This is to certify that the

dissertation entitled

GENETIC DETERMINANTS OF mRNA STABILITY
IN PLANTS

presented by

Mark Aikens Johnson

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Major professor

Date 1/4/00

THE GENETIC DETERMINANTS OF mRNA STABILITY IN PLANTS

By

Mark Aikens Johnson

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Microbiology

Program in Cellular and Molecular Biology

1999

ABSTRACT

GENETIC DETERMINANTS OF mRNA STABILITY IN PLANTS

By

Mark Aikens Johnson

Regulation of mRNA stability is now known to be an important means of controlling gene expression. Despite many exciting advances in recent years, there are still many unresolved questions about the mechanisms responsible for mRNA degradation. The aim of this thesis was to address how plant cells recognize unstable messages and to understand the mechanisms the cell uses to degrade them. The work presented here provides important information as well as a foundation for mechanistic insights that will be made in the future.

In Section One, Sequence-Specific mRNA Degradation, the focus is on the DST element, which targets certain plant transcripts for rapid mRNA degradation. Mutants of Arabidopsis were isolated that do not fully recognize this instability signal. At least two genes, *dst1* and *dst2*, are shown to be involved in the recognition of the DST sequence. The cloning of these genes will be of tremendous interest and will likely be very informative about the recognition of unstable mRNAs. Also in this section, a dissection of the *SAUR-AC1* 3' untranslated region (UTR) is described. This 3'UTR contains one DST element and several DST-like subdomains. Studies presented here indicate that redundant DST-like sequences located upstream of the DST element may play an important role in the instability function of the *SAUR-AC1* 3'UTR. In the future it will be

interesting to analyze the effect of these redundant DST-like sequences on mRNA stability in the *dst1* and *dst2* mutants.

In Section Two, General Mechanisms of mRNA Degradation, two sets of experiments address how the plant cell degrades mRNA. To get a glimpse into this process, poly(G) tracts were inserted into reporter transcripts and these were introduced into plant cells in an attempt to stabilize intermediates in the process of mRNA degradation. Such intermediates were not observed, which may indicate that there are some differences between the mechanisms of mRNA turnover in plants and yeast, where poly(G) tracts are known to stabilize mRNA decay intermediates. The second set of experiments addresses the role of a potential plant poly(A) ribonuclease, AtPARN, in plant mRNA decay. It has been shown that removal of the poly(A) tail leads to rapid mRNA degradation in yeast and mammals. Preliminary experiments indicate that AtPARN has poly(A) ribonuclease activity in vitro, suggesting that it may play a role in the degradation of mRNA in plants.

ACKNOWLEDGMENTS

I would like to thank Pam Green for being my mentor. The experiments described in this thesis would not have been possible if not for her creativity, support, guidance, encouragement, and enthusiasm. Pam has fostered a laboratory environment that helps to make our work enjoyable and intellectually rewarding.

Many people have contributed to this work and while specific acknowledgements are given at the end of each chapter there are a few people who deserve special thanks. Miguel Pérez-Amador and I have worked together throughout the course of my Ph.D. He has been an extremely helpful and caring colleague without whom the selection of *dst* mutants would have been even more difficult. Likewise, Michael Sullivan was extremely helpful when I first joined the laboratory and laid the groundwork for the mutant selection. Linda Danhof has kept our laboratory well organized and has maintained an army of undergraduate helpers. Jonathon Vogel, who started out in Linda's army, has made countless contributions to my work and has provided me with the pleasure of helping him to learn how to do science. Preet Lidder and I worked together during her rotation. She has and will continue to make important contributions to our study of the *dst* mutants and to AtPARN. Thanks also to Ellen Baker and Jim Colbert who, along with Pam, were my co-authors on the review article that became the introduction to this thesis. I am also grateful for my thesis committee: Frans deBruijn, Donna Koslowsky, and Mike Thomashow; who have taken time out of their schedules to give me excellent advice at several important stages in this process.

Members of the Green Lab, past and present, have had a tremendous impact on these experiments and on the way I think and communicate about science. They have

been a great bunch of friends and colleagues. My thanks go to Jim Kastenmayer, Nikki Lebrasseur, Michael Feldbrügge, Rodrigo Gutiérrez, Gustavo MacIntosh, Mike Abler, Pauline Bariola, Jay De Rocher, Scott Diehn, Pedro Gil, Christy Howard, Deb Thompson, and Ambro van Hoof.

My thesis is dedicated to Carol, who has made this tremendous learning experience a time of great joy for me, and to my entire family (my grand parents: Henry and Virginia Aikens, Wilhelm and Hilda Johnson; my parents: Tom and Kathy Johnson, Scott and Sherie Barnes; my brothers and sisters: Suzy, Jenny, Kate, and Andy Johnson, and Bill Barnes; and all of my aunts, uncles, and cousins) who have been with me the whole way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

SECTION 1: SEQUENCE-SPECIFIC mRNA DEGRADATION

CHAPTER 1-1: A PROCEDURE TO SELECT MUTANTS OF ARABIDOPSIS DEFECTIVE INSEQUENCE-SPECIFIC mRNA DEGRADATION

CHAPTER 1-2: MUTANTS OF ARABIDOPSIS DEFECTIVE IN A SEQUENCE-SPECIFIC mRNA DEGRADATION PATHWAY

CHAPTER 1-3: ANALYSIS OF THE DST ELEMENT AND DST-LIKE SUBDOMAINS WITHIN THE *SAUR-AC1* 3' UNTRANSLATED REGION

SECTION 2: GENERAL MECHANISMS OF mRNA DEGRADATION

CHAPTER 2-1: USE OF POLY(G) INSERTIONS TO GAIN INSIGHT INTO THE STEPS INVOLVED IN PLANT mRNA DEGRADATION

CHAPTER 2-2: CLONING AND INITIAL CHARACTERIZATION OF A POLY(A) RIBONUCLEASE FROM ARABIDOPSIS

INTRODUCTION

DETERMINANTS OF MESSENGER RNA STABILITY IN PLANTS

In its original form, this introduction was published in "A Look Beyond Transcription: Mechanisms Determining mRNA stability and Translation in Plants", Julia Bailey-Serres, Daniel R. Gallie, eds. Copyright 1998, American Society of Plant Physiologists (Johnson et al., 1998). Changes have been made to update the content and bring the text into the larger context of this dissertation.

INTRODUCTION

The abundance of an RNA molecule depends both upon the frequency with which it is transcribed and the rate at which it is degraded. This statement holds true regardless of whether one is considering mature mRNA molecules encoded by nuclear genes, plastid-encoded mRNAs, nuclear-encoded rRNAs, or the abundance of a particular species of primary transcript in the nuclear compartment. In each of these instances degradative processes play an important role in the amount of RNA that is present, and potentially, in the final level of gene expression.

The focus of this introduction will be on the degradative events affecting the abundance of mature mRNAs encoded by nuclear genes in plants. It is generally assumed that these types of degradative events occur within the cytosolic portion of the cell. However, it also seems clear that nuclear degradative events may play a critical role in the establishment of cytosolic mRNA levels in particular organs, tissues, or cells (Kamalay and Goldberg, 1984; Okamuro and Goldberg, 1989). The significance of mRNA degradation to gene expression is aptly demonstrated by attempts to express the *Bacillus thuringiensis* toxin genes (*cry* genes) in transgenic plants. Wild-type versions of *cry* genes are typically not expressed at high levels in transgenic plants (Adang et al., 1993), even when fused to the strong cauliflower mosaic virus 35S (35S) promoter. High level expression has been obtained by use of synthetic, plant-like versions of the *cry* genes that contain codon usage and other changes (Adang et al., 1993; Koziel et al., 1993; Stewart et al., 1996; reviewed in Diehn et al., 1996). The plant-like *cry* genes are generally thought to produce mRNAs that exhibit longer half-lives in plant cells (Diehn et al., 1996), leading to higher level accumulation of the toxin. There is direct evidence

for this in the case of a plant-like synthetic *cryI* gene which encodes a transcript that is considerably more stable than the wild-type *cryIA(c)* transcript (De Rocher et al., 1998).

The half-lives of mRNA molecules can vary over a wide range. Typical plant mRNA molecules appear to exhibit half-lives of several hours (Siflow and Key, 1979; Sullivan and Green, 1993; Taylor and Green, 1995). Relatively unstable plant mRNAs, with half-lives of an hour or less, have also been described (McClure and Guilfoyle, 1989; Braam and Davis 1990; Seeley et al., 1992; Taylor and Green, 1995; van Hoof and Green, 1996). On the other end of the spectrum are very stable mRNAs with half-lives of days or more, some of which may be stored or sequestered from the mRNA decay machinery. In addition, there are some well-characterized examples of mRNAs whose half-lives can vary in response to specific stimuli. In mammalian cells, the transferrin receptor mRNA is stabilized 20- to 30-fold under conditions of low intracellular iron concentration (Casey et al., 1989; Koeller et al., 1991). Mammalian histone and β-tubulin mRNAs also exhibit regulated variations in mRNA half-life (Ross, 1995). In plants, a good example is the *PvPRP1* mRNA which is destabilized in the presence of fungal elicitor (Zhang et al., 1993; Mehdy and Brodl, 1998).

