles. An appealing hypothesis to explain this moderate stabilization would be that the circadian clock promotes mRNA degradation. In the absence of the zeitgeber (time-giver), the clock-induced degradation could dampen or shift phase over time leading to the observed partial stabilization. Dampening and shift in the phase of circadian phenotypes is commonly observed under free running conditions. In this scenario, regulation of mRNA stability could still be achieved if the decay machinery but not the circadian mechanism

that triggers the decay is altered. Alternatively, continuous light or the absence of a dark period might repress or otherwise impair the normal function of the mRNA decay machinery involved in *Ccr*-like and *SEN1* transcript degradation. Further research is required to distinguish between these possibilities.

Ccr-like and *SEN1* mRNAs are thought to be targets of the DST-mediated mRNA degradation pathway (Pérez-Amador *et al.*, 2001; Gutiérrez *et al.*, 2002). To evaluate the relevance of this sequence-specific mRNA decay pathway for the expression of *Ccr*-like and *SEN1* genes, the diurnal oscillation of their transcripts was studied in *dst1*, a mutant defective in DST-mediated mRNA degradation (Johnson *et al.*, 2000). We showed that *Ccr*-like and *SEN1* mRNA oscillations were altered in *dst1* mutant as compared to parental plants. In contrast, oscillation of *SAUR-AC1* transcript, another target of this mRNA decay pathway, was comparable in *dst1* mutant and 1519 parental line. These results indicate that *DST1* function is necessary for the normal diurnal oscillation of selected targets of the DST-mediated decay pathway. The reason for this selectivity is not clear at this point. No obvious bias in the 3'UTR sequences could be detected. In addition, two copies of the DST sequence element failed to confer diurnal oscillation to a reporter mRNA (data not shown). Therefore sequence requirements can not be predicted. However previous studies have suggested different modes of recognition of the DST sequence in different cell types (Feldbrügge *et al.*, 2002; Sullivan and Green, 1996). There is also precedent for differences in DST-element function depending upon the sequence context in which is present (Newman *et al.*, 1993). Further experiments are needed to understand the function of the DST-element and the molecular basis of this selectivity.

Based on the characteristics of the *dst1* mutant, a plausible explanation for the molecular phenotypes discussed above is defective mRNA decay. In fact, preliminary experiments argue that *DST1* function is important for proper timing of mRNA degradation. *SEN1* mRNA was stable in the afternoon and very unstable in the morning in the *dst1* mutant, a pattern of regulation that was completely opposite to what is seen in parental plants (Preet, P. and Green, P.J. unpublished results). This alteration in the pattern of regulation of *SEN1* mRNA decay can explain the difference in mRNA levels throughout the day in *dst1* mutant as compared to parental. In wild type plants, accumulation of mRNA during the morning is preceded by stabilization of the message and decrease in the transcript levels during the afternoon is preceded by *SEN1* mRNA rapid turnover. In contrast, in the *dst1* mutant, rapid decay of *SEN1* transcript in the morning would prevent the message from accumulating early during the day. The increase in *SEN1* mRNA stability in the afternoon in the *dst1* mutant should lead to accumulation of the transcript with a peak later during the day. However no accumulation of *SEN1* is evident in *dst1* mutant during the 16 h light period. A possible explanation for this result is that *SEN1* mRNA accumulates towards the end of the day and it is masked by the strong accumulation induced during the dark period in normal day/night cycles. Alternatively, the stabilization of *SEN1* mRNA could be temporally shorter in the *dst1* mutant as compared to parental and insufficiently long for detectable increase in mRNA levels. Additional experiments conducted in continuous light conditions should help distinguish between these two possibilities.

With regard to the relationship between *DST1* and the circadian clock, one interpretation of the data is that *DST1* functions downstream of the master circadian

oscillator, receives signals from the clock and affects the expression of specific CCGs at the post-transcriptional level. This model would predict that *dst1* plants do not have a severe clock defect. Normal oscillation of *SAUR-AC1* and *At*GRP7/*CCR2* are consistent with this model. In addition, preliminary mapping experiments of *dst1* are also in accordance with such explanation. *DST1* would be located at the bottom of Chromosome I, far from the genes known to be important for clock function (Lidder, P. and Green, P.J. unpublished results). However, mutants in clock-associated genes can affect diurnal oscillation of CCGs (Somers *et al.*, 2000; Alabadi *et al.*, 2002; Mizoguchi *et al.*, 2002). The clock phenotypes of such mutants, are often more evident under free-running conditions (Harmer *et al.*, 2001). Hence, some circadian alterations in the *dst1* mutant might not be readily detected in the presence of the zeitgeber, due to resetting of the phase every morning. This could explain that despite reaching similar levels and peaking at the same time of the day, *LHY* and *CCA1* mRNAs appeared to start the accumulation phase later in *dst1* than in 1519 plants. In this model, *dst1* would be associated with or affect the function of the central oscillator. This in turn would affect downstream circadian output pathways including oscillation of CCGs. To better understand the relationship between the *dst1* mutation and circadian rhythms, classical circadian phenotypes are currently being analyzed in collaboration with Dr. C Robertson McClung (Dept. of Biological Sciences, Dartmouth College).

Regardless of the mechanistic relationship between *DST1* and the circadian clock, we have uncovered a previously unknown connection between a sequence-specific mRNA decay pathway and expression of CCGs. Our results also strengthen the idea that DST sequences are more functionally versatile than previously anticipated. Recognition

and mode of action of the DST-mediated decay pathway might go beyond simple rapid turnover. Precise regulation of decay might be essential for proper expression of selected CCGs in Arabidopsis. Although DST-sequence recognition appears to be unique to plants, the relationship between post-transcriptional control and circadian rhythms might be of significance to other eukaryotes.

References

Alabadi,D., Oyama,T., Yanovsky,M.J., Harmon,F.G., Mas,P., and Kay,S.A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science *293*, 880-883.

Alabadi,D., Yanovsky,M.J., Mas,P., Harmer,S.L., and Kay,S.A. (2002). Critical role for CCA1 and LHY in maintaining circadian rhythmicity in Arabidopsis. Curr. Biol. *12*, 757-761.

Chen,Y., Hunter-Ensor,M., Schotland,P., and Sehgal,A. (1998). Alterations of per RNA in noncoding regions affect periodicity of circadian behavioral rhythms. J. Biol. Rhythms *13*, 364-379.

Chung,B.C., Lee,S.Y., Oh,S.A., Rhew,T.H., Nam,H.G., and Lee,C.H. (1997). The promoter activity of sen1, a senescence-associated gene of Arabidopsis, is repressed by sugars. J. Plant Physiol. *151* , 339-345.

Crawford,N.M., Wilkinson,J.Q., and Labrie,S.T. (1992). Control of nitrate reduction in plants. Aust. J Plant Physiol. *19*, 377-385.

Elliott,R.C., Dickey,L.F., White,M.J., and Thompson,W.F. (1989). cis-acting elements for light regulation of pea ferredoxin I gene expression are located within transcribed sequences. Plant Cell *1*, 691-698.

Feldbrügge,M., Arizti,P., Sullivan,M.L., Zamore,P.D., Belasco,J.G., and Green,P.J. (2002). Comparative analysis of the plant mRNA-destabilizing element, DST, in mammalian and tobacco cells. Plant Mol. Biol. *49*, 215-223.

Frisch,B., Hardin,P.E., Hamblen-Coyle,M.J., Rosbash,M., and Hall,J.C. (1994). A promoterless period gene mediates behavioral rhythmicity and cyclical per expression in a restricted subset of the Drosophila nervous system. Neuron *12*, 555-570.

Glossop,N.R., Lyons,L.C., and Hardin,P.E. (1999). Interlocked feedback loops within the Drosophila circadian oscillator. Science *286*, 766-768.

Green,R.M., Tingay,S., Wang,Z.Y., and Tobin,E.M. (2002). Circadian rhythms confer a higher level of fitness to Arabidopsis plants. Plant Physiol *129*, 576-584.

Gutiérrez,R.A., Ewing,R.M., Cherry,J.M., and Green,P.J. (2002). Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: Rapid decay is associated with a group of touch- and specific clock-controlled genes. Proc. Natl. Acad. Sci. USA *99*, 11513-11518.

Gutiérrez,R.A., Ewing,R.M., Cherry,J.M., and Green,P.J. (2002). Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: rapid decay is

associated with a group of touch- and specific clock-controlled genes. Proc. Natl. Acad. Sci. U. S. A *99*, 11513-11518.

Harmer,S.L., Hogenesch,J.B., Straume,M., Chang,H.S., Han,B., Zhu,T., Wang,X., Kreps,J.A., and Kay,S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science *290* , 2110-2113.

Harmer,S.L., Panda,S., and Kay,S.A. (2001). Molecular bases of circadian rhythms. Annu. Rev. Cell Dev. Biol. *17*, 215-253.

Heintzen,C., Nater,M., Apel,K., and Staiger,D. (1997). *At*GRP7, a nuclear RNA-binding protein as a component of a circadian- regulated negative feedback loop in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A *94*, 8515-8520.

Johnson,M.A., Pérez-Amador,M.A., Lidder,P., and Green,P.J. (2000). Mutants of Arabidopsis defective in a sequence-specific mRNA degradation pathway. Proc. Natl. Acad. Sci. U. S. A *97*, 13991-13996.

McClung,C.R. (2001). Circadian rythms in plants. Annu. Rev. Plant Physiol Plant Mol. Biol. *52*, 139-162.

Millar,A.J., Carre,I.A., Strayer,C.A., Chua,N.H., and Kay,S.A. (1995). Circadian clock mutants in Arabidopsis identified by luciferase imaging. Science *267*, 1161-1163.

Mizoguchi,T., Wheatley,K., Hanzawa,Y., Wright,L., Mizoguchi,M., Song,H.R., Carre,I.A., and Coupland,G. (2002). *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Dev. Cell *2*, 629-641.

Newman,T.C., Ohme-Takagi,M., Taylor,C.B., and Green,P.J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell *5*, 701-714.

Oh, S.A., Lee, S.Y., Chung, I.K., Lee, C.H. and Nam, H.G. (1996). A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol. Biol. *30*, 739-754.

Ouyang,Y., Andersson,C.R., Kondo,T., Golden,S.S., and Johnson,C.H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. Proc. Natl. Acad. Sci. U. S. A *95*, 8660-8664.

Panda,S., Hogenesch,J.B., and Kay,S.A. (2002). Circadian rhythms from flies to human. Nature *417*, 329-335.

Pérez-Amador,M.A., Lidder,P., Johnson,M.A., Landgraf,J., Wisman,E., and Green,P.J. (2001). New molecular phenotypes in the dst mutants of Arabidopsis revealed by DNA microarray analysis. Plant Cell *13*, 2703-2717.

Pilgrim,M.L., Caspar,T., Quail,P.H., and McClung,C.R. (1993). Circadian and light-regulated expression of nitrate reductase in Arabidopsis. Plant Mol. Biol. *23*, 349-364.

Schaffer,R., Landgraf,J., Accerbi,M., Simon,V., V, Larson,M., and Wisman,E. (2001). Microarray Analysis of Diurnal and Circadian-Regulated Genes in Arabidopsis. Plant Cell *13*, 113-123.

Schaffer,R., Ramsay,N., Samach,A., Corden,S., Putterill,J., Carre,I.A., and Coupland,G. (1998). The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell *93*, 1219-1229.