There are a number of ways to measure mRNA decay rates in plant systems. These include measuring the half-lives of endogenous or in vitro synthesized mRNAs in protoplasts (Gallie et al., 1989) or in vitro (Byrne et al., 1993; Tanzer and Meagher, 1994). Alternatively, mRNA decay rates can be monitored in transformed or nontransformed cultured cells or intact plants, following treatment with a transcriptional inhibitor (Newman et al., 1993; Byrne et al., 1993). Recently, repressible promoters have also been used to shut off transcription of the genes of interest in cultured cells or plants

so that the stability of the corresponding mRNA can be measured without the use of general transcriptional inhibitors (Weinman, et al., 1994; Gil and Green, 1996; Petracek et al., 1998). For a more detailed description of these methods and a discussion of the advantages and limitations of each, readers are referred to reviews by Abler and Green (1996) and Ross (1995).

The refinement of methods for measuring mRNA stability, as well as mounting evidence that many plant genes are regulated at this level, has led to considerable progress in recent years. Information has been obtained about the contribution of the cap and poly(A) tail, and it has been shown that plants have multiple poly(A) binding proteins that can be differentially regulated. Another important step has been the identification of specific sequences that control mRNA decay rates. In particular, several sequences that cause rapid mRNA degradation have been characterized. These and other studies have increased our perception of the types of cellular factors that may be involved in mRNA decay and the mechanisms that may be involved. Finally, it has become apparent that mRNA decay mechanisms may play a prominent role in certain forms of gene silencing such as the phenomenon of post-transcriptional cosuppression that is sometimes observed in transgenic plants. All of these topics will be discussed in this introduction in an effort to present our current understanding of the components and mechanisms that determine the inherent stabilities of different mRNAs in plants. Emphasis will be placed on describing the most recent findings, many of which pertain to rapid mRNA decay mechanisms that allow plants to respond quickly to internal and external stimuli. The purpose of this introduction is to highlight what is known about

mRNA turnover in plants and to point out how the work presented in this dissertation addresses some of the many important questions that are being addressed in this field.

mRNA INSTABILITY SEQUENCES

Rapid turnover of mRNA is thought to be an active process. Indeed, *cis*-acting sequences have been identified that target transcripts for rapid turnover in plants as well as in other systems (reviewed in Ross, 1995; Abler and Green, 1996; Caponigro and Parker, 1996; van Hoof and Green, 1997). The DST element, found in the 3' untranslated region (UTR) of the unstable small auxin up RNAs (*SAURs*), is one such instability sequence that so far appears to be unique to plants (McClure et al., 1989; Newman et al., 1993; Gil et al., 1994; Gil and Green, 1996; Sullivan and Green, 1996). Another element that can target transcripts for rapid turnover in plants consists of repeats of AUUUA pentamers (Ohme-Takagi et al., 1993). Multiple repeats of this pentamer are a common feature of many highly unstable mammalian transcripts (reviewed in Chen and Shyu, 1995). Premature stop codons have also been shown to target transcripts for rapid decay in plants as well as in yeast, *Caenorhabditis elegans*, and mammalian cells (reviewed in Maquat, 1995; van Hoof and Green, 1997; Jacobson and Peltz, 1996). In this section, the plant instability sequences that have been most thoroughly characterized will be described. These are summarized in Table 1.

Table 1. Sequences that have been demonstrated to control mRNA stability in plants.

Sequence	Description	Experimental System	Ref
AUUUA	AUUUA repeats are a common feature of AU-rich instability determinants found in labile mammalian cytokine and protooncogene transcripts. 11 repeats of the AUUUA pentamer caused instability.	$t_{1/2}$ measured during an ActD time course in stably transformed BY-2 cells*	1
DST	A highly conserved 40 base sequence found downstream of the stop codon in unstable SAUR transcripts. A tandem dimer of DST caused instability.	$t_{1/2}$ measured during an ActD time course in stably transformed BY-2 cells*	2,3
SAUR-AC1 3' UTR	Approximately 140 bases, includes one highly conserved DST element and several repeated ATAGAT-like and GTA-like subdomains.	$t_{1/2}$ measured using Top10 promoter in stably transformed BY-2 cells*	4
Premature Stop Codon	Naturally occurring and in vitro generated premature stop codons in the coding region of the PHA transcript.	$t_{1/2}$ measured during an ActD time course in stably transformed BY-2 cells*	5
iLRE	5' UTR plus the first 14 codons of *Fed-1*; controls light regulation of mRNA stability.	$t_{1/2}$ measured using Top10 promoter in transgenic tobacco plants	6

ActD, actinomycin D; UTR, untranslated region; Ref, References; PHA, Phytohemagglutinin; BY-2, bright yellow-2 (NT-1, tobacco cell line); $t_{1/2}$, half-life
References: 1) Ohme-Takagi et al., 1993; 2) Newman et al., 1993; 3) Sullivan and Green, 1996; 4) Gil and Green, 1996; 5) van Hoof and Green, 1996; 6) Petracek et al., 1998) *Confirmed in transgenic plants by measuring mRNA accumulation

Instability Sequences in *SAUR* genes

mRNAs encoded by the auxin induced *SAUR* genes are among the most unstable plant transcripts and therefore offer an excellent model with which to study rapid mRNA turnover. The half-lives of these transcripts have been estimated to be between 10 and 50 minutes depending upon the method used in the analysis (McClure and Guilfoyle, 1989; Franco et al., 1990). Seven *SAUR* genes have been cloned from soybean, mungbean, and *Arabidopsis thaliana* all of which contain a highly conserved, 40 base pair sequence motif in their 3' UTRs (McClure et al., 1989; Yamamoto et al., 1992; Gil et al., 1994).

This sequence was termed DST because of its location downstream of the *SAUR* stop codon (McClure et al., 1989). The highly conserved nature of this sequence and its placement in the 3' UTR of the *SAUR* transcripts led to the suggestion that the DST element may control the stability of the *SAUR* transcripts (McClure et al., 1989; Franco et al., 1990).

The destabilizing function of the DST element was tested directly by Newman et al. (1993). They demonstrated that a synthetic dimer of the DST element, when placed in the 3' UTR of the β-glucuronidase (*GUS*) or the *β-globin* reporter transcript, is able to target these reporter transcripts for rapid turnover relative to controls lacking DST sequences. In these experiments, half-lives of reporter mRNAs were measured in stably transformed tobacco (BY-2) cell suspension cultures by inhibiting new transcription using actinomycin D and monitoring the decay of the reporter transcript over a two-hour time course. It is likely that DST sequences act as potent instability determinants in intact plants as well as in cultured cells because DST elements also cause a marked decrease in mRNA abundance relative to controls in transgenic tobacco plants.

The DST element consists of three highly conserved subdomains separated by two variable regions. Site-directed mutagenesis experiments have been performed in order to determine which features of the DST element are most important for its function as an instability determinant (Sullivan and Green, 1996). The results of these experiments are illustrated in Figure 1. Highly conserved subdomains are in boxes. The second and third subdomains contain residues that are invariant among all DST elements and are termed ATAGAT and GTA, respectively. Both of these subdomains have been found to be necessary for DST function. This was shown by constructing mutated DST

elements in which either the ATAGAT or GTA subdomains were disrupted with five or six base substitutions, respectively. These mutant elements were tested as dimers placed in the 3' UTR of the *β-globin* reporter transcript. The effects of the mutations were analyzed by measuring mRNA accumulation and half-life in stably transformed tobacco cell cultures and mRNA accumulation in leaves of transgenic tobacco plants. These mutations resulted in mRNA half-lives that were nearly as long as a control lacking DST sequences and in the restoration of *β-globin* mRNA abundance to control levels in stably transformed tobacco cell cultures and transgenic tobacco leaves. In order to pinpoint critical bases within these two invariant subdomains, two base pair (CC) substitution mutations were analyzed. The results indicated that the first four bases of the ATAGAT subdomain are most important for DST function in tobacco cell culture. Interestingly, a CC substitution in the invariant GTA sequence led to inactivation of the DST element in transgenic tobacco leaves but not in cultured cells. This finding may indicate that the DST element is recognized differently in different cell types.

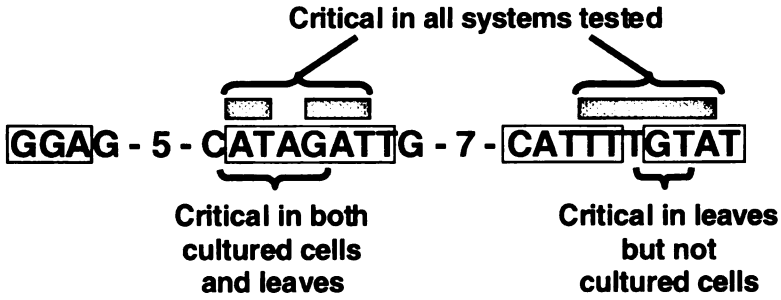

Figure 1. Critical regions of the DST sequence. DST sequences, found in unstable *SAUR* transcripts, consist of three highly conserved regions (boxed) separated by two variable regions. The second and third regions contain the invariant sequences ATAGAT and T--GTA, respectively. Sequence requirements for function of the DST element were established using a series of substitution mutations introduced into a dimer of the soybean *SAUR 15A* DST element. All mutations were tested in both copies of the dimer. Sequences shown to be critical using five and six-base substitution mutations are highlighted above the sequence. Critical sequences identified with two-base pair substitution mutations are highlighted below the sequence.

Although a function for *SAUR* gene products has not been demonstrated, the expression patterns of these transcripts indicate that they may have a role in early events in auxin-induced cell elongation. The *SAUR-AC1* gene of *A. thaliana* has been carefully analyzed in order to determine which regions of the gene are responsible for *SAUR* expression characteristics, namely auxin induction and rapid mRNA turnover (Gil et al., 1994; Gil and Green, 1996). By constructing chimeric genes that consisted of the *SAUR-AC1* promoter, coding region, or 3' UTR fused to reporter genes, it was shown that the promoter is the site of auxin action, the 3' UTR is largely responsible for rapid mRNA turnover and the coding region contributes to low mRNA abundance but not by decreasing message half-life (Gil and Green, 1996). It was shown that the 3' UTR of *SAUR-AC1* functions as a determinant of rapid mRNA turnover by fusing this region of the *SAUR* gene to the *β-globin* coding region whose expression was driven by a tetracycline-repressible promoter known as Top10 (Weinman et al., 1994). Following tetracycline treatment, mRNA stability can be measured without the use of general transcriptional inhibitors such as actinomycin D.

The *SAUR-AC1* 3' UTR contains one canonical DST element which is located 80 bases downstream of the stop codon and 10 bases upstream of the poly(A) addition site (Gil et al., 1994). It is notable that experiments with the synthetic DST element, described above, indicated that two copies were required for instability function. It is not clear whether this apparent difference reflects an absolute DST recognition requirement or if it reflects a necessary context within the *SAUR-AC1* 3' UTR. Interestingly, there are several ATAGAT-like and GTA-like subdomains of the DST sequence located just upstream of the classically defined DST element within the *SAUR-AC1* 3' UTR. These

redundancies may be serving as multiple recognition sites for DST mediated mRNA decay within the *SAUR-AC1* 3' UTR.

AUUUA sequences

AU-rich elements are found in the 3' UTRs of several of the most unstable mammalian transcripts (Chen and Shyu, 1995). The genes that encode these transcripts have functions involved in cell division and differentiation and their expression is very stringently controlled. Indeed, overexpression of some of these genes can lead to oncogenesis and it is thought that AU-rich elements serve to limit expression by targeting their transcripts for rapid decay. Repeats of the pentamer, AUUUA, are often found in these AU-rich elements and have been shown to be important for their function (Caput et al., 1986; Shaw and Kamen., 1986; Vakalopoulou et al., 1991; Shyu et al., 1991). A repeat of eleven AUUUA pentamers has been shown to target reporter transcripts for rapid degradation in plants (Ohme-Takagi et al., 1993). This was demonstrated by fusing the repeated AUUUA element downstream of the *β-globin* or *GUS* coding region and comparing the half-life of these transcripts to that of controls lacking AUUUA repeats during actinomycin D time courses. The sequence of the element rather than its A+U content appears to be most important because an AU-rich control lacking AUUUA repeats had little effect on *β-globin* or *GUS* mRNA stability.

These results indicate that the mRNA decay pathway mediated by AUUUA repeats may be conserved between animals and plants. There is no evidence that this pathway functions in yeast. The natural targets of the plant AUUUA mediated decay

pathway are unknown but one possible candidate is *PvPRP1* from *Phaseolus vulgaris*. The *PvPRP1* transcript, which appears to encode a cell wall protein, is rapidly degraded (t1/2≅45') following the addition of fungal elicitor to bean cell cultures (Zhang et al., 1993). Zhang and Mehdy have reported a 50 kd protein that can be specifically UV cross-linked to the 3' UTR of the *PvPRP1* transcript (Zhang and Mehdy, 1994). The binding of this 50 kd polypeptide, termed PRP-BP, has been mapped to a 27 bp sequence that contains an AUUUA motif (Zhang and Mehdy, 1994). Although the 3' UTR of this transcript has not been shown to be responsible for its rapid turnover, it is interesting to consider the possibility that the PRP-BP is involved in the recognition of an AUUUA motif that is involved in regulating the stability of this transcript. This speculation does, however, beg the question of minimal sequence requirements for AUUUA repeats in plants. Although this has not yet been established, recent data in mammalian cells indicate that as few as three copies (Lagnado et al., 1994) or even one copy (Zubiaga et al., 1994) of the sequence UUAUUUAUU are sufficient to destabilize a reporter transcript.

Premature stop codons and other translational influences on mRNA stability

Premature stop codons function as *cis*-acting instability determinants in yeast, *C. elegans*, mammalian cells and plants, and offer compelling evidence for a link between translation and mRNA turnover that is widespread among eukaryotes (Maquat, 1995; Jacobson and Peltz, 1996; Sullivan and Green, 1993; van Hoof and Green, 1997; Marcotte, 1998). This was first recognized in plants when naturally occurring mutant

alleles of phytohemagglutinin (PHA) and the Kunitz trypsin inhibitor were studied. These alleles contain early stop codons and result in very little mRNA accumulation (Voelker et al., 1986; Jofuku et al., 1989). Run-on transcription experiments showed that the accumulation of the Kunitz trypsin inhibitor was being limited post-transcriptionally (Jofuku et al., 1989). Recently, a study of the effects of premature stop codons in plants has been conducted using the initially isolated PHA allele and other PHA alleles that were constructed in vitro (van Hoof and Green, 1996). These experiments demonstrated that premature stop codons, when present in the first 60% of the PHA coding region, caused rapid mRNA turnover. This effect was measured by generating stably transformed tobacco cell lines with mutant and control PHA alleles and monitoring mRNA decay over an actinomycin D time course. mRNA accumulation studies carried out in transgenic tobacco leaves confirmed that premature stop codons cause mRNA instability in plants as well as in cell culture. Interestingly, a premature stop codon placed 80% of the way through the coding region did not decrease mRNA stability in stably transformed tobacco cells. One explanation for this finding is that mRNA turnover is triggered by a certain length between start and stop codons or between the stop codon and the poly(A) tail. This explanation is somewhat unsatisfying because coding regions and 3' UTRs vary greatly in length and no correlation has been found between transcript length and stability.

Alternatively, nonsense mediated mRNA decay in plants may require an additional *cis*-acting element located downstream of the premature stop codon as has been found in yeast. In yeast and in *C. elegans*, genetic analysis has led to the elucidation of a pathway that is believed to have evolved to eliminate mRNAs with

premature stop codons so that the cell does not produce truncated and potentially harmful polypeptides. This pathway is referred to as the nonsense mediated mRNA decay pathway. In yeast, this pathway requires an early stop codon, a downstream *cis*-acting element and *trans*-acting cellular factors (reviewed in Jacobson and Peltz, 1996). The region between 60 and 80% of the PHA transcript may contain a *cis*-acting element that is required for the function of the nonsense mediated decay pathway. This region does not contain sequences similar to the downstream element that has been determined in yeast (Zhang et al., 1995), however, it may contain a *cis*-acting element recognized by plant nonsense mediated decay machinery.

The internal light regulatory element (iLRE) which comprises the 5' UTR and the first 14 codons of the *Fed-1* transcript appears to be another *cis*-acting determinant of mRNA stability that is closely linked to translation (Dickey et al., 1992). The pea *Fed-1* gene encodes ferredoxin I which is a major photosynthetic electron carrier. Like other nuclear encoded genes involved in photosynthesis, *Fed-1* expression increases in response to light. Levels of *Fed-1* mRNA are about five fold higher in light-grown versus dark-adapted plants (Elliot et al., 1989). The iLRE was identified because of its ability to confer light responsiveness to reporter genes (Dickey et al., 1992; 1994). The iLRE's location in the transcribed portion of *Fed-1* and the observation that nuclear run-on transcription experiments revealed no differences in transcription rates between light-grown and dark adapted plants, provide a strong argument that the iLRE functions post-transcriptionally at the level of mRNA stability (Dickey et al., 1992). Recently, direct evidence that the iLRE modulates *Fed-1* gene expression at the level of mRNA stability was obtained using the Top10 promoter system in tobacco plants. This system

enabled Thompson and coworkers, to measure half-lives of reporter transcripts with or without the iLRE in transgenic plants in light versus dark conditions (Petracek et al., 1998). This system was also used to analyze the effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport, on iLRE-mediated mRNA degradation. The results of these experiments indicate that the photosynthetic apparatus mediates light signaling that results in degradation of iLRE-containing transcripts (Petracek et al., 1998). Previously it was shown that the iLRE requires an open reading frame in order to modulate *Fed-1* mRNA levels in response to light, which indicates that control of *Fed-1* mRNA decay may be dependent upon translation (Dickey et al., 1994). Further experiments have shown that iLRE-containing messages are loaded on polyribosomes in illuminated plants but are on monosomes in the dark (Dickey et al., 1998). Therefore it seems clear that translation plays a major role in determining the stability of these transcripts. It would appear that active translation protects the transcript from degradation in this case.