Schaffer,R., Landgraf,J., Accerbi,M., Simon,V., V, Larson,M., and Wisman,E. (2001). Microarray Analysis of Diurnal and Circadian-Regulated Genes in Arabidopsis. Plant Cell *13*, 113-123.

Seeley,K.A., Byrne,D.H., and Colbert,J.T. (1992). Red Light-Independent Instability of Oat Phytochrome mRNA in Vivo. Plant Cell *4*, 29-38.

Silverthorne,J. and Tobin,E.M. (1990). Posttranscriptional regulation of organ-specific expression of individual *rbc-S* messenger-RNAs in *Lemna-gibba*. Plant Cell *2*, 1181-1190.

So,W.V. and Rosbash,M. (1997). Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. EMBO J. *16* , 7146-7155.

Somers,D.E., Schultz,T.F., Milnamow,M., and Kay,S.A. (2000). ZEITLUPE encodes a novel clock-associated PAS protein from Arabidopsis. Cell *101*, 319-329.

Stanewsky,R. (2002). Clock mechanisms in Drosophila. Cell Tissue Res. *309*, 11-26.

Stanewsky,R., Jamison,C.F., Plautz,J.D., Kay,S.A., and Hall,J.C. (1997). Multiple circadian-regulated elements contribute to cycling period gene expression in Drosophila. EMBO J. *16*, 5006-5018.

Strayer,C., Oyama,T., Schultz,T.F., Raman,R., Somers,D.E., Mas,P., Panda,S., Kreps,J.A., and Kay,S.A. (2000). Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science *289*, 768-771.

Sullivan,M.L. and Green,P.J. (1996). Mutational analysis of the DST element in tobacco cells and transgenic plants: Identification of residues critical for mRNA instability. RNA *2*, 308-315.

Vorst,O., Vandam,F., Weisbeek,P., and Smeekens,S. (1993). Light-regulated expression of the *Arabidopsis-thaliana* ferrodoxin-A gene involves both transcriptional and posttranscriptional processes. Plant J. *3*, 793-803.

Wang,Z.Y. and Tobin,E.M. (1998). Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell *93*, 1207-1217.

CHAPTER 4

Mammalian determinants of long mRNA half-life and their significance to plants

Introduction

Plant transcripts decay with rates similar to those in other multicellular eukaryotes. The spectrum of mRNA half-lives extends from an hour or less for unstable messages to days or more for very stable transcripts. Most mRNAs however, appear to decay with half-lives in the order of hours (Siflow and Key, 1979; Hargrove *et al.*, 1991). The rate with which individual mRNA species are degraded is determined by a combination of general and specific structural features (Sachs, 1993; Ross, 1995; Chen and Shyu, 1995; Caponigro and Parker, 1996; Gutiérrez *et al.*, 1999). Structures that are common to virtually all eukaryotic mRNAs are the 5' cap and the 3' polyadenylate (poly(A)) tail. Although it is not yet clear how the cap and poly(A) tail removal are regulated in plants, these structures are thought to play a general role in the overall stability of plant transcripts. Transcript-specific sequence elements that can affect mRNA stability in plants and other eukaryotes have been identified (reviewed in Ross, 1995; Caponigro and Parker, 1996; Gutiérrez *et al.*, 1999). Adenylate/uridylate-rich elements (AREs) a common determinant of mRNA stability in mammalian cells are one of the best characterized. AREs are approximately 50-150 nucleotides long, usually contain multiple copies of the AUUUA motif and a high content of uridine. AREs are located in the 3'UTR of mRNAs encoding a variety of proto-oncoproteins, cytokines and transcription factors (Chen and Shyu, 1995). Transcripts that contain them are selectively targeted for rapid decay (Chen and Shyu, 1995). Due to the significance of AUUUA elements in mammals, a synthetic AUUUA repeat was tested for the ability to act as instability determinant in plants. Reporter transcripts containing 11 repeats of the AUUUA motif in their 3'UTRs were degraded much more rapidly in stably transformed tobacco cells and

accumulated to a lower level in transgenic tobacco plants than those of control constructs (Ohme-Takagi *et al.*, 1993). These results suggest that the mRNA decay pathway mediated by AUUUA repeats is conserved between animals and plants. In plants, the DST or downstream element has been best characterized. Originally identified as a conserved sequence in the 3' untranslated region (UTR) of the unstable small auxin-up RNA (*SAUR*) transcripts (McClure *et al.*, 1989) DST sequences were first showed to target reporter transcripts for rapid decay in BY-2 cells (Newman *et al.*, 1993).

It is now clear that unstable mRNAs in plants contain instability sequences, however it is still unclear whether stable ones contain discrete stabilizing determinants, conditional stabilization elements or are stable by default. In fact comparatively little attention has been devoted to the long-lived end of the mRNA stability spectra in plants or other systems. However the relevance of stable mRNAs is well justified from a cellular economy perspective (Russell *et al.*, 1997) (Mehdy and Brodl, 1998) and is perhaps most evident in cases where it is lost (below) (Russell *et al.*, 1997). The best understood case of mRNA stabilization occurs during red blood cell differentiation. The α-globin mRNA is selectively stabilized during erythroid cell development and at the late reticulocyte stage accounts for 95-98% of the total mRNA (Russell *et al.*, 1997). A single sense mutation in the translation termination codon of the α-globin mRNA severely destabilizes the transcript (Weiss and Liebhaber, 1994). This destabilization results in greater than 95% reduction in α-globin expression and accounts for one of the most common non-deletional α-thalassemias throughout the world (Russell *et al.*, 1997). Efforts to understand the molecular basis of α-globin mRNA longevity identified a pyrimidine-rich sequence in the 3' untranslated region as essential for the transcript's

stability (Weiss and Liebhaber, 1995). Several proteins can bind to this region and form a ribonucleoprotein complex in vitro (Wang *et al.*, 1995). Mutations that affect the formation of this complex correlated with destabilization effect on the α-globin transcript, suggesting that the complex is a major determinant of α-globin mRNA stability (Wang *et al.*, 1995). Formation of the α-complex was also observed in mouse erythroleukemia cells (MEL), human erythroleukemia cells (K562), fibroblasts (C127), human epithelioid carcinoma cells (HeLa) and rat adrenal pheochromocytoma cells (PC-12) (Wang *et al.*, 1995; Holcik and Liebhaber, 1997). Furthermore, other stable transcripts (*e.g.* β-globin, 15-lipoxygenase, tyrosine hydroxylase and α(I)-collagen) possess similar sequences in their 3' UTR. These sequences are capable of forming related ribonucleoprotein complexes suggesting that the mechanism of α-globin stabilization might have a broader significance in mammalian cells (Holcik and Liebhaber, 1997).

Besides their crucial role in the regulation of endogenous genes, post-transcriptional mechanisms that promote long-lived mRNAs are also becoming important considerations in plant biotechnology. Attaching a strong promoter to a gene will not always guarantee high expression in plants (Diehn *et al.*, 1996). Expression of a foreign gene can be limited at the mRNA level through mechanisms such as aberrant splicing, aberrant polyadenylation, and rapid mRNA degradation (Diehn *et al.*, 1998; De Rocher *et al.*, 1998).

As a first step to gain insight into the molecular principles that govern slow mRNA decay rates in plants we investigated the effect of the pyrimidine-rich sequence derived from the mammalian α-globin transcript and a related synthetic sequence on mRNA stability in three different plant expression systems.

Materials and methods

Plant materials and culture

Arabidopsis thaliana ecotype Columbia plants were grown in soil under a 12-h light/12-h dark cycle at 20°C. *Nicotiana tabacum* cv Bright Yellow 2 (BY-2) cells (An, 1985; NAGATA *et al.*, 1992) were cultured as described by Newman *et al.* (1993). Maize (*Zea mays* cv BMS) cells (a gift from Pioneer Hi-Bred International, Inc., Des Moines, IA) were grown as described previously (DeRocher *et al.*, 1998).

Gene constructions

In order to test the activity of the putative stability sequences in plants, expression cassettes consisting of a double enhanced 35S promoter followed by the dihydrofolate reductase coding region (DHFR), and the 3'UTR of the pea rubisco small subunit E9 gene (E9 tail) were made (p1801; Figure 4.1). A duplication of the 42 nt long stabilization sequence from the α-globin 3'UTR was then cloned downstream of the DHFR coding region and upstream of the E9 tail to generate construct p1802 (DHFR-gSE). In a separate construct, a synthetic sequence (84 nt long) derived from the consensus for the stability element (Holcik and Liebhaber, 1997) was inserted instead to generate construct p1803 (DHFR-cSE). The plant expression cassettes from p1801, p1802 and p1803 were then moved into the *Hind III* site of plasmid pBI121 (Jefferson *et al.*, 1987) to generate constructs p1804, p1805, p1814 respectively. pBI121 posses a

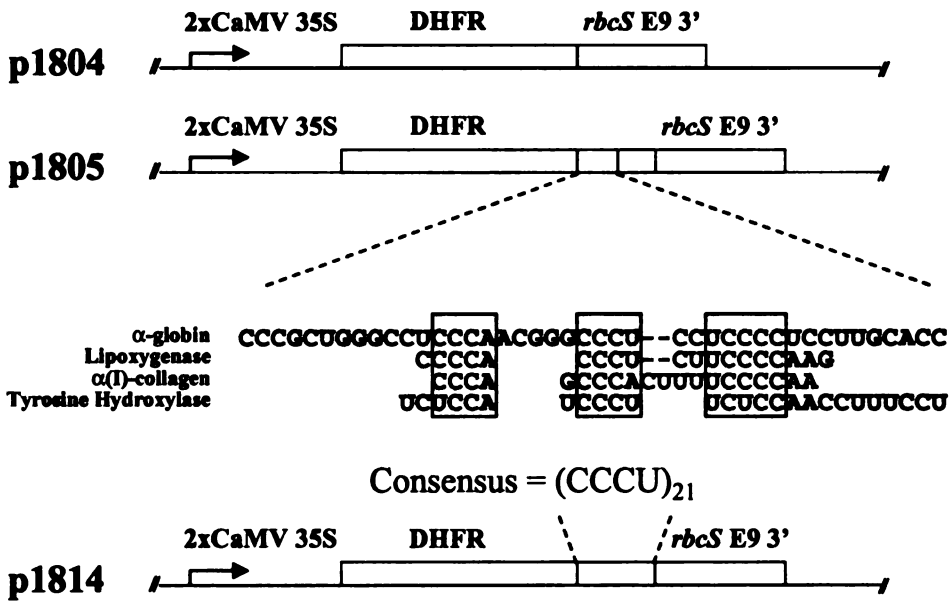


Figure 4.1. Reporter system for testing putative stabilization elements derived from mammalian transcripts. Test sequences were introduced into a plant expression cassette as illustrated, downstream of the dihydrofolate reductase coding region, and upstream of the 3'UTR of the pea rubisco small subunit E9 gene. All chimeric genes were under the control of a double enhanced CaMV 35S promoter. p1804: DHFR control (DHFR), p1805: DHFR and α-globin stability determinant (DHFR-gSE). p1814: DHFR and $(CCCU)_{21}$, a synthetic sequence based on the sequence alignment of α-globin, Lipoxygenase, α(I)-collagen and Tyrosine Hydroxylase 3'UTRs (see text for details) (DHFR-cSE).

nopaline synthase driven neomycin phosphotransferase II gene that confers kanamicin resistance to transgenic plants.