Sequences involved in regulated mRNA degradation.

PvPRP1 and *Fed-1*, discussed above, are examples of genes that are regulated at the level mRNA stability in response to a stimulus. There are many more reports in the literature of message stability that responds to various stimuli including high fluences of blue light (Anderson et al., 1999), cytokinin treatment (Downes and Crowell, 1998), and heat shock (Belanger, et al., 1986). This mode of regulation provides the plant with a

means to rapidly tailor gene expression, which may be particularly important for plants because they are firmly rooted in a dynamic environment.

Changes in α-amylase message stability in response to sucrose starvation is a well characterized example. The expression of several α-amylase genes is induced when rice suspension cells are cultured without sucrose (Yu et al., 1991). Gene-specific probes were designed that could differentiate between the eight members of this gene family and it was shown by a combination of Northern blot analysis, nuclear run-on experiments, and mRNA t(1/2) analysis that three α-amylase genes are controlled at the level of mRNA stability (Sheu et al., 1996). The 3'UTR of *αAmy3* was studied in greater detail and was shown to be largely responsible for the sugar regulation of this gene (Chan and Yu, 1998a, Chan and Yu, 1998b). The *αAmy3* 3'UTR was divided into three sections and each was introduced into the 3'UTR of the same reporter gene. Each construct was introduced stably into rice cells and the effect on message stability was assayed during an actinomycin D time course with or without the addition of sugar. Sections I and III were shown to destabilize reporter transcripts in the presence of sugar (Chan and Yu, 1998b). This 3'UTR has a remarkable predicted secondary structure with several proposed stem-loops. In fact, sections I and III are predicted to form stem-loops that have the same sequence (AUAUAU) at the end of the loop (Chan and Yu, 1998a). This may be just an intriguing coincidence because the contribution of these sequences to message stability has not been tested. However, there is a precedent for stem-loop structures being important for regulated mRNA stability. The transferrin receptor of mammalian cells has multiple stem-loops in its 3'UTR that serve as a binding site for the iron-responsive element binding protein (IRE-BP). The IRE-BP is thought to protect the message from

degradation in iron limiting conditions. These same stem-loops appear to be the recognition sequences for an endonuclease that cleaves the transcript when iron concentration is high and the IRE-BP is no longer present (Mullner and Kuhn, 1988, Casey et al., 1989, Binder et al., 1994).

TRANS-ACTING FACTORS INVOLVED IN mRNA STABILITY

Trans-acting factors involved in mRNA stability include both the actual ribonucleases involved in the degradative process and regulatory factors which can be supposed to act both positively (to stimulate rapid degradation) and negatively (to protect from degradation). The close relationship between mRNA degradation and translation for at least some mRNAs suggests that some factors may have dual roles in translation and turnover (Jacobson and Peltz, 1996). There are three major approaches used in the identification of new *trans*-acting factors: (1) cloning of genes encoding proteins with known RNA-binding motifs and subsequent assignment of function; (2) identification of proteins that bind to known *cis*-determinants of stability by gel mobility shift and/or cross-linking assays; and (3) biochemical purification of proteins with ribonuclease activity.

RNA binding proteins

The only factors with a likely role in stability to be identified by the first method are the *Arabidopsis* poly(A)-binding proteins (Belostotsky and Meagher, 1993; 1996;

Hilson et al., 1993). Poly(A)-binding proteins (PABPs) from diverse organisms have a characteristic set of four RNA-binding domains (RBDs) and a highly conserved region which can be used as a tentative first identification (Burd and Dreyfuss, 1994). One of the *Arabidopsis* PABPs (*PAB5*) has been further identified by its abilities to bind poly(A) with high affinity (Belostotsky and Meagher, 1993) and to complement certain PABP functions in yeast (Belostotsky and Meagher, 1996). The presumed function of the PABPs in mRNA turnover is discussed below. A large number of other plant RNA-binding protein cDNAs have been cloned and sequenced, using primers and probes whose design was based on other related RBD-type sequences (e.g. see Guiltinan and Niu, 1996 for a recent summary). To date, no definite functions have been assigned to any of these proteins. Many are clearly not involved in cytoplasmic mRNA stability because they localize to the chloroplast or nucleus. Since the great majority of yeast and animal RBD-containing proteins whose functions are known are involved in nuclear processing and transport events (Burd and Dreyfuss, 1994), seeking RBD-containing proteins may not be the most effective way to uncover *trans*-acting factors involved in turnover. At least ten RNP motifs have been distinguished (Mattaj, 1993), some of which may turn out to be more prevalent in stability-related RNA-binding proteins.

The most direct approach to identifying *trans*-acting factors involved in stability is to seek proteins that bind directly to, or participate in complexes which bind to, known *cis*-acting stability determinants. For example, the iron response element binding protein (IRE-BP) and a number of AU-rich element binding proteins in mammalian cells have been identified in this way (e.g., Gillis and Malter, 1991; Rouault et al., 1989). Of course, the identification of such proteins is a long step away from understanding how

they actually participate in degradation. Although a number of plant stability determinants have been characterized (discussed above), no specific binding proteins have been identified to date. The identification of a stability determinant does not guarantee the future finding of a *trans*-acing factor, since not all stability determinants necessarily function by serving as binding sites. One plant mRNA-binding protein which may function in controlling stability is a 50-kD protein that binds to a sequence in the 3' UTR of the bean *PvPRP1* mRNA (Zhang and Mehdy, 1994) The protein binding site, a 27-nt U-rich sequence, has not yet been demonstrated to be a determinant involved in this destabilization event, but the protein binding activity in extracts increases after elicitor treatment, consistent with this scenario. Interestingly, the binding activity of the protein is strongly influenced by its redox state. Redox regulation of RNA binding has been described for a number of other proteins, some with known functions in the control of stability and/or translation (Hentze et al., 1989; Malter and Hong, 1991; Chu et al., 1994), and could turn out to be a widespread *modus operandi* for such regulators (Hentze, 1994).

RNA-degrading activities

Ribonucleases are expected to figure prominently among the protein factors that participate in mRNA decay mechanisms. As discussed below, current models for mRNA decay pathways in plants predict the involvement of both endo- and exoribonucleases, although the relative importance of these two types of activities appears to differ depending on the mRNA in question. Unfortunately, the plant RNases that we know the most about are the least likely to be involved in mRNA degradation because these

enzymes enter the secretory pathway and are targeted to the extracellular space or vacuole (see Bariola and Green, 1997, for a review). It should be noted that a role for the vacuole in mRNA decay, particularly in the later stage of the process, can not be ruled out because small fragments of RNA have been shown to exist in the vacuole (Abel et al., 1990). However, it seems most likely that this RNA enters the vacuole by autophagy or some other bulk process rather by mechanisms that target specific mRNAs to the vacuole for degradation. The most common assumption is that mRNA decay mainly occurs in the cytosol. The fact that translation is often coupled to mRNA decay supports this idea. Whether some mRNAs are primarily degraded in the nucleus is, nevertheless, an open question. Clearly RNA-degrading activities must exist in the nucleus to turnover introns and 3' ends of precursor RNAs. Virtually nothing is known about these enzymes in any system but it seems possible that some may be capable of degrading fully processed transcripts prior to or during export.

Recently it was shown that RNase III, an enzyme involved in pre-rRNA processing as well as the decay of some specific mRNAs in *Escherichia coli* (Court, 1993), also participates in pre-rRNA processing in yeast nucleoli (Elela et al., 1996). Examining plant genes with homology to RNase III (e.g. an *Arabidopsis* -expressed sequence tag accession number Z18464, Höfte et al., 1993) may be of particular interest because most current models for antisense RNA-mediated inhibition (Nellen and Lichtenstein, 1993) and some cosupression models argue for the involvement of a dsRNase (discussed below). Other yeast activities known to be involved in mRNA decay may also have plant homologues such the 5' to 3' exoribonuclease *XRN1*, which is a major participant in the decay of most yeast mRNAs. Indeed, three *XRN*-like genes have

been cloned from *Arabidopsis* (James Kastenmayer and Pamela J. Green, unpublished). *XRN*s have also been cloned from flies and mammals, indicating that these genes have been conserved throughout eukaryotic evolution (Till et al., 1998, Bashkirov et al., 1997; Shobuike et al., 1997). However, one potentially relevant observation is that insertion of a poly(G) tract into an mRNA blocks the progression of *XRN1* in yeast giving rise to a prominent intermediate that includes sequences 3' of the poly(G) tract (Vreken and Raué, 1992; Decker and Parker, 1993; Muhlrad et al., 1994). A 3' degradation intermediate also results from poly(G) insertion into messages in *Chlamydomonas*, suggesting the occurrence of a similar exonuclease activity in this organism (Gera and Baker, 1998; Drager et al., 1998). Poly(G) intermediates have been looked for but not seen in both higher plant and mammalian cell systems (Mark A. Johnson and Pamela J. Green, Chapter 2-1 of this dissertation; Goddall, G., personal communication; Maquat, L., personal communication; Shyu, A.-B., personal communication) indicating that the role of *XRN*-like genes may be less prominent, or different in higher eukaryotes. In any event, additional efforts to characterize RNases that may participate in mRNA decay pathways are certainly warranted.