Protoplast preparation and transformation

Protoplasts from BY-2 or BMS cells were prepared and transformed as described previously (Diehn, 1998). Briefly, the cells from a 3-4 days subculture were incubated in 2 % cellulysin, 1 % cytolase and 0.2 % pectolyase in buffer KMC 700 (8.65g KCl, 16.47g $MgCl_2.6H_2O$, 12.5g $CaCl_2.2H_2O$, 5g MES in 900ml ddH_2O, pH 6 with KOH;

osmolarity 700mOsm) for 3-5 h at 28°C with gentle agitation. Hydrolytic enzymes were prepared as described previously (van Hoof and Green, 1996). The cells were monitored under microscope periodically to evaluate protoplast formation. The protoplasts were then sieved, washed in KMC 650 (like KMC 700 except osmolarity was adjusted to 650mOsm), washed in KMC 600 (like KMC 700 except osmolarity was adjusted to 600mOsm) and then counted. For transformation, 0.5 mL of the final protoplast suspension ($8x10^6$ cells/mL) were incubated with 100 µL of plasmid DNA (1 µg/µL) in the presence of 0.5 mL PEG solution (40% PEG 8000 (w/v), 0.4M mannitol, 0.1M $Ca(NO_3)_2.4H_2O$, pH 9.0). After 30 min at room temperature, W6 solution (0.38g KCl, 9g $CaCl_2.2H_2O$, 9g NaCl, 9g glucose, 1g MES (2[N-morpholino]ethanesulfonic acid) in 600ml ddH_2O, pH 6.0, osmolarity 550-600mOsm) was added stepwise in 1, 2, and 5 mL volumes 5 min apart. After centrifugation, protoplasts were resuspended in FW media (4.3g l^{-1} MS salts (Gibco BRL, Gaithersburg, MD), 5ml l^{-1} 200X B5 vitamins, 30g l^{-1} sucrose, 1.5g l^{-1} proline, 54g l^{-1} mannitol, 3mg l^{-1} 2,4-D, pH 5.7 with KOH) and transferred to 100 mm Petri plates, where they were incubated at 28°C in the dark for 12 or 16h before harvesting.

Plant transformation

Arabidopsis plants were transformed using the infiltration method described in Chapter 5. Trangenic plants were selected by germinating the seeds of infiltrated plants on agar plates containing 1x Murashige and Skoog salts, 1x Gamborg's vitamins, 1% sucrose and 50 µg/mL kanamycin as selectable marker.

RNA isolation and northern blot analysis

RNA was extracted from plant tissue as described previously (Newman *et al.*, 1993). RNA was isolated from BY-2 or BMS protoplasts using the same method except that the protoplasts were not grounded under liquid N_2, but thawed directly in 2.5 mL of guanidinium thiocyanate solution while vortexing. Aliquots of total RNA from transiently transformed protoplasts were treated with DNaseI for 15 min at 37°C to remove residual plasmid DNA previous to northern blot analysis. Equal amounts of total RNA (20 μg/lane) were used for RNA gel blots and hybridization using standard techniques.

.

Results

α-Globin stabilization element and a related sequence do not increase abundance of a reporter transcript in maize or in tobacco protoplasts

To test if the α-globin pyrimidine-rich element is recognized in plants as an stability determinant, two copies of the 42 nt sequence from the α-globin transcript 3'UTR (Holcik and Liebhaber, 1997) was cloned between the DHFR coding region and the 3' UTR of the pea rubisco small subunit E9 (p1805, DHFR-gSE. Figure 4.1). Sequences similar to the α-globin stability element have been identified in three other stable mammalian mRNAs (Figure 4.1; (Holcik and Liebhaber, 1997)). A synthetic "stabilization" element was then synthesized based on the consensus in the sequence comparison and introduced in a separate plant expression cassette (p1814, DHFR-cSE. Figure 4.1). The relative mRNA abundance for the different constructs was studied in maize and tobacco cells. Protoplasts from maize and tobacco cells were prepared and transiently transformed with plasmids p1804 (DHFR), p1805 (DHFR-gSE) and p1814 (DHFR-cSE). Total RNA was isolated from samples harvested 12 and 16 h after transformation and analyzed by northern blot hybridization using ^{32}P-labeled DHFR coding region as probe. As shown in Figure 4.2*a-b*, the reporter transcripts DHFR-gSE and DHFR-cSE did not accumulate to higher levels than the control DHFR mRNA in maize or tobacco protoplasts. In fact, the sequences appeared to decrease mRNA accumulation of the reporter mRNAs. These results suggest that the sequences tested are not recognized as stability determinants in maize and tobacco cell cultures.

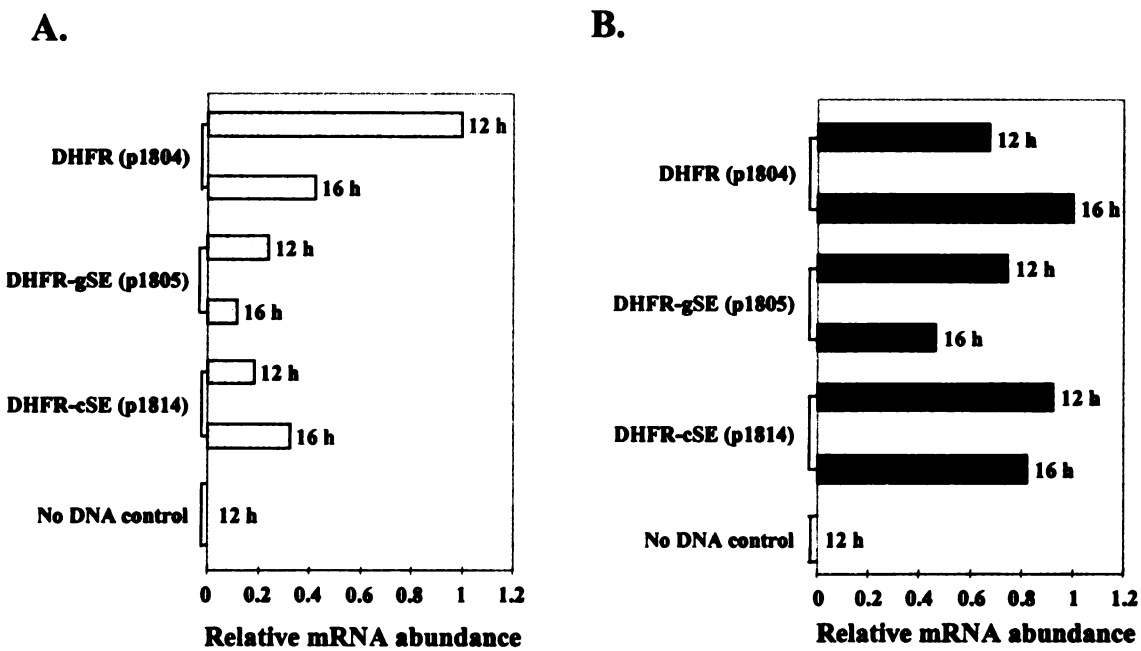


Figure 4.2. The α-globin stabilization element and the synthetic poly(CCCU) sequence do not increase the abundance of a reporter transcript in maize or in tobacco cells. (A) Maize protoplasts were transiently transformed with plasmids p1804 (DHFR control), p1805 (DHFR-gSE) and p1814 (DHFR-cSE) as described in the text. Cells were harvested 12 and 16h after transformation and 20 μg of total RNA was used for northern blot analysis using the DHFR coding region as a probe. The relative abundance of the reporter transcripts is expressed as a fraction of the control construct (p1804), which lacks the putative stabilization sequence. (B) Similar experiments were performed in tobacco protoplasts.

α-Globin stabilization element and a related sequence do not increase abundance of a reporter mRNA in transgenic Arabidopsis seedlings

As an alternative to the cell culture expression system, the same constructs were stably introduced into *Arabidopsis thaliana* via Agrobacterium-mediated transformation. In order to have a representative population of transgenic plants, approximately 100 seedlings were randomly selected from the first generation of transformed plants and pooled for total RNA isolation and northern blot analysis as before. As shown in Figure 4.3, steady state levels of the DHFR-gSE and DHFR-cSE did not accumulate to higher

levels than the control DHFR transcript. Similar to what observed in protoplasts, DHFR-gSE and DHFR-cSE reporters appeared to accumulate to lower levels than the control reporter transcript. These results are consistent with that observed in maize and tobacco cells and suggest that in the conditions tested the α-globin stability determinant and the synthetic poly(CCCU) sequence do not confer higher stability to the reporter transcript.

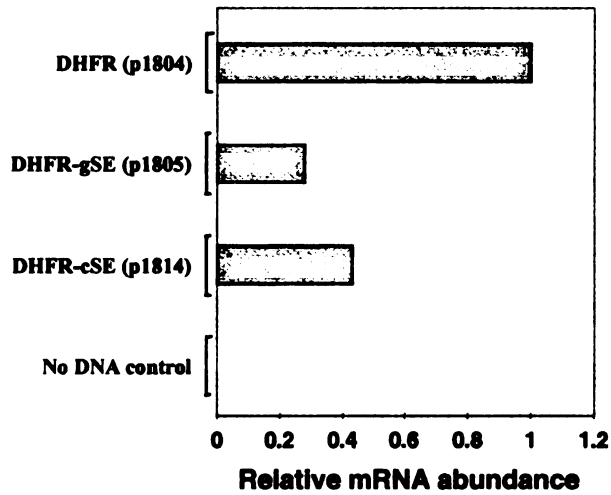


Figure 4.3. The α-globin stabilization element and the synthetic poly(CCCU) sequence do not increase the abundance of a reporter transcript in transgenic Arabidopsis plants. Arabidopsis plants were transformed with the constructs p1804 (control), p1805 (DHFR-gSE) and p1814 (DHFR-cSE). Kanamycin resistant seedlings from independent transformation events were pooled for total RNA isolation. 20 μg of total RNA was used for northern blot analysis using the DHFR coding region as a probe. The relative abundance of the reporter transcript is expressed as a fraction of the control construct (p1804), which lacks a putative stabilization sequence.

Discussion

Plant transcripts exhibit a wide spectrum of cytoplasmic half-lives, from as short as a few minutes to as long as several days. While a significant amount of information is available regarding mechanisms underlying rapid mRNA turnover (Ross, 1995; McCarthy, 1998; Gutiérrez *et al.*, 1999), the mechanisms responsible for the long half-life of transcripts in plants and other systems are largely unknown. An important first step to understand the mechanisms of regulation of mRNA stability is the identification of *cis*-acting sequences.

In an effort to gain insight into mRNA stabilization in plants, we tested the best understood stabilization sequence element, the pyrimidine-rich element found in the human α-globin transcript, in three plant expression systems. A similar strategy was used to demonstrate that AUUUA repeats, a common instability determinant in mammalian transcripts, can target mRNA for rapid degradation in plants (Ohme-Takagi *et al.*, 1993). Because sequence motifs can function better when multimerized (Kuhlemeier *et al.*, 1987; Lam and Chua, 1990; Newman *et al.*, 1993), two copies of the α-globin stabilization element were placed between a reporter DHFR coding region and the E9 polyadenylation signal. Sequences similar to the α-globin stability element have been found in other stable mammalian transcripts (Holcik and Liebhaber, 1997). Therefore a synthetic poly(CCCU) element derived from the consensus in the sequence comparison (Holcik and Liebhaber, 1997) was also tested.

The results obtained in the three systems consistently indicated that the α-globin and the synthetic sequence do not increase the abundance of reporter transcripts in plants. In

fact, the sequences tested appear to diminish reporter transcript accumulation. These results strongly argue that at least in the conditions tested, these sequence elements are not recognized as mRNA stability determinants. It is not clear what caused reduced mRNA accumulation. It is possible that these sequences are recognized as determinants for rapid mRNA degradation, but influence over other post-transcriptional processes (*e.g.* polyadenylation) can not be ruled out with the available data. It is also possible that under different conditions the elements could manifest their mRNA stabilization function. However, because no obvious plant condition appears to mirror the red blood cell environment, testing would have to be conducted by trial and error. Such approach might be challenging and has no guaranteed success because these sequences might not have a functional significance in plants and stability elements might be different. Because of these considerations, this strategy was not pursued any further.

As an alternative for the future, a directed evolution approach as that described by Chrzanowska-Lightowlers and Lightowlers (2001) to find sequences that increase mRNA stability in mammalian cells could be adapted for plants. Briefly, in such approach a randomized sequence cassette is placed in the 3'UTR of a reporter transcript and a library is made with the heterogeneous population of transcripts. Several rounds of expression and selection for high levels of the reporter are then performed, for example, by extracting RNA several hours after transient transformation experiments as described in this Chapter. The putative stability sequences are then rescued using PCR and fused to the same original reporter to prepare new "enriched" libraries. This process is iterated until individual sequences that confer long life to the reporter mRNA are identified. Though this strategy proved successful in mammalian cells (Chrzanowska-Lightowlers

and Lightowlers, 2001), one drawback is that the biological significance of the sequences identified is not readily determined. This might not be of concern for applied purposes. However the most attractive strategy to find stabilization sequences that are relevant to plant mRNAs might be to combine deletion analysis of long-lived transcripts with half-life measurements using the promoter system described in the next Chapter. Candidate stable transcripts for analysis can be obtained from the global study of mRNA stability described in Chapter 2. Table 4.1 contains examples of very stable transcripts detected in the microarray experiments and that have been confirmed by northern blot analysis in at least one extended cordycepin time course. In addition, sequences located downstream of the coding region of plant genes have been shown to increase the abundance of reporter transcripts via an unknown mechanism (summarized in Table 4.2). Fusion of these sequences to unstable reporter transcripts and mRNA half-life measurements in transcriptional pulse type experiments could also lead to the identification of signals that protect transcripts from degradation, the first step towards understanding mechanisms of long mRNA life in plants.

Table 4.1. Examples of Arabidopsis genes with stable transcripts. The genes indicated showed no detectable difference when comparing the 0 and 120 min time point of cordycepin time courses in a microarray experiment (described in Chapter 2) and were also stable by northern blot in at least one extended cordycepin time course (data not shown).

Locus	Description
At1g08360	Putative ribosomal protein L10
At1g09780	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase
At3g13920	Eukaryotic protein synthesis initiation factor 4A
At3g47340	Asparagine synthetase 1
At5g60390	Translation elongation factor eEF-1 alpha chain

Table 4.2. Sequence elements located downstream of the translation stop codon can increase mRNA abundance of endogenous as well as reporter genes in different plant systems.

Organism	Gene	Expression pattern	Studies	Effect on expression	Reference
Flaveria bidentis	*Me1*	Leaves	GUS chimeras, deletion analysis	Increase in leaves	(Ali and Taylor, 2001)
Sesbania rostrata	*SrEnod2*	Nodule parenchyma	GUS chimeras, deletion analysis	Increase in nodule parenchyma.	(Chen *et al.*, 1998)
Potato	*SUS4*	Developing tubers; basal tissues of axillary buds and shoots; root cap and meristem..	GUS chimeras, deletion analysis	increase in tuber, root, leaves and stems.	(Fu *et al.*, 1995)
Arabidopsis thaliana	*Glabrous1*	Developing trichomes, leaf primordia and stipules.	GUS chimeras, deletion analysis	Increase in leaf primordia, trichomes and stipules.	(Larkin *et al.*, 1993)
Brassica napus	*AX92*	Root cortex of embryos and seedlings.	GUS chimeras	Increase in root cortex of embryos and seedlings.	(Dietrich *et al.*, 1992)
Petunia	*SSU301*	Leaves	Chimeras in tobacco plants	Increase in leaves	(Dean *et al.*, 1989)
Potato	PI-II	Leaves, wound inducible	CAT chimeras, deletion analysis in tobacco plants.	Increase in leaves	(An *et al.*, 1989)

References

Ali,S. and Taylor,W.C. (2001). The 3 ' non-coding region of a C-4 photosynthesis gene increases transgene expression when combined with heterologous promoters. Plant Mol. Biol. *46*, 325-333.

An,G.H. (1985). High-efficiency transformation of cultured tobacco cells. Plant Physiol. *79*, 568-570.

An,G.H., Mitra,A., Choi,H.K., Costa,M.A., An,K.S., Thornburg,R.W., and Ryan,C.A. (1989). Functional-analysis of the 3' control region of the potato wound-inducible proteinase inhibitor-II gene. Plant Cell *1*, 115-122.

Caponigro,G. and Parker,R. (1996). Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol. Rev. *60*, 233-249.

Chen,C.Y.A. and Shyu,A.B. (1995). AU-rich elements - Characterization and importance in messenger-RNA degradation. Trends Biochem. Sci. *20*, 465-470.

Chen,R., Silver,D.L., and de Bruijn,F.J. (1998). Nodule parenchyma-specific expression of the Sesbania rostrata early nodulin gene SrEnod2 is mediated by its 3 ' untranslated region. Plant Cell *10*, 1585-1602.

Chrzanowska-Lightowlers,Z. and Lightowlers,R.N. (2001). Fending off decay: a combinatorial approach in intact cells for identifying mRNA stability elements. RNA *7*, 435-444.

De Rocher,E.J., Vargo-Gogola,T.C., Diehn,S.H., and Green,P.J. (1998). Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol. *117*, 1445-1461.

Dean,C., Favreau,M., Bondnutter,D., Bedbrook,J., and Dunsmuir,P. (1989). Sequences downstream of translation start regulate quantitative expression of 2 petunia *rbcS* genes. Plant Cell *1*, 201-208.

DeRocher,E.J., Vargo-Gogola,E.C., Diehn,S.H., and Green,P.J. (1998). Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol. *117*, 1445-1461.

Diehn, S. H. (1998). *B.t.* toxin gene expression and differential utilization of polyadenylation signals in gramineous and dicotyledonous plants. PhD Dissertation. Michigan State University.

Diehn,S.H., Chiu,W.L., De Rocher,E.J., and Green,P.J. (1998). Premature polyadenylation at multiple sites within a *Bacillus thuringiensis* toxin gene-coding region. Plant Physiol. *117*, 1433-1443.

Diehn,S.H., De Rocher,E.J., and Green,P.J. (1996). Problems that can limit the expression of foreign genes in plants: lessons to be learned from *B.t.* toxin genes. In Genetic Engineering: Principles and Methods, J.K.Setlow, ed. (New York: Plenum Press), pp. 83-99.

Dietrich,R.A., Radke,S.E., and Harada,J.J. (1992). Downstream DNA-sequences are required to activate a gene expressed in the root cortex of embryos and seedlings. Plant Cell *4*, 1371-1382.

Fu,H.Y., Kim,S.Y., and Park,W.D. (1995). High-level tuber expression and sucrose inducibility of a potato *SUS4* sucrose synthase gene requires 5'-flanking and 3'-flanking sequences and the leader intron. Plant Cell *7*, 1387-1394.

Gutiérrez,R.A., MacIntosh,G.C., and Green,P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. *4*, 429-438.

Hargrove,J.L., Hulsey,M.G., and Beale,E.G. (1991). The kinetics of mammalian gene-expression. Bioessays *13*, 667-674.

Holcik,M. and Liebhaber,S.A. (1997). Four highly stable eukaryotic mRNAs assemble 3' untranslated region RNA-protein complexes sharing cis and trans components. Proc. Natl. Acad. Sci. USA *94*, 2410-2414.

Jefferson,R.A., Kavanagh,T.A., and Bevan,M.W. (1987). GUS fusions-beta-glucoronidase as a sensitive and versatile gene fusion marker in higher-plants. EMBO J. *6*, 3901-3907.

Kuhlemeier,C., Fluhr,R., Green,P.J., and Chua,N.-H. (1987). Sequences in the pea rbsS-3A gene have homology to constitutive mammalian enhancers but function as negative regulatory elements. Genes Dev. *1*, 247-255.

Lam,E. and Chua,N.H. (1990). GT-1 binding-site confers light responsive expression in transgenic tobacco. Science *248*, 471-474.

Larkin,J.C., Oppenheimer,D.G., Pollock,S., and Marks,M.D. (1993). Arabidopsis GLABROUS1 gene requires downstream sequence for function. Plant Cell *5*, 1739-1748.

McCarthy,J.E.G. (1998). Posttranscriptional control of gene expression in yeast. Microbiol. Rev. *62*, 1492-1553.

McClure,B.A., Hagen,G., Brown,C.S., Gee,M.A., and Guilfoyle,T.J. (1989). Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. Plant Cell *1*, 229-239.

Mehdy,M.C. and Brodl,M.R. (1998). The role of stress in regulating mRNA stability. In A look beyond transcription: Mechanisms determining mRNA stability and translation in plants, J.Bailey-Serres and D.R.Gallie, eds. American Society of Plant Physiologists), pp. 54-63.

Nagata,T., Nemoto,Y., and Hasezawa,S. (1992). Tobacco BY-2 cell-line as the Hela-cell in the cell biology of higher-plants. Int. Rev. Cytol. *132*, 1-30.

Newman,T.C., Ohme-Takagi,M., Taylor,C.B., and Green,P.J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell *5*, 701-714.

Ohme-Takagi,M., Taylor,C.B., Newman,T.C., and Green,P.J. (1993). The effect of sequences with high AU content on mRNA stability in tobacco. Proc. Natl. Acad. Sci. USA *90*, 11811-11815.

Ross,J. (1995). Messenger-RNA stability in mammalian-cells. Microbiol. Rev. *59*, 423-450.

Russell,J.E., Morales,J., and Liebhaber,S.A. (1997). The role of mRNA stability in the control of globin gene expression. Prog. Nucleic Acid Res. Mol. Biol. Vol 57 *57*, 249-287.

Sachs,A.B. (1993). Messenger-RNA degradation in eukaryotes. Cell *74*, 413-421.

Siflow,C.D. and Key,J.L. (1979). Stability of polysome-associated polyadenylated RNA from soybean suspension culture cells. Biochemistry *18*, 1013-1018.

van Hoof,A. and Green,P.J. (1996). Premature nonsense codons decrease the stability of phytohemagglutinin mRNA in a position-dependent manner. Plant J. *10*, 415-424.

Wang,X.M., Kiledjian,M., Weiss,I.M., and Liebhaber,S.A. (1995). Detection and characterization of a 3' untranslated region ribonucleoprotein complex-associated with human alpha-globin messenger-RNA stability. Mol. Cell. Biol. *15*, 2331.

Weiss,I.M. and Liebhaber,S.A. (1994). Erythroid cell-specific determinants of alpha-globin messenger-RNA stability. Mol. Cell. Biol. *14*, 8123-8132.

Weiss,I.M. and Liebhaber,S.A. (1995). Erythroid cell-specific messenger-RNA stability elements in the alpha-2-globin 3' nontranslated region. Mol. Cell. Biol. *15*, 2457-2465.