As will be discussed in the next section, the 5' cap and poly(A) tail play an important role in determining mRNA stability. Therefore, it will be important to characterize enzymes responsible for removing these structures during the process of mRNA degradation. Recently, a poly(A) ribonuclease (PARN) was purified from calf thymus which led to the isolation of a human cDNA encoding this enzyme (Korner and Wahle, 1997; Korner et al., 1998). This is an intriguing activity because the enzyme responsible for removing the poly(A) tail, the first step in the degradation of most yeast

mRNAs, has not yet been identified. An Arabidopsis *PARN*-like gene (*AtPARN*) has been cloned and will be the subject of Chapter 2-2 of this dissertation. A decapping complex has been described in yeast (Dunckley and Parker, 1999; Beelman et al., 1996). There are also putative *Arabidopsis* homologues of these genes and the role that these play in plant mRNA turn-over will be an interesting area of future work (Gustavo MacIntosh and Pamela J. Green, unpublished).

The role of the cap and poly(A) tail in mRNA stability

A variety of primarily in vitro studies over many years have suggested a function for the poly(A) tail and 7-methyl guanosine cap as mRNA-stabilizing structures (reviewed in Baker, 1993; Stevens, 1993). However, the ability to definitively demonstrate a stabilizing function in cells has been limited, in part, by an inability to generate uncapped and unadenylated mRNA species in vivo. New genetic approaches in yeast have finally conclusively demonstrated stabilizing functions for these sequences in this organism. Decapping has been shown to be an early, sometimes rate-limiting, step in the major degradation pathway of many mRNAs in yeast (Hsu and Stevens, 1993; Muhlrad et al., 1994, 1995). Mutations that affect an essential component of the decapping enzyme cause a marked stabilization of both normally unstable and stable mRNAs (Beelman et al., 1996). In yeast, decapping is followed by rapid 5' to 3' exonucleolytic digestion of the rest of the mRNA (by the *XRN1* nuclease discussed below), and this sequence of events is likely to be the major degradative pathway for yeast mRNAs. Because decapped mRNA is extremely labile in yeast, it could be

detected only in mutant cells with defective 5' to 3' exonuclease activity (*xrn1* cells) (Hsu and Stevens, 1993; Muhlrad et al., 1994). The unavailability of similar mutants in other organisms has so far precluded a demonstration of an analogous decay pathway. The ability of an inserted tract of guanine nucleotides to trap 3' degradation intermediates by impeding exonuclease progress has allowed the demonstration that the substrate for decapping is, for many or most yeast mRNAs, a molecule which has undergone substantial poly(A) shortening (Decker and Parker, 1993). Thus, a longer poly(A) tail protects the mRNA from decapping, almost certainly via its ability to bind at least one poly(A)-binding protein (Caponigro and Parker, 1995). A similar deadenylation-dependent pathway has not yet been demonstrated to occur in other organisms. On the other hand, observation of the poly(A) status of naturally stable degradation intermediates has provided evidence against such a pathway for certain mRNAs (discussed below).

Alternative experimental options to directly test for stabilizing functions for the cap and poly(A) tail in other organisms are limited. The best experimental approach to date has been the study of in vitro transcribed mRNAs introduced into cells by transfection, electroporation or injection. Only the evidence pertaining to plant cells will be discussed here. Gallie and colleagues have conducted extensive analyses of the roles of the cap and poly(A) tail in mRNA translation, using synthetic reporter mRNAs electroporated into plant protoplasts (Browning et al., 1998). Comparisons of the chemical and functional stability of capped versus uncapped and polyadenylated versus unadenylated transcripts were included in some of these reports. Chemical stability was measured simply by following the disappearance of the full length mRNA with time by northern blot. A modest stabilizing effect of a 5' cap was observed: a cap increased the

half-lives of either unadenylated or polyadenylated luciferase transcripts about 2-fold, from 31 and 44 minutes to 53 and 100 minutes, respectively. While all of these electroporated transcripts can be considered relatively unstable, the rather small stabilizing effect of a cap could be interpreted to mean that 5' to 3' exonuclease digestion does not represent the major pathway for degradation of electroporated mRNAs. Alternatively, it is possible that most capped transcripts are rapidly decapped following delivery, and the small stability differential reflects the decapping rate. In either case, the uncapped state does not dictate immediate degradation by a 5' to 3' exonuclease, since uncapped mRNA is detectable for several hours. Given the extremely rapid degradation that apparently follows decapping of natural mRNAs in yeast, it would be interesting to know whether uncapped transcripts electroporated into yeast spheroplasts are subject to a similar fate, or whether introduced transcripts are degraded by a different or slower mechanism (Russell et al., 1991; Everett and Gallie, 1992). The addition of a 50-nt poly(A) tail to electroporated reporter mRNAs was shown to also confer a modest 1.5 to 3-fold increase in chemical half-life (Gallie et al, 1989; Gallie, 1991). Again, it is not clear how rapidly these reporter RNAs are deadenylated following delivery, or, in this case, what fraction of delivered RNA acquires poly(A)-binding proteins (discussed below). The stabilizing effects of a cap and poly(A) tail together are consistently additive, unlike their contributions to translation, which are strongly synergistic.

Another possible explanation for the failure of a cap or poly(A) tail to act as strong stabilizers of electroporated mRNAs is that the major substrates for putative mRNase activities may be messenger RNPs, not naked RNA molecules. The extent to which electroporated mRNAs associate with RNA-binding proteins is not known. It has

been determined, however, that only about 2% of electroporated mRNAs are polysomal 1.5 hours after delivery into carrot protoplasts (Gallie et al., 1995). It could be that only this small subset of actively translated mRNAs are substrates for the usual mRNase activities. This possibility was addressed by measuring the functional half-life of electroporated mRNAs, defined as the time required to reach 50% of the final level of protein produced. The functional half-life could be a more accurate measure of the stability of those mRNAs that assemble into an mRNP form. In fact, when functional half-lives were compared, the cap and poly(A) tail each did confer a somewhat stronger stabilizing effect than their effects on chemical half-lives (3 to 5-fold versus 1.5 to 3-fold). However, the relatively modest protection suggests that the electroporated RNAs are not substrates for potent deadenylation-dependent or decapping-dependent pathways in these cells. It is not yet clear whether this finding reflects the absence of a dominant role for the cap and poly(A) tail in plant mRNA stability or is the result of our inability to evaluate the effect of these elements on the decay of transcripts synthesized in vivo.

Poly(A)-binding proteins.

Poly(A)-binding proteins (PABPs) are ubiquitous and multifunctional RNA-binding proteins that have been implicated in several aspects of mRNA metabolism and translation (reviewed in Jacobson and Peltz, 1996; Baker, 1997). The yeast poly(A)-binding protein (pab1p) has recently been assigned an unexpected stabilizing function: it apparently prevents decapping of polyadenylated mRNAs, presumably in *cis*. The evidence supporting this role is that decapping is normally triggered by shortening of

a poly(A) to a length too short to bind pab1p (Decker and Parker, 1993; Muhlrad et al., 1994) and in *pab1Δ* cells, decapping occurs without prior poly(A) shortening (Caponigro and Parker, 1995). There are multiple PABPs in plants; in *Arabidopsis*, at least four different *PABP* genes are expressed in a tissue and organ-specific manner (Belostotsky and Meagher, 1993; 1996; Hilson et al, 1993). Whether these genes encode functionally distinct or functionally redundant proteins is not yet known. To begin to address that important question, Belostotsky and Meagher (1996) have expressed one of the *Arabidopsis PABP* genes *(PAB5)* in yeast strains depleted of endogenous PAB1p to determine what functions could be complemented by the plant protein. Remarkably, although the two proteins are only 44% identical at the amino acid level, the PAB5 protein was able to rescue inviability and at least partially restore two functions: normal poly(A) shortening and translation initiation. However, expression of the *PAB5* gene did not restore deadenylation-dependent decapping of yeast mRNAs. Had it done so, this would have strongly suggested the occurrence of a similar PABP function, and thus a similar mRNA degradation pathway, in *Arabidopsis*. However, the inability of *PAB5* to complement this function in yeast does not imply that it lacks this function in *Arabidopsis*. It will be important to see how other *Arabidopsis* PABPs behave in this regard.