CHAPTER 5

Promoter region of the *At-EXPL1* gene drives transient expression of reporter transcripts: A new regulated promoter system to study mRNA degradation in Arabidopsis[IV].

[IV] Part of the initial cloning of the *At-EXPL1* promoter and part of the analysis of reproducibility of *At-EXPL1* gene expression (Figure 5.2) were carried out by the undergraduate student Mr. Chris Behrens as part of his professorial assistantship.

Introduction

The process of mRNA degradation is an important regulatory point for gene expression. In multicellular eukaryotes, turnover rates can vary from minutes, for the most rapidly degraded mRNAs, to several days for those transcripts that are most stable (Gutiérrez *et al.*, 1999). In addition, modulation of these rates can play a significant role during the transition to new mRNA steady state levels triggered by developmental, environmental, or physiological cues. The faster the mRNA is degraded the faster it reaches a new steady state level (Hargrove *et al.*, 1991).

Determining the turnover rate of a transcript and how that rate changes in response to internal or external signals is key in evaluating the contribution of this post-transcriptional control mechanism to the expression of any gene. Moreover, accurate knowledge of the kinetics of mRNA degradation is essential in order to understand the molecular mechanism that underlies this level of control of gene expression.

Methods that monitor the rate of loss of mRNA after transcription is halted are the most popular to investigate the process of mRNA degradation (Parker *et al.*, 1991). Primarily because they provide information on mRNA integrity, they can be used to study high- and low-abundance mRNAs, require little amounts of radioactivity, and are technically simple (Parker *et al.*, 1991). Typically, a chemical that inhibits global transcription is utilized and time courses are generated by extracting tissue samples at regular intervals after the tissue has been exposed to the inhibitor. Different chemicals have been used in different model organisms to inhibit the RNA polymerase. For example, the DNA interchalating agent actinomycin D has been widely applied in studies in mammalian systems (Brawerman, 1993) while the chain terminator cordycepin has

been common in plants (Seeley *et al.*, 1992; Johnson *et al.*, 2000). The main disadvantage of using drugs to stop global transcription is that prolonged incubation with inhibitors can have a severe impact on the cellular physiology (Brawerman, 1993). As a consequence, usually only short incubation periods are possible, which limits the application of this approach to the study of unstable mRNAs. However even with short incubation times, normal decay of cellular mRNAs can be altered in the presence of the inhibitor (*e.g.* Shyu *et al.*, 1989; Gil and Green, 1996; Petracek *et al.*, 1998). In *S. cerevisiae* another option to stop global transcription is to thermally inactivate the temperature sensitive RNA polymerase II (*rpb1-1*) (Nonet *et al.*, 1987). Although this approach has yielded comparable results with other methods for selected mRNAs (Herrick *et al.*, 1990) and has been used in global studies of mRNA turnover (Wang *et al.*, 2002), it is not yet clear to what extent normal degradation is affected by the elevated temperature.

As an alternative to global transcriptional repression, a regulated promoter system can be used to stop mRNA synthesis. Although turnover rate of only individual transcripts can be measured with this approach, it has the main advantage that cell physiology is not severely compromised. Examples of regulated promoters systems include the *GAL1* promoter in yeast (Parker *et al.*, 1991) and the *c-fos* promoter in mammalian systems (Shyu *et al.*, 1989)

In plants, promoter systems that are positively regulated by chemical ligands have been developed (*e.g.* Aoyama and Chua, 1997; Salter *et al.*, 1998; Bohner *et al.*, 1999; Martinez *et al.*, 1999; Granger and Cyr, 2001). Although these are good inducible systems, they are not well suited to determine mRNA stability, primarily because transcriptional repression is not fast or easily achieved.

Efforts to develop negatively regulated promoters have yielded far fewer options in plant systems. In fact the only alternatives are variants of the tetracycline(Tc)-repressible system initially developed in tobacco plants (Weinmann *et al.*, 1994). This system has been useful for mRNA degradation studies in tobacco cell cultures (Gil and Green, 1996) and tobacco plants (Petracek *et al.*, 1998). It has also been adapted to Arabidopsis (Love *et al.*, 2000), but it is not yet clear whether this "Top10 promoter system" would be useful to study mRNA degradation in this plant. Transcriptional down-regulation is achieved in the presence of Tc in Arabidopsis, but repression kinetic studies were carried out at the protein level and detectable changes were observed only after several hours (Love *et al.*, 2000). Nevertheless, the Top10 promoter system is not a strong promoter (approximately 10-fold lower than the CaMV 35S promoter. Gil & Green, unpublished data) which makes difficult the study of labile mRNAs. Furthermore, due to the heterogeneity in the mRNA population that results from Top10 constitutive transcription, this approach will not readily allow the study of critical mechanistic aspects of mRNA degradation such as deadenylation and decay kinetics and precursor/product relationship for mRNAs with stable decay intermediates.

For successful mRNA degradation studies an ideal regulated promoter system should exhibit at least the following properties: (i) Extremely low or no basal levels of expression in the absence of inducing conditions (ii) A high level of expression in the induced stage (iii) Rapid transcriptional induction and repression to generate a synchronized population of mRNAs (iv) Highly reproducible expression patterns (v) No external chemical signal necessary to activate and/or repress transcription. Recently, Shimizu-Sato *et al.* (2002) described a light controlled promoter system that appears to

comply with these rules. Although promising, its potential for mRNA turnover measurements remains to be investigated.

Here we describe a new regulated promoter system that meets all the ideal requirements for mRNA stability studies. We have successfully used this system to recapitulate the instability of a well studied unstable reporter mRNA. The system makes use of the natural transcriptional regulatory properties of the *Arabidopsis thaliana EXPL1* gene (*At-EXPL1*). *At-EXPL1* is member of a small group of genes known as expansin-like which belong to the expansin superfamiliy (http://www.bio.psu.edu/expansins/). In the conditions described, the promoter region of the Arabidopsis *At-EXPL1* gene behaves as a strong promoter system that is transiently activated to produce a synchronized population of transcripts.

Materials and Methods

Arabidopsis strain and growth conditions

Arabidopsis thaliana ecotype Columbia was used for all experiments. Plants were germinated on agar plates containing 1x Murashige and Skoog salts, 1x Gamborg's vitamins and 1% sucrose at 22 °C and 16h light and 8h dark cycles. After two weeks they were either used for the half-life experiments described below or transferred to soil to complete their life cycle.

Plasmid constructs

The polymerase chain reaction (PCR) was used to amplify a 2650 bp region of Chromosome III upstream of the expansin-like gene *At-EXPL1* (locus number At3g45970). The DNA fragment was amplified with primers pg956 (TCGTCACAATGGAGTTACATGAGTAGAAG) and pg957 (CTTTAAGATCTAATAAGAGAGAGAGATATGTG) and cloned into the pGEM-T Easy Vector (Promega. Madison, WI) according to instructions provided by the manufacturer. Sequence of the PCR fragment was carried out to discard any mistake introduced during the amplification. The sequence verified upstream region of *At-EXPL1* (pEXPL1) was excised from the pGEM-T Easy Vector as a fragment *Sac I* / pEXPL1 / *Bgl II*. This DNA fragment replaced the CaMV 35S promoter present in the plant expression cassette in plasmids p1088 and p1060 at corresponding restrictions sites. p1088 originally contained an expression cassette consisting of *Pvu II* / *Sac I* / CaMV

35S promoter / *Bgl II* / human β-globin coding sequence / 2 DST instability sequences / 3'UTR from the pea Rubisco small subunit *rbcS-E9* / *Pvu II* (Newman *et al.*, 1993). Plasmid p1060 was identical to p1088 except that there were two copies of a control spacer instead of the DST instability sequence (Newman *et al.*, 1993). The new derivative of p1088 was termed p2102 and the corresponding derivative of p1060 was termed p2103. The complete expression cassette from plasmids p2102 and p2103 was then removed with *Pvu II* and inserted into the *Sma I* site of the vector pCAMBIA3301 (CAMBIA. Canberra, Australia) to generate plasmids p2112 and p2113 respectively. Orientation of plant expression cassettes in the CAMBIA vectors was verified to be the same. p2112 and p2113 were then introduced into *A. thaliana* via Agrobacterium mediated transformation as described below. To ensure integrity of the plasmid constructions, restriction mapping with at least 3 restriction enzymes and sequence verification of the junctions was carried out at each cloning step.

Arabidopsis transformation

Transformation of Arabidopsis plants was carried out with the *Agrobacterium tumefaciens* infiltration method as detailed previously (Bariola *et al.*, 1999). Briefly, individual plasmids were electroporated into *A. tumefaciens* strain GV3101 pMP90 (Koncz and Schell 1986) using a Gene-Pulser (BioRad). The aerial portion of soil grown 4 to 5-week old Arabidopsis plants was then submerged in a solution of the transformed Agrobacterium. Vacuum of 18 in Hg was applied for 5 min and the plants were then allowed to set seed under normal growth conditions. The transformed seeds were selected by germination on agar plates containing 1x Murashige and Skoog salts, 1x Gamborg's

vitamins, 1% sucrose and 50 μg/mL of gluphosinate ammonium (C14030000, Crescent Chemical Co.). The herbicide resistant plants were transferred after two weeks to soil or used for further studies.

Half-life measurements with a transcriptional inhibitor

Half-lives were determined as described by Seeley *et al.* (Seeley *et al.*, 1992) with the following modifications. Two-week old Arabidopsis plants grown on plates were transferred to a flask with incubation buffer (Seeley *et al.*, 1992). After a 30 min incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and quickly frozen in liquid nitrogen. Total RNA was isolated and analyzed by northern blot using standard techniques.

Half-life measurements using the *At-EXPL1* promoter:

To determine half-lives using the *At-EXPL1* promoter system we followed the same protocol described above for half-life measurement with a transcription inhibitor except that the drug was never added. Plants were transferred to the incubation buffer and tissue samples were harvested at regular time intervals thereafter and quickly frozen in liquid nitrogen. Total RNA was isolated and analyzed by northern blot using standard techniques.

Results

At-EXPL1 gene is transiently induced

We investigated the expression of the expansin-like gene *At-EXPL1* (At3g45970), which encodes one of the most unstable Arabidopsis mRNAs recently identified through microarray analysis (Gutiérrez *et al.*, 2002). *At-EXPL1* exhibited an unusual pattern of gene expression. Initially undetectable in two-week old Arabidopsis plants grown on agar plates, it was rapidly and transiently up-regulated following submersion of the plants in incubation buffer during our routine mRNA half-life experiments (Figure 5.1*a*). Interestingly, this pattern of gene expression occurred irrespective of the presence or absence of cordycepin (Compare Figure 5.1*a* to 5.1*b*) and was unique among 14 genes with unstable and moderately unstable transcripts (data not shown). Similar transient induction of the *c-fos* promoter following the addition of serum to serum-starved

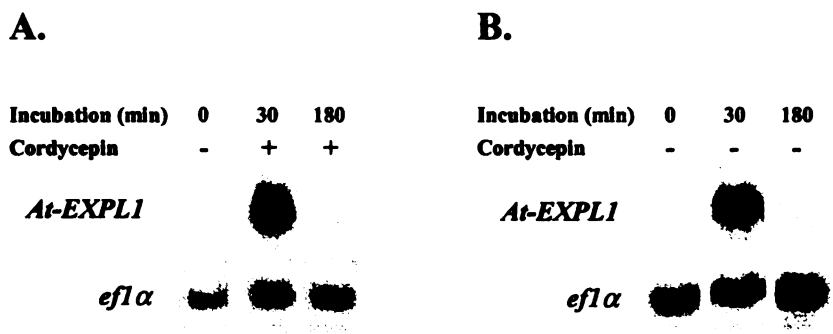


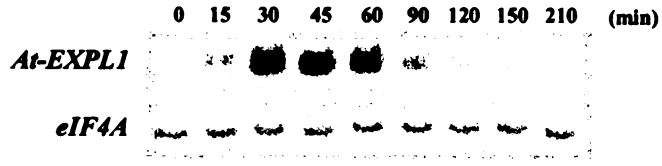
Figure 5.1. Transient induction of the At-EXPL1 gene. (A) Northern blot analysis of *At-EXPL1* mRNA at the indicated time points after transferring two-week old plants to an incubation buffer. Cordycepin was added after 30 min of incubation. Samples consisted of 10 µg of total RNA isolated from the indicated time points. (B) As (A) but in the absence of cordycepin. The stable *eIF4A* mRNA was used as a loading control.