PATHWAYS OF mRNA DEGRADATION

Pathways by which mRNA molecules are degraded have been proposed for both mammalian and yeast mRNAs. Mammalian mRNAs have generally been proposed to be

degraded in a 3' to 5' direction. Degradation of the body of message is thought to be preceded by either stepwise shortening of the poly(A) tail (e.g., MYC mRNA), or by endonucleolytic removal of the poly(A) tail (e.g., transferrin receptor mRNA) (Ross, 1996). Whether poly(A) removal is an essential rate-limiting step in the degradation of mammalian mRNAs is not known, although the biphasic degradation kinetics of *c-fos* and other ARE-containing mRNAs is compatible with that idea (Chen and Shyu, 1994). In yeast, a major pathway of degradation appears to be the deadenylation-dependent decapping pathway (Decker and Parker, 1993; Decker and Parker, 1994). After decapping, degradation of some mRNAs (e.g., PGK1 mRNA) appears to proceed principally in the 5' to 3' direction. However, a more limited 3' to 5' degradation of the *PGK1* mRNA has also been observed following deadenylation (Muhlrad and Parker, 1994). A deadenylation-independent decapping pathway also occurs (Muhlrad and Parker, 1994). In this pathway, decapping and 5' to 3' degradation occur in the absence of significant poly(A) shortening. mRNAs carrying premature nonsense codons, and perhaps other mRNAs, are targeted for rapid degradation via this route. It would be premature to conclude that yeast and mammalian cells degrade mRNAs by fundamentally distinct pathways. An equally likely possibility is that eukaryotic cells have multiple mRNA degradation pathways, with the pathway used being mRNA species specific. In some species, one or more pathways may predominate and the predominant pathways may differ among organisms.

Identification and analysis of in vivo produced mRNA degradation intermediates would greatly facilitate the elucidation of mRNA degradation pathways. Unfortunately, such intermediates are often not readily apparent in RNA samples from mammalian, yeast, or plant cells. However, two plant mRNAs (encoded by the soybean *SRS4* and oat *PHYA* genes), for which putative in vivo mRNA degradation products have been reported, have provided some insight into the pathways of mRNA degradation in plant cells. It should be noted that mRNA degradation intermediates may be more prevalent than is often assumed. It is common to observe hybridizing RNA fragments smaller than the full-length mRNA in RNA blot analysis. Trimming of RNA blots prior to publication to focus attention on the full-length band or to save journal space may obscure the true frequency with which such RNA fragments occur. The problem, of course, is to determine whether the RNA fragments are degradation intermediates produced in vivo, or the result of degradation in vitro during isolation of the RNA samples. Control experiments useful to address this question have been described (Seeley et al., 1992; Thompson et al., 1992; Tanzer and Meagher, 1994).

A series of discrete fragments of the soybean ribulose-1,5-bisphosphate carboxylase small subunit (*SRS4*) mRNA are detected on RNA gel blots (Thompson et al., 1992). Similar mRNA fragments are present in transgenic petunia transformed with the soybean *SRS4* gene under the control of the 35S promoter. These fragments appear to be bona fide in vivo degradation intermediates. This interpretation is supported by the observation that tracer RNAs are not degraded in homogenized samples during RNA isolation (Thompson et al., 1992). In addition, degradation of in vitro synthesized *SRS4* RNA in cell-free systems generates the same RNA fragments observed in vivo (Tanzer

and Meagher, 1994; 1995). The discrete nature of the *SRS4* mRNA fragments suggests the activity of an endonuclease. S1 nuclease and primer extension mapping of the *SRS4* RNA fragments indicate that the fragments were indeed derived from endonucleolytic cleavage of the full-length *SRS4* mRNA (Tanzer and Meagher, 1995). The endonucleolytic cleavage appears to be independent of both deadenylation and decapping. Tanzer and Meagher (1995) have proposed that the endonucleolytic cleavages are catalyzed by a stochastic endonuclease, cleaving at various sites within the *SRS4* mRNA, followed by 3' to 5' or 5' to 3' exonucleolytic cleavage of the resulting fragments. The proximal (5') and distal (3') fragments would be degraded while possessing either a 5' cap or poly(A) tail, respectively. As such, this model is distinct from those proposed for mammalian and yeast mRNAs. Although, for example, a 5' capped fragment of an mRNA could be thought of as being analogous to a deadenylated mRNA, similar to deadenylation of mammalian transferrin receptor mRNA prior to degradation (Ross, 1996).

RNA fragments have also been observed in RNA blot analysis of oat phytochrome A (*PHYA*) mRNA. These fragments also appear to be in vivo degradation products. This interpretation is based on several lines of evidence including the observation that the *PHYA* fragments are present in RNA samples isolated by various distinct procedures (Seeley et al., 1992). Other endogenous or exogenously-added tracer RNAs remain intact under the same isolation conditions. Unlike the discrete fragments observed for soybean *SRS4* mRNA, the *PHYA* fragments form a continuous distribution ranging from full-length (approximately 4 kb) down to about 200 bp (Seeley et al, 1992; Higgs and Colbert, 1994; Higgs et al, 1995). Analysis of these fragments, using distinct

probes corresponding to various regions of the *PHYA* mRNA molecule, has led to the proposal that these fragments are generated by exonucleases (Higgs and Colbert, 1994). RNase H mapping fails to reveal endonucleolytic cleavages near the 5' or 3' ends of the mRNA (Higgs and Colbert, 1994). In addition, analysis of the polyadenylation status of *PHYA* mRNA suggests that about 25% of the apparently full-length *PHYA* mRNA lacks a poly(A) tail. *PHYA* RNA fragments are present in both the polyadenylated RNA fraction and in the deadenylated RNA fraction. These observations have been incorporated into a model (Higgs and Colbert, 1994) proposing that about 75% of the *PHYA* mRNA population is degraded by a 5' to 3' exonuclease, with the poly(A) tail still attached to the RNA fragment undergoing degradation. The deadenylated portion of the *PHYA* mRNA population is proposed to be degraded by exonucleases acting at both the 3' and 5' ends of the molecule. If this model is correct, the bulk of the *PHYA* mRNA would be degraded by a pathway similar to the deadenylation-independent decapping pathway of yeast (Muhlrad and Parker, 1994).

The degradation pathway of mRNA molecules is a complex process about which we have rather limited knowledge. It does seem clear that multiple pathways for mRNA degradation may be present within eukaryotic cells. Even individual mRNA species may be degraded by more than one pathway (Muhlrad and Parker, 1994; Higgs and Colbert, 1994). There are numerous unresolved questions regarding the pathway(s) of degradation of nuclear-encoded mRNAs within plant cells. Perhaps the most pressing basic question is the location of selective mRNA degradation. Two lines of evidence suggest that degradation may occur on polysomes. First, putative in vivo degradation products of both *SRS4* mRNA and *PHYA* mRNA are present in polysomal RNA fractions

(Thompson et al., 1992; Byrne et al., 1993; Higgs and Colbert, 1994). Second, cell-free mRNA degradation systems, based on isolated polysomes, have been described for both plant (Byrne et al., 1993; Tanzer and Meagher, 1994) and animal (Brewer and Ross, 1990; Ross, 1995) cells. These cell-free systems appear to accurately reflect at least some aspects of in vivo mRNA degradation. In addition, polysome-associated ribonuclease activities have been reported (Green, 1994). However, other compartments within plant cells are also known to possess ribonuclease activity. As discussed above, plant vacuoles have high levels of ribonuclease activity (Abel and Glund, 1987; Green, 1994). The possibility that some nuclear-encoded mRNAs are targeted for decay by regulated transport into the vacuole is difficult to rule out. There is, however, only limited evidence for the movement of RNA molecules across organellar membranes (Chang and Clayton, 1987; Oda et al., 1992).

Another area that warrants further development is the use of in vitro systems to dissect mRNA decay pathways. Already, the two systems described above have been shown to produce mRNA decay intermediates that mimic those observed in vivo. An important next step will be to test whether *cis*-acting instability sequences can be recognized in these and additional in vitro systems. Further characterization of the process by which mRNA degradation occurs may also be possible using in vitro systems. For example, recent evidence suggests that ATP is not required for the decay of *PHYA* mRNA in vitro (Byrne and Colbert, unpublished). Clearly, additional exploration of current in vitro systems and the development of new ones will provide further mechanistic insights.

POST-TRANSCRIPTIONAL GENE SILENCING

Cosuppression is a form of gene silencing that in many cases, appears to involve degradation of the target mRNA. It was first identified during attempts by plant molecular biologists to overexpress various cloned plant genes. These experiments usually involved the generation of transgenic plants expressing the gene of interest driven by the strong, constitutive, 35S promoter. Much to the surprise of these early investigators, in a few of these transgenic lines, rather than over expression of the gene of interest, both the transgene and the corresponding endogenous gene were coordinately silenced (Napoli et al., 1990; van der Krol, 1990, Smith et al., 1990). This phenomenon is not limited to transgenes with endogenous counterparts because it appears that multiple copies of a transgene can suppress one another through similar mechanisms. In addition, homology dependent virus resistance, a technique which employs portions of a viral genome to generate transgenic plants that are resistant to the virus, may also function in the same manner (Lindbo et al., 1993).

Many cases of cosuppression have been explained by transcriptional repression that appears to be similar to other epigenetic phenomena described in fungi, plants, and animals (Matzke and Matzke, 1996). In other cases, however, transcription rates of transgenes and endogenous genes are normal, yet very little mRNA accumulates in the cytoplasm. In these cases it has been assumed that expression is suppressed either by blocking export of mature mRNA to the cytoplasm and eventual degradation in the nucleus or by cytoplasmic mRNA degradation. Studies using cytoplasmically replicating RNA viruses as targets for homology dependent virus resistance support the idea that

gene silencing is occurring in the cytoplasm via rapid degradation of the suppressed mRNA (Lindbo et al., 1993). Further support for this idea comes from the finding that nuclear mRNA abundance of a silenced transgene is unaffected (De Carvalho Niebel et al., 1995).

Post-transcriptional gene silencing (PTGS) of this type was once thought to be limited to plants, but is now considered to be a mechanism that many organisms employ to ward off invasive nucleic acid. The phenomena known as RNA interference and quelling that have been studied in *Caenorhabditis elegans* and *Neurospora crassa*, respectively, are probably examples of PTGS (Fire et al., 1998; Romano and Mancino, 1992). Most models that describe the initiation, maintenance, and spread of PTGS in plants and other organisms call for the formation of a double-stranded RNA (dsRNA) molecule. This is an attractive idea because it accounts for the sequence-specificity that is observed in PTGS. This model implies the existence of an RNA-dependent RNA polymerase (RDRP) that would be responsible for the synthesis of RNA complementary to the target RNA and a dsRNA-specific RNase that would degrade the target RNA. RDRPs have been cloned from plants and recently an RDRP was identified in a screen for *N. crassa* mutants defective in quelling (Schiebel et al., 1998; Cogoni and Macino, 1999). RDRPs may generate small RNAs from specific target messages that would be able to move from one cell to another within the organism to silence the target gene. Recently evidence in support of this hypothesis has come by displaying small RNAs that are complementary to silenced viral targets in plants (Hamilton and Baulcombe, 1999). These studies represent dramatic progress in our understanding of how the PTGS signal is generated. However, how dsRNA is degraded is still an open question. Insights into

these questions and other lingering questions about how PTGS works may come by studying the mechanisms of rapid mRNA turnover. It is possible that the activities involved in the final steps of both of these processes, namely rapid RNA degradation, will be similar.

FUTURE DIRECTIONS

The development of reliable methods to measure mRNA half-lives in plants has resulted in the identification of *cis*-acting sequences that target transcripts for rapid turnover and in the identification of a *trans*-acting factor potentially involved in the recognition of one of these elements. It is also now clear that alterations in gene expression in response to extra and intra-cellular stimuli is in some cases controlled at the level of mRNA stability in plants. In addition, understanding of the pathways involved in mRNA turnover in plants has improved recently and points to possible differences between plants and animals, and yeast. The future of this work must include increased efforts aimed at the elucidation of mRNA degradation pathways and at identifying cellular factors that carry out the steps in these pathways. By characterizing the decay of a greater number of mRNAs, it will be possible to determine whether sets of mRNAs are funneled into one or more distinct pathways and to determine whether there are any common themes conserved among plants, fungi and animals. Molecular analysis of cellular factors involved in mRNA degradation will improve efforts to characterize mRNA turnover pathways and will provide insights into intriguing problems in plant molecular biology such as post-transcriptional gene silencing.

The progress outlined in this introduction represents a foundation upon which a rich future can be built. One exciting avenue of research will be the application of molecular genetics in *Arabidopsis thaliana* in order to gain insight into the mechanisms of mRNA turnover in plants and to identify cellular factors involved in this process. Knowledge of *cis*-acting instability sequences should allow the isolation of mutants in specific mRNA decay pathways. This may be particularly interesting in the case of AU-rich elements because genetic approaches have been lacking in mammalian systems in which the pioneering work on such elements has been done. Although most studies have thus far dealt with unstable mRNAs, it is now possible to measure long mRNA half-lives through the use of regulated promoters rather than prolonged incubation with transcriptional inhibitors. This coupled with the finding of active stabilization mechanisms in other systems opens the door for elucidating how the stabilities of long-lived plant mRNAs are controlled. Genetic studies combined with biochemical approaches aimed at purifying mRNA binding and degrading activities should lead to an era of great expansion in our knowledge of fundamental mechanisms of mRNA degradation in plants.

THE SCOPE OF THIS THESIS

The members of our laboratory we have begun to think of mRNA degradation as a hierarchy consisting of three tiers (Gutierez et al., 1999). At the bottom of this scheme are the basic mRNA degrading enzymes which dismantle and digest the message. Above the basal mRNA degradation machinery is sequence-specific mRNA degradation which

determines the degradation rate of particular mRNAs. In this scheme, instability determinants can be thought of as flags that target transcripts to the mRNA decay machinery. Above sequence-specific decay is regulated mRNA degradation which is more complex because the function of an instability sequence is determined by extra and intracellular stimuli.

In my thesis, I have addressed the first two levels of this hierarchy and have therefore divided this dissertation into two sections. In Section One, I report my work aimed at understanding the mechanisms responsible for sequence-specific mRNA degradation in plants. In Chapters 1-1 and 1-2 an approach is presented that resulted in the isolation of two mutants that are defective in sequence-specific mRNA degradation mediated by the DST element. In Chapter 1-3, I report on a series of experiments that were designed to identify the critical instability sequences within the 3'UTR of *SAUR-AC1*, an Arabidopsis transcript that is likely to be regulated by DST-mediated degradation.

Section Two is devoted to my work on understanding the basic machinery involved in degrading mRNA in plants. In a set of experiments described in Chapter 2-1, a stable secondary structure was introduced into plant reporter transcripts in an attempt to analyze intermediates in the mRNA decay process. In Chapter 2-2, preliminary work on the characterization of a potential poly(A) ribonuclease of Arabidopsis is described.

The studies reported here represented steps along the way to answering some of the important questions that have been laid out in this introduction. The major contribution of this thesis is likely to be the *dst* mutants. These are the first mutants of

their kind to be isolated in any eukaryote and may be very useful in understanding the mechanisms of sequence-specific mRNA degradation.

REFERENCES

Abel S, Blume B, Glund K (1990) Evidence for RNA-oligonucleotides in plant vacuoles isolated from cultured tomato cells. Plant Physiol. 94: 1163-1171

Abel S, Glund K (1987) Ribonuclease in plant vacuoles: purification and molecular properties of the enzyme from cultured tomato cells. Planta 172: 71-78

Abler ML, Green PJ (1996) Control of mRNA stability in higher plants. Plant Mol. Biol. 32: 63-78

Adang MJ, Brody MS, Cardineau G, Eagan N, Roush RT, Shewmaker CK, Jones A, Oakes JV, McBride KE (1993) The reconstruction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. Plant Mol. Biol. 21: 1131-1145

Anderson,M.B., Folta,K., Warpeha,K.M., Gibbons,J., Gao,J., and Kaufman,L.S. (1999). Blue light-directed destabilization of the pea *Lhcb1*4* transcript depends on sequences within the 5' untranslated region. Plant Cell *11*, 1579-1589.

Baker EJ (1993) Control of poly(A) length. *In* J Belasco, G Brawerman, eds, Control of Messenger RNA Stability, Academic Press, inc. San Diego, pp 367-415

Baker EJ (1997) mRNA polyadenylation: functional implications. *In* D Morris, JB Harford, eds, mRNA Metabolism and Post-Transcriptional Gene Regulation. Wiley-Liss, Inc. New York, pp 85-104

Bariola PA and Green PJ (1997) Plant Ribonucleases. *In* JF Riordan, G D'Alessio, eds, Ribonucleases: Structure and Function, Academic Press, Orlando, FL, pp 163-190

Bashkirov,V.I., Scherthan,H., Solinger,J.A., Buerstedde,J.-M., and Heyer,W.-D. (1997). A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. Journal of Cell Biology *136* , 761-733.

Baulcombe DC, English JJ (1996) Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. Curr. Opin. Biotechnol. 7: 173-180

Beelman CA, Stevens A, Caponigro G, LaGrandeur TE, Hatfield L, Fortner DM, Parker R (1996) An essential component of the decapping enzyme required for normal rates of mRNA turnover. Nature 382: 642-646

Belanger,F.C., Brodl,M.R., and Ho,T.D. (1986). Heat shock causes destabilization of specific mRNAs and destruction of endoplasmic reticulum in barley aleurone cells. Proc. Natl. Acad. Sci. USA *83*, 1354-1358.

Belostotsky DA, Meagher RB (1993) Differential organ-specific expression of three poly(A)-binding protein genes from *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 90: 6686-6690

Belostotsky DA, Meagher RB (1996) A pollen-, ovule-, and early embryo-specific poly(A) binding protein from Arabidopsis complements essential functions in yeast. Plant Cell 8: 1261-1275

Binder,R., Horowitz,J.A., Basilion,J.P., Koeller,D.M., Klausner,R.D., and Harford,J.B. (1994). Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. EMBO J. *13*, 1969-1980.