NIH-3T3 cells has been successfully exploited to determine mRNA degradation rates in mammalian cells (Shyu *et al.*, 1989). In fact, such transient-induction system it is now a mainstay of mammalian mRNA decay research. As noted above, such a system is unavailable for plants. If the observed *At-EXPL1* transient induction is transcriptionally controlled, it could be used as a regulated promoter system to study the mRNA degradation process in *Arabidopsis thaliana*.

Transient induction of *At-EXPL1* is highly reproducible

As a prerequisite for developing *At-EXPL1* as a regulated promoter system, we evaluated the reproducibility of its expression pattern in biological replicate experiments. Two-week old Arabidopsis plants were used to perform extended time course experiments in the absence of cordycepin as described in the *Materials and Methods*. A representative time course and summary data are shown in Figure 5.2. In the three independent experiments performed, *At-EXPL1* mRNA levels were undetectable at the beginning of the time course, accumulated very rapidly to easily detectable levels and reached maximum at 45 min. *At-EXPL1* transcript levels dropped very rapidly afterwards to nearly undetectable levels by 150 min (Figure 5.2). The transient induction pattern of *At-EXPL1* was highly reproducible as evidenced by the small standard error bars shown in Figure 5.2.

A.



B.

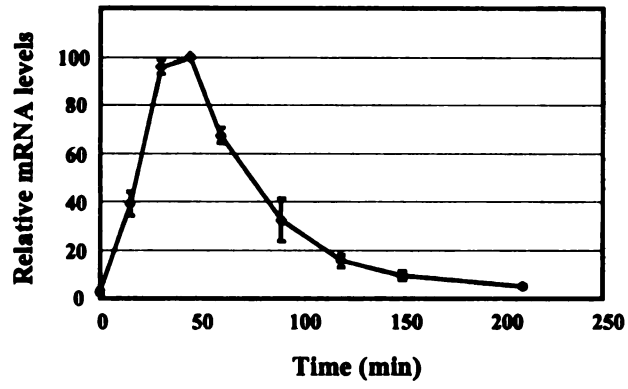


Figure 5.2. At-EXPL1 gene expression is highly reproducible. (A) Representative northern blot analysis of *At-EXPL1* mRNA at the indicated time points after transferring two-week old Arabidopsis plants to an incubation buffer (see text for details). Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Average and standard error of mRNA levels for three independent experiments are shown. All values were normalized to the corresponding *eIF4A* mRNA levels. The signal for *eIF4A* does not change significantly during the time courses and is used as a reference for equal loading.

At-EXPL1 gene transcription is severely down-regulated 45 min after its induction

To evaluate the extent of transcriptional down-regulation of *At-EXPL1* gene during the declining phase we monitored *At-EXPL1* mRNA levels in the presence of the general transcription inhibitor cordycepin and compared them to those in the absence of the drug. Two-week old Arabidopsis plants were used to perform time course experiments in the presence and in the absence of cordycepin as described in the *Materials and Methods*. As

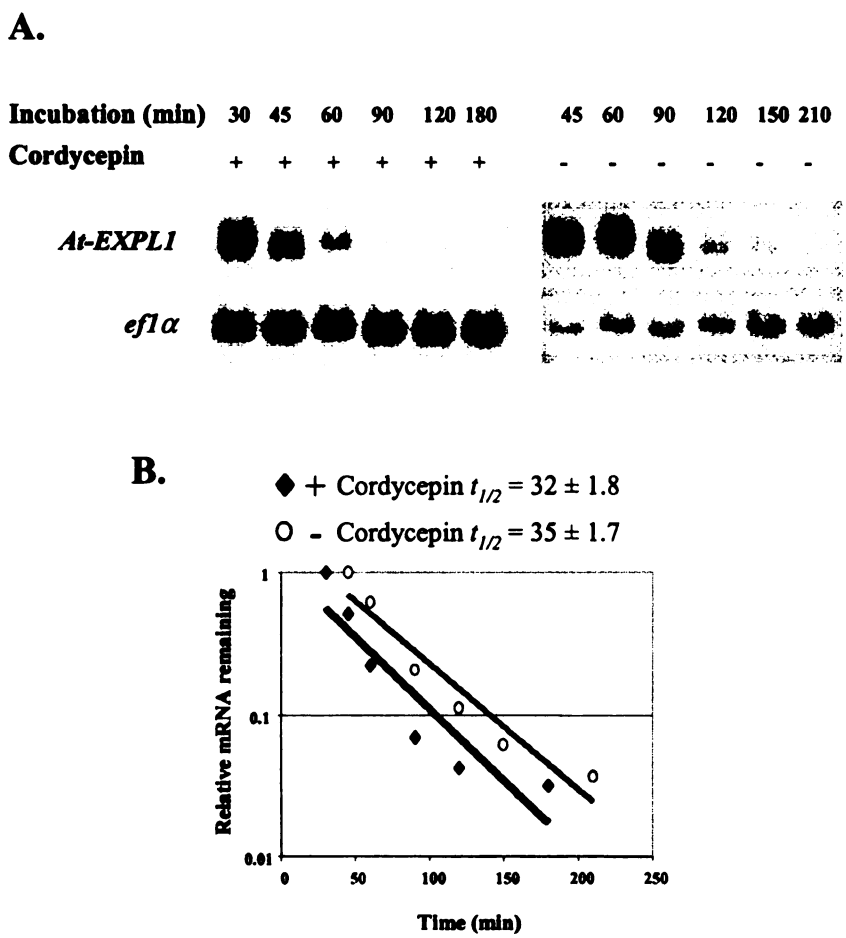


Figure 5.3. At-EXPL1 mRNA disappears with similar speed in the presence or absence of cordycepin. (A) Representative northern blot analysis of time course experiments in the presence and in the absence of cordycepin for *At-EXPL1* and *efl α* mRNA. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of the decrease in mRNA abundance and half-life estimation. The stable *efl α* transcript was used as a reference for equal loading. Values are representative of three independent cordycepin time courses.

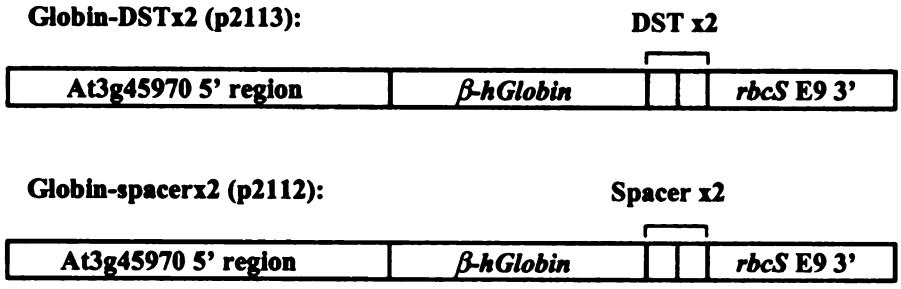
shown in Figure 5.3 the rate of disappearance of *At-EXPL1* mRNA was indistinguishable in the two conditions. The only detectable difference was that decline in the presence of cordycepin began upon addition of the drug, 15 min earlier than the natural decline of *At-EXPL1* mRNA in the absence of the inhibitor (Figure 5.3*b*). We conclude from these results that *At-EXPL1* gene expression is down-regulated after 45 min, reaching levels

comparable to those in the presence of the general transcription inhibitor cordycepin. This data suggest mRNA transcription from the *At-EXPL1* promoter ceases naturally approximately 45 min after the plants have been submerged in the incubation buffer. This data also indicates that the RNA chain terminator cordycepin does not interfere with the mRNA decay machinery that degrades the *At-EXPL1* mRNA. These results encourage the use of this chemical as a global transcriptional inhibitor for mRNA decay studies in plants.

Promoter region of *At-EXPL1* gene drives transient expression of reporter transcripts

To demonstrate that the DNA regulatory elements that control the transient induction of the *At-EXPL1* gene lay within its promoter region, the upstream sequence of *At-EXPL1* was fused to reporter transcripts of known stability. The polymerase chain reaction (PCR) was used to amplify a 2650 bp region of Chromosome III upstream of the predicted *At-EXPL1* coding sequence. The PCR amplification product was directly cloned into a commercial vector and sequence verified. A *Bgl II* site was engineered via the downstream primer pg957 to facilitate cloning into the plant expression cassettes. The promoter region was then excised as a 2584 fragment that included 37 bp of the 5'untranslated region of *At-EXPL1* using a natural *Sac I* restriction site and the new *Bgl II* site. This fragment was fused to a chimeric reporter previously built by Newman *et al.* (1993) consisting of the human β-globin coding sequence, two copies of the DST instability sequence and the E9 polyadenylation signal (globin-DSTx2; Figure 5.4*a*).

A.



B.

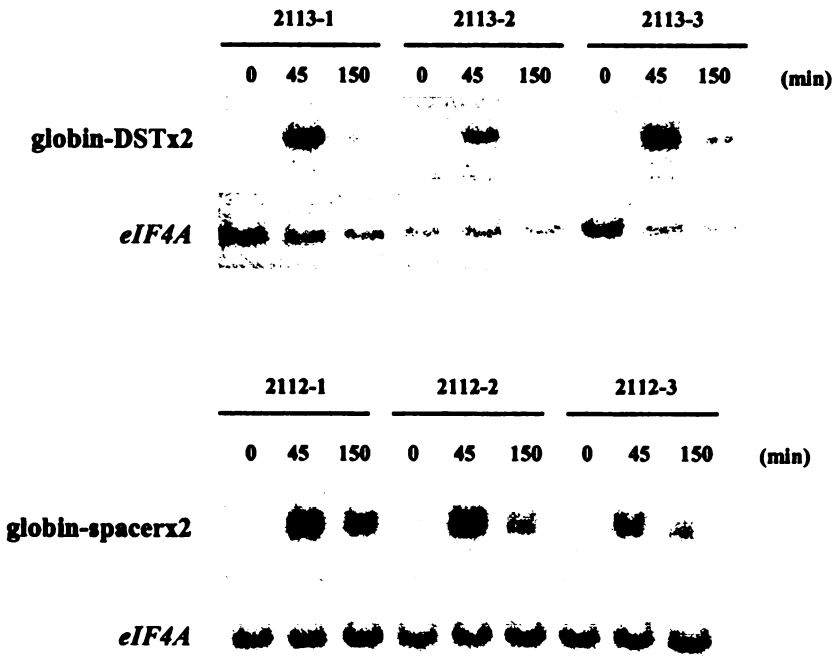


Figure 5.4. At-EXPL1 promoter region drives transient expression of two globin reporter mRNAs in transgenic Arabidopsis plants. (A) A 2584 bp DNA fragment containing the promoter region of the *At-EXPL1* gene (At3g45970) was fused to a reporter consisting of the human β-globin coding region, two copies of the DST instability sequence and the E9 polyadenylation signal (globin-DSTx2. p2113). A second construction was made in which a control spacer sequence replaced the DST element (globin-spacerx2. p2112) as illustrated. (B) Northern blot analysis of time course experiments for three independent transgenic lines expressing the globin-DSTx2 or the globin-spacerx2 reporter transcript. *eIF4A* was used as a reference for equal loading. Samples consisted of 10 μg of total RNA isolated from the indicated time points in the absence of cordycepin.

A second construction was made by fusing the *At-EXPL1* promoter fragment to a control chimeric reporter in which a spacer sequence between the β-globin coding sequence and the E9 polyadenylation signal replaced the DST element (globin-spacerx2; Figure 5.4*a*) (Newman *et al.*, 1993). Transgenic Arabidopsis plants carrying these two constructs were made via Agrobacterium mediated transformation. Seeds from independent T1 plants were germinated on agar plates in the presence of gluphosinate ammonium. Two-week old herbicide-resistant plants were then transferred to leaf incubation buffer and time courses in the absence of cordycepin were performed. As shown in Figure 5.4*b* the three lines tested for each construct exhibited reporter mRNA profiles comparable to those of the endogenous *At-EXPL1* gene. Reporter transcripts in all lines tested were near background levels at the beginning of the experiment, accumulated to easily detectable levels by 45 min and dropped afterwards to levels proportional to their stability (Figure 5.4*b*). These results demonstrate that the promoter region of *At-EXPL1* is sufficient to drive transient expression of sequences fused downstream.

***At-EXPL1* promoter system allows measurement of mRNA stability.**

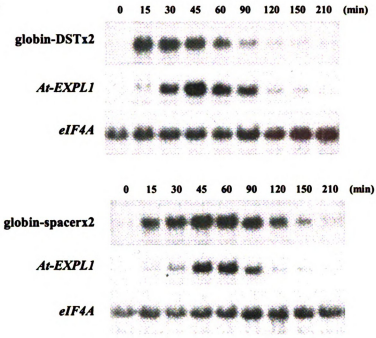
In order to test the utility of the *At-EXPL1* promoter system to study mRNA degradation in plants, lines carrying the *At-EXPL1* promoter globin-DSTx2 and globin-spacerx2 fusions were used for half-life experiments in the absence of transcription inhibitor. Seeds from three independent T1 plants per construct were used for these experiments. As expected, reporter mRNA levels were near background levels at the beginning of the experiment, accumulated rapidly to detectable levels and peaked at 30 min for globin-

DST2 and 45 min for the globin-spacerx2 mRNA (Figure 5.5*a,c*). Reporter transcript levels dropped afterwards with a rate proportional to their mRNA stability. Figure 5.5*a-b* shows a representative time course experiment. Time course experiments extended for up to 5 hrs failed to detect resumption of *At-EXPL1* promoter activity (data not shown). Summary data for the three lines studied per construct are presented in Table 5.1. The stability of the globin-DSTx2 transcript (35 ± 1.5 min) agreed well with that previously reported in tobacco cells for the same mRNA (33 ± 2.0 min) (Newman *et al.*, 1993). Surprisingly, we found a 1.6-fold difference in mRNA stability between the two reporters in Arabidopsis plants (Table 5.1), in contrast to the 4.8-fold difference previously reported in studies in tobacco cell cultures (Newman *et al.*, 1993).

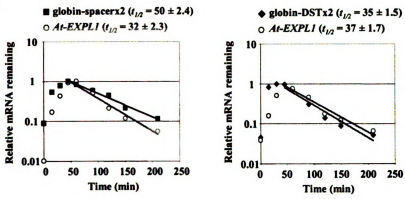
Assuming zero order mRNA synthesis and first order mRNA degradation kinetics, theoretical calculations predict that given the same transcriptional activity, unstable mRNAs will reach a new steady state level faster than stable mRNAs (Hargrove *et al.*, 1991). Our experimental data support this mathematical model in plants. On average the more unstable globin-DSTx2 reporter mRNA reached maximum accumulation 15 min earlier than the globin-spacerx2 mRNA did under the same conditions (Figure 5.5*c*). In contrast endogenous *At-EXPL1* mRNA levels peaked at 45 min regardless of the genetic background (data not shown). Given the enormous range of mRNA stabilities observed in cellular mRNAs in comparison with the less than 2-fold difference in the transcripts studied, this data emphasizes the impact that mRNA decay rates can have on plant gene expression.

Figure 5.5. At-EXPL1 promoter system can be used to study mRNA degradation in Arabidopsis. (A) Representative northern blot analysis of time courses in the absence of cordycepin. Transgenic Arabidopsis plants expressing the globin-DSTx2 or globin-spacerx2 reporter mRNAs under the control of the *At-EXPL1* promoter were used for these experiments. Endogenous *At-EXPL1* was used as internal reference and *eIF4A* mRNA as a loading control in each time course experiment. Samples consisted of 10 μg of total RNA isolated from the indicated time points. (B) Quantitation of mRNA abundance and half-life estimation in a representative experiment. The signal for *eIF4A* was used for normalization and the values were made relative to the maximum mRNA accumulation in the time course. Half-lives were calculated using the 45 min time point and thereafter as illustrated. (C) Average mRNA levels and standard error for three transgenic lines for each construct.

A.


0 15 30 45 60 90 120 150 210 (min)
globin-DSTx2
At-EXPL1
eIF4A
0 15 30 45 60 90 120 150 210 (min)
globin-spacerx2
At-EXPL1
eIF4A

B.


globin-spacerx2 ($t_{1/2}$ = 50 ± 2.4)
At-EXPL1 ($t_{1/2}$ = 32 ± 2.3)
globin-DSTx2 ($t_{1/2}$ = 35 ± 1.5)
At-EXPL1 ($t_{1/2}$ = 37 ± 1.7)
Relative mRNA remaining
Time (min)

C.

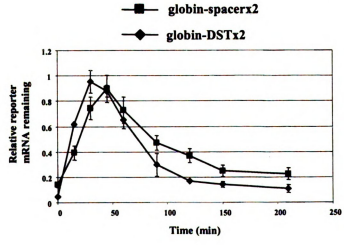

globin-spacerx2
globin-DSTx2
Relative reporter mRNA remaining
Time (min)

Table 5.1. Summary of mRNA stability measurements with regulated promoter system.

Construct		Mean Absolute Half-Life (min)			
Test gene	Ref. Gene	Test mRNA	Ref. mRNA	Mean Test/Ref.[a]	Relative Half-Life[a]
Globin-spacerx2	***At-EXPL1***	**50 ± 2.4**	**32 ± 2.3**	**1.60 ± 0.11**	**1.00 ± 0.07**
Globin-DSTx2	***At-EXPL1***	**35 ± 1.5**	**37 ± 1.7**	**0.94 ± 0.01**	**0.59 ± 0.006**

a. Calculations were made as previously described (Newman *et al.*, 1993)

Discussion

It is now acknowledged that regulation of the stability of cytoplasmic mRNAs is an important step in the control of gene expression. To understand the molecular mechanism underlying this level of modulation, a detailed analysis of the steps involved in mRNA degradation is essential. Methods to study the decay of mRNAs have been devised. Most commonly, direct measurements of mRNA stability are performed by stopping transcription with chemical inhibitors and monitoring mRNA disappearance thereafter. However transcriptional pulsing approaches are perhaps best suited for uncovering critical mechanistic aspects of the mRNA degradation process such as deadenylation and decay kinetics and precursor/product relationship. Such approaches are not available for plant systems.

Here we describe a new regulated promoter system that allows for transcriptional pulse experiments in Arabidopsis. This system takes advantage of the natural regulatory properties of the *Arabidopsis thaliana EXPL1* promoter region. We showed that *At-EXPL1* gene expression was transiently activated after submerging two-week old Arabidopsis plants in incubation buffer. *At-EXPL1* expression pattern was highly reproducible in our experimental conditions and the main regulatory elements that control its transient induction are contained within the promoter region of the gene. We took advantage of these characteristics to study the stability of the globin-DSTx2 and globin-spacerx2 reporter mRNAs. The *At-EXPL1* regulated promoter system recapitulated the instability of the globin-DSTx2 transcript (35 ± 1.5) previously studied in tobacco cell culture systems (33 ± 2 min) (Newman *et al.*, 1993). Unexpectedly, the globin-spacerx2 mRNA decayed faster in Arabidopsis (50 ± 2.4) than anticipated based on the previous

measurements performed in tobacco (113 ± 6 min) with actinomycin D (Newman *et al.*, 1993). Although sequences resembling known instability determinants can not be identified in the control spacer used in these experiments, we can not rule out the possibility that the Arabidopsis degradation machinery recognizes some features of this artificial transcript as instability determinants. Other sequences in the chimeric transcript, *e.g.* within the globin coding sequence, might also contribute to its increased turnover in Arabidopsis plants. In addition, it is known that mRNA stability studies with actinomycin D can lead to underestimation of decay rates in plants and other systems (Belasco and Brawerman, 1993; Gil and Green, 1996; Petracek *et al.*, 1998). Furthermore, the instability function of distinct sequences within the same transcript can be affected to different degrees by actinomycin D treatment (Shyu *et al.*, 1989). Therefore the function of additional instability elements in the globin-spacerx2 reporter could have been masked in the tobacco experiments by the use of actinomycin D, leading to an overestimation of the transcript stability.

It is important to mention that the turnover rate of the *At-EXPL1* mRNA was indistinguishable in time course experiments performed in the presence or in the absence of cordycepin. This data indicates that this general transcription inhibitor does not interfere with the mRNA decay machinery that normally degrades the *At-EXPL1* mRNA. This is in contrast to what obtained with other chemicals (Gil and Green, 1996; Petracek *et al.*, 1998) and encourage the use of cordycepin for mRNA decay studies in plants.

A great advantage of the system described is that the endogenous *At-EXPL1* mRNA can be used as a control for the transient induction of *At-EXPL1* promoter activity as well as an mRNA stability internal reference to compare across multiple experiments.

Previous studies have shown that the use of a reference transcript as an internal standard is a very effective strategy to normalize for experimental variation that may occur due to small differences in growth conditions, developmental stage, physiological status, etc (Newman *et al.*, 1993).

It is not yet known what stimulus or stimuli control the transient transcriptional activation of *At-EXPL1*. General knowledge about the expansin-like gene family is very limited. However, semi-quantitative RT-PCR experiments indicated *At-EXPL1* is expressed in most Arabidopsis organs (Dan Cosgrove, personal communication). In addition, promoter β-glucuronidase fusion experiments suggest *At-EXPL1* is induced by wounding (Dan Cosgrove, personal communication). Preliminary studies in our laboratory are consistent with *At-EXPL1* being induced in response to mechanical damage (data not shown). Based on our experimental design, we can propose mechanical stimulation/damage and perhaps submersion are the most likely signals involved in regulating *At-EXPL1* promoter activity. Previous studies indicate that mechanical stimulation (touch) can induce transient gene expression. In fact the touch gene transcripts initially characterized were all detected within minutes of treatment and disappeared very rapidly afterwards, similar to what observed for the *At-EXPL1* mRNA (Braam and Davis, 1990). Transient gene expression in response to wounding has also been reported (*e.g.* (Rojo *et al.*, 1999)). Submergence could also be implicated in *At-EXPL1* expression as it is known that submersion alone can effectively induce expression of two α-expansin genes in *Oryza sativa* (Cho and Kende, 1997). Additional experiments will be necessary to determine the signals(s) that regulate *At-EXPL1* promoter activity. Nevertheless, under the conditions described for our assays it reproducibly behaves as a

strong promoter system that is transiently activated. This transient induction results in a synchronized population of transcripts that can be monitored overtime. Hence, this tool will be most useful to study mRNA decay kinetics and specific aspects of the mRNA degradation process such as deadenylation. The strength of this promoter will greatly facilitate the study of labile mRNAs. In addition, because no toxic chemicals are needed to activate or represses the *At-EXPL1* promoter, time courses can be extended allowing the study of stable plant transcripts.

References

Aoyama,T. and Chua,N.H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J. *11*, 605-612.

Belasco,J.G. and Brawerman,G. (1993). Experimental Approaches to the Study of mRNA Decay. In Control of Messenger RNA Stability, J.Belasco and G.Brawerman, eds. Academic Press, Inc. San Diego, CA), pp. 475-493.

Bohner,S., Lenk,I., Rieping,M., Herold,M., and Gatz,C. (1999). Transcriptional activator TGV mediates dexamethasone-inducible and tetracycline-inactivatable gene expression. Plant J. *19*, 87-95.

Braam,J. and Davis,R.W. (1990). Rain-induced, wound-induced, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell *60*, 357-364.

Brawerman,G. (1993). mRNA degradation in eukaryotic cells:an overview. In Control of Messenger RNA Stability, J.Belasco and G.Brawerman, eds. Academic Press, Inc. San Diego, CA), pp. 149-160.

Cho,H.T. and Kende,H. (1997). Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell *9*, 1661-1671.

Gil,P. and Green,P.J. (1996). Multiple regions of the *Arabidopsis SAUR-AC1* gene control trascript abundance: the 3' untranslated region functions as an mRNA instability determinant. EMBO J. *15*, 1678-1686.

Granger,C.L. and Cyr,R.J. (2001). Characterization of the yeast copper-inducible promoter system in Arabidopsis thaliana. Plant Cell Reports *20*, 227-234.

Gutiérrez,R.A., Ewing,R.M., Cherry,J.M., and Green,P.J. (2002). Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: rapid decay is associated with a group of touch- and specific clock-controlled genes. Proc. Natl. Acad. Sci. U. S. A *99*, 11513-11518.

Gutiérrez,R.A., MacIntosh,G.C., and Green,P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci. *4*, 429-438.

Hargrove,J.L., Hulsey,M.G., and Beale,E.G. (1991). The kinetics of mammalian gene expression. Bioessays *13*, 667-674.

Herrick,D., Parker,R., and Jacobson,A. (1990). Identification and comparison of stable and unstable messenger-RNAs in *Saccharomyces-cerevisiae*. Mol. Cell. Biol. *10*, 2269-2284.

Johnson,M.A., Pérez-Amador,M.A., Lidder,P., and Green,P.J. (2000). Mutants of Arabidopsis defective in a sequence-specific mRNA degradation pathway. Proc. Natl. Acad. Sci. U. S. A *97*, 13991-13996.

Love,J., Scott,A.C., and Thompson,W.F. (2000). Stringent control of transgene expression in Arabidopsis thaliana using the Top10 promoter system. Plant J. *21*, 579-588.

Martinez,A., Sparks,C., Hart,C.A., Thompson,J., and Jepson,I. (1999). Ecdysone agonist inducible transcription in transgenic tobacco plants. Plant J. *19*, 97-106.

Newman,T.C., Ohme-Takagi,M., Taylor,C.B., and Green,P.J. (1993). DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell *5*, 701-714.

Nonet,M., Scafe,C., Sexton,J., and Young,R. (1987). Eukaryotic RNA polymerase conditional mutant that rapidly ceases messenger RNA synthesis. Mol. Cell. Biol. *7*, 1602-1611.

Parker,R., Herrick,D., Peltz,S.W., and Jacobson,A. (1991). Measurement of messenger RNA decay rates in *Saccharomyces cervisiae*. Methods Enzymol. *194*, 415-423.

Petracek,M.E., Dickey,L.F., Nguyen,T.T., Gatz,C., Sowinski,D.A., Allen,G.C., and Thompson,W.F. (1998). Ferredoxin-1 mRNA is destabilized by changes in photosynthetic electron transport. Proc. Natl. Acad. Sci. USA *95*, 9009-9013.

Rojo,E., Leon,J., and Sanchez-Serrano,J.J. (1999). Cross-talk between wound signalling pathways determines local versus systemic gene expression in Arabidopsis thaliana. Plant J. *20*, 135-142.

Salter,M.G., Paine,J.A., Riddell,K.V., Jepson,I., Greenland,A.J., Caddick,M.X., and Tomsett,A.B. (1998). Characterisation of the ethanol-inducible alc gene expression system for transgenic plants. Plant J. *16*, 127-132.

Seeley,K.A., Byrne,D.H., and Colbert,J.T. (1992). Red light-independent instability of oat phytochrome mRNA *in vivo*. Plant Cell *4*, 29-38.

Shimizu-Sato,S., Huq,E., Tepperman,J.M., and Quail,P.H. (2002). A light-switchable gene promoter system. Nature Biotechnol. *20*, 1041-1044.

Shyu,A.B., Greenberg,M.E., and Belasco,J.G. (1989). The c-*fos* transcript is targeted for rapid decay by 2 distinct messenger-RNA degradation pathways. Genes Dev. *3*, 60-72.

Wang,Y.L., Liu,C.L., Storey,J.D., Tibshirani,R.J., Herschlag,D., and Brown,P.O. (2002). Precision and functional specificity in mRNA decay. Proc. Natl. Acad. Sci. USA *99*, 5860-5865.

Weinmann,P., Gossen,M., Hillen,W., Bujard,H., and Gatz,C. (1994). A chimeric transactivator allows tretracycline-responsive gene expression in whole plants. Plant J. *5*, 559-569.

CHAPTER 6

Final remarks and challenges ahead

At the onset of this work, genomic approaches using DNA microarrays were common only for comparing mRNA steady-state levels between two conditions. They have been rarely applied to the study of mRNA decay or other post-transcriptional processes. In this dissertation we used the Arabidopsis Functional Genomics Consortium cDNA microarrays to identify the genes with the most unstable mRNAs in Arabidopsis plants. Our study provided basic information regarding rapid mRNA turnover in Arabidopsis and constituted the foundation for further research.

This study, together with genomic studies in other systems, underscores the relevance of a fundamental and yet not fully answered question in the field: What are the *cis*-acting molecular determinants of rapid turnover? It is clear that discrete sequences within can act as signals for accelerated mRNA degradation. However those stability determinants that are known can at best explain a small fraction of the labile mRNA population. Deletion analysis experiments of new unstable transcripts coupled with mRNA half-life measurements should help us identify novel molecular determinants of mRNA stability. The identification of a high number of transcripts with similar turnover rates prompts the analysis of structural features. However although computational approaches have been useful for detecting DNA sequences involved in transcriptional regulation (*e.g.* (Harmer *et al.*, 2000)), they are not the best to detect sequences that control gene expression at the mRNA stability level. Perhaps due to the complexity of these sequence motifs and/or the presence of secondary structures, stability determinants are difficult to detect above background noise with the current sequence analysis tools. In the future, and as more stability determinants are known, optimized algorithms might

better predict mRNA stability determinants and thus guide experimental research. We should also consider that perhaps different biological principles play a role in governing mRNA decay rates as well. Recently, a class of micro-RNAs (miRNAs) was shown to direct cleavage of Scarecrow-like mRNAs in Arabidopsis (Llave *et al.*, 2002). miRNAs are a numerous class of short ~21 to 22 RNAs present in plants, animals and microorganisms most of which have unknown function. Perhaps miRNAs play a more general role in controlling mRNA degradation. Comparing genome-wide patterns of mRNA decay between Arabidopsis WT and KO mutants impaired in miRNA synthesis or in the post-transcriptional gene silencing pathway should help evaluate the significance of this mechanism for global mRNA degradation in plants. Similar studies can be also carried out to investigate the role of new candidate regulators of the mRNA degradation process as well as known components of the decay machinery. Because mRNAs will decay with slower rates in the relevant KO mutants, this analysis should help categorize transcripts on the basis of their decay strategy.

Another intriguing question for future studies that emerges from this dissertation research relates to the *AtGUTs* regulated by touch, light and other signals. Are these *AtGUTs* "constitutively" unstable, or is their mRNA stability regulated in response to light, touch, or other signals? It is clear that at least in some cases regulation of mRNA stability occurs. However by comparing global turnover rates under different conditions, post-transcriptional regulatory networks might be unraveled. This large-scale approach for analysis of post-transcriptional control mechanisms should help uncover the extent and significance of this level of regulation in plants in response to a variety of stimuli.

The studies of mRNA decay and circadian rhythms opened an exciting avenue for future research. We observed a new relationship between expression of clock-controlled genes and the sequence specific mRNA decay pathway mediated by the DST element. Determining the mechanistic relationship between *DST1* and the circadian clock should further our understanding of the importance of this level of control for circadian rhythms in plants. Isolation of the *DST1* gene should be a priority. But further physiological studies, *e.g.* circadian phenotypes, should help us narrow the context for DST1 function and its relationship with circadian gene expression. Such knowledge should aid current mapping efforts. Additional mutants impaired in DST-mediated decay have been isolated. The study of circadian phenotypes in these mutants, *e.g. dst2*, and the relationship to the *dst1* mutant in this context should provide further insight into the role of DST-mediated mRNA degradation for circadian rhythms.

Similarities between the mRNA decay process in plants and other eukaryotic systems have been shown before. However it is also clear that differences exist. This may be especially true with respect to determinants of long mRNA life, which are typical of transcripts expressed in terminally differentiated and specialized cells. Deletion analysis of stable transcripts and mRNA half-life measurements should help identify the signals that protect transcripts from degradation in plants. In addition, the availability of a regulated promoter system in plants should facilitate the study of specific aspects of the mRNA degradation process in stable and unstable transcripts, such as deadenylation. Deadenylation is an important control point for transcript decay. This step often initiates the degradation process in yeast (Caponigro and Parker, 1996) and mammals (Wilusz, *et al.*, 2001) and its rate can impact mRNA stability. Comparing deadenylation rates in

stable and unstable transcripts should help evaluate the significance of this step in the degradation of plant mRNAs.

References

Caponigro,G. and Parker,R. (1996). Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol. Rev. *60*, 233-249.

Harmer,S.L., Hogenesch,J.B., Straume,M., Chang,H.S., Han,B., Zhu,T., Wang,X., Kreps,J.A., and Kay,S.A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science *290* , 2110-2113.

Llave,C., Xie,Z.X., Kasschau,K.D., and Carrington,J.C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science *297*, 2053-2056.

Wilusz,C.J., Wormington,M., and Peltz,S.W. (2001). The cap-to-tail guide to mRNA turnover. Nature Rev. Mol. Cell Biol. *2*, 237-246.