Braam J, Davis RW (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell 60: 357-364

Brewer G, Ross J (1990) Messenger RNA turnover in cell-free extracts. Methods Enzymol. 181: 202-209

Burd CG, Dreyfuss G (1994) Conserved structures and diversity of functions of RNA-binding proteins. Science 265: 615-621

Byrne DH, Seeley KA, Colbert JT (1993) Half-lives of oat mRNAs in vivo and in a polysome-based in-vitro system. Planta 189: 249-256

Caponigro G, Parker R (1995) Multiple functions for poly(A)-binding protein in mRNA decapping and deadenylation in yeast. Genes Dev. 9: 2421-2432

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

Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A (1986) Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. USA 83: 1670-1674

Casey JL, Koeller DM, Ramin VC, Klausner RD, Harford JB (1989) Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. EMBO J. 8: 3693-3699

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

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

Chang DD, Clayton DA (1987) A mammalian mitochondrial RNA processing activity contains nucleus-encoded RNA. Science 235: 1178-1184

Chen C-YA, Shyu A-B (1994) Selective degradation of early-response-gene mRNAs: Functional analyses of sequence features of the AU-rich elements. Mol. Cell. Biol. 14: 8471-8482

Chen CYA, Shyu AB (1995) AU-rich elements: Characterization and importance in mRNA degradation. Trends Biochem. Sci. 20: 465-470

Chu S, Archer RH, Zengel JM, Lindahl L (1994) The RNA of RNase MRP is required for normal processing of ribosomal RNA. Proc. Natl. Acad. Sci. USA 91: 659-663

Cogoni,C. and Macino,G. (1999). Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature *399*, 166-169.

Court D (1993) RNA processing and degradation by RNaseIII. *In* G Brawerman, JG Belasco, eds, Control of mRNA stability. Academic Press, New York, pp 70-116

De Carvalho Niebel F, Frendo P, Van Montagu M, Cornelissen M (1995) Post-transcriptional cosuppression of ∃-1,3-glucanase genes does not affect accumulation of transgene nuclear mRNA. Plant Cell 7: 347-358

De Carvalho F, Gheysen G, Kushnir S, Van Montagu M, Inzé D, Castresana C (1992) Suppression of ∃-1,3-glucanase transgene expression in homozygous plants. EMBO J. 11: 2595-2602

Decker CJ, Parker R (1993) A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev. 7: 1632-1643

Decker CJ, Parker R (1994) Mechanisms of mRNA degradation in eukaryotes. Trends Biochem. Sci. 19: 336-340

Dehio C, Schell J (1994) Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible transgene silencing. Proc. Natl. Acad. Sci. USA 91: 5538-5542

Dickey LF, Gallo-Meagher M, Thompson WF (1992) Light regulatory sequences are located within the 5' portion of the *Fed-1* message sequence. EMBO J. 11: 2311-2317

Dickey LF, Nguyen T-T, Allen GC, Thompson WF (1994) Light modulation of ferredoxin 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.

Diehn SH, De Rocher EJ, Green PJ (1996) Problems that can limit the expression of foreign genes in plants: lessons to be learned from *B.t.* toxin genes. *In* JK Setlow, ed, Genetic Engineering: Principles and Methods, Vol 18. Plenum Press, New York, pp 83-99

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

Drager,R.G., Girard-Bascou,J., Choquet,Y., Kindle,K.L., and Stern,D.B. (1998). *In vivo* evidence for 5'-->3' exoribonuclease degradation of an unstable chloroplast mRNA. Plant J. *13*, 85-96.

Dunckley,T. and Parker,R. (1999). The DCP2 protein is required for mRNA decapping in saccharomyces cerevisiae and contains a functional MutT motif [In Process Citation]. EMBO J. *18*, 5411-5422.

Elela SA, Igel H, Ares M (1996) RNaseIII cleaves eukaryotic preribosomal RNA at a U3 snoRNP-dependent site. Cell 85: 115-124

Elliott RC, Dickey LF, White MJ, Thompson WF (1989) cis-acting elements for light regulation of pea ferredoxin I gene expression are located within transcribed sequences. Plant Cell 1: 691-698

English JJ, Mueller E, Baulcombe DC (1996) Suppression of Virus Accumulation in Transgenic Plants Exhibiting Silencing of Nuclear Genes. Plant Cell 8: 179-188

Everett JG, Gallie DR (1992) RNA delivery in *Saccharomyces cerevisiae* using electroporation. Yeast 8: 1007-1014

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA 91: 3490-3496

Franco AR, Gee MA, Guilfoyle TJ (1990) Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. J. Biol. Chem. 265: 15845-15849

Gallie DR, Lucas WJ, Walbot V (1989) Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. Plant Cell 1: 301-311

Gallie DR (1991) The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev. 5: 2108-2116

Gallie DR (1993) Posttranscriptional regulation of gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44: 77-105

Gallie DR, Caldwell C, Pitto L (1995) Heat shock disrupts cap and poly(A) tail function during translation and increases mRNA stability of introduced reporter mRNA. Plant Physiol. 108: 1703-1713

Gera,J.F. and Baker,E.J. (1998). Deadenylation-dependent and -independent decay pathways for α1-tubulin mRNA in *Chlamydomonas reinhardtii*. Mol. Cell. Biol. *18*, 1498-1505.

Gil P, Liu Y, Orbovic V, Verkamp E, Poff KL, Green PJ (1994) Characterization of the auxin-inducible *SAUR-AC1* gene for use as a molecular genetic tool in *Arabidopsis*. Plant Physiol. 104: 777-784

Gil P, Green PJ (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

Gillis P, Malter JS (1991) The adenosine-uridine binding factor recognizes the AU-rich elements of cytokine, lymphokine, and oncogene mRNAs. J. Biol. Chem. 266: 3172-3177

Goodwin J, Chapman K, Swaney S, Parks TD, Wernsman EA, Dougherty WG (1996) Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. Plant Cell 8: 95-105

Green PJ (1993) Control of mRNA stability in higher plants. Plant Physiol. 102: 1065-1070

Green PJ (1994) The ribonucleases of higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45: 421-445

Guiltinan MJ, Niu XP (1996) cDNA encoding a wheat (*Triticum aestivum* cv Chinese Spring) glycine-rich RNA-binding protein. Plant Mol. Biol. 30: 1301-1306

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.

Hamilton,A.J. and Baulcombe,D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950-952.

Hentze MW, Rouault TA, Harford JB, Klausner RD (1989) Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction. Science 244: 357-359

Hentze MW (1994) Enzymes as RNA-binding proteins: a role for (di)nucleotide-binding domains? Trends Biochem. Sci. 18: 101-103

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

Higgs DC, Barnes LJ, Colbert JT (1995) Abundance and half-life of the distinct oat phytochrome A3 and A4 mRNAs. Plant Mol. Biol. 29: 367-377

Higgs DC, Colbert JT (1994) Oat phytochrome A mRNA degradation appears to occur via two distinct pathways. Plant Cell 6: 1007-1019

Hilson P, Carroll KL, Masson PH (1993) Molecular characterization of PAB2, a member of the multigene family coding for poly(A)-binding proteins in *Arabidopsis thaliana*. Plant Physiol. 103: 525-533

Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. 15: 851-864

Hobbs SLA, Warkentin TD, DeLong CMO (1993) Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21: 17-26

Höfte H, Desprez T, Amselem J, Chiapello H, Caboche M, Moisan A, Jourjon M-F, Charpenteau J-L, Berthomieu P, Guerrier D, Giraudat J, Quigley F, Thomas F, Yu D-Y, Mache R, Raynal M, Cooke R, Grellet F, Delseny M, Parmentier Y, de Marcillac G, Gigot C, Fleck J, Philipps G, Axelos M, Bardet C, Tremousaygue D, Lescure B (1993) An inventory of 1152 expressed sequence tags obtained by partial sequencing of cDNAs from *Arabidopsis thaliana*. Plant J. 4: 1051-1061

Hsu CL, Stevens A (1993) Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. Mol. Cell. Biol. 13: 4826-4835

Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA 91: 10502-10506

Jacobson A, Peltz SW (1996a) Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. Annu. Rev. Biochem. 65:

Jacobson A and Peltz SW (1996b) Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *In* C Richardson, J Abelson, and C Raetz, eds, Annual Review of Biochemistry, Ed 65 pp 693-739

Jofuku KD, Schipper RD, Goldberg RB (1989) A frameshift mutation prevents Kunitz trypsin inhibitor mRNA accumulation in soybean embryos. Plant Cell 1: 427-435

Kakutani T, Jeddeloh JA, Richards EJ (1995) Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant. Nucleic Acids Res. 23: 130-137

Kamalay JC, Goldberg RB (1984) Organ-specific nuclear RNAs in tobacco. Proc. Natl. Acad. Sci. USA 81: 2801-2805

Koeller DM, Horowitz JA, Casey JL, Klausner RD, Harford JB (1991) Translation and the stability of mRNAs encoding the transferrin receptor and c-*fos*. Proc. Natl. Acad. Sci. USA 88: 7778-7782

Körner,C.G. and Wahle,E. (1997). Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J. Biol. Chem. *272*, 10448-10456.

Körner,C.G., Wormington,M., Muckenthaler,M., Schneider,S., Dehlin,E., and Wahle,E. (1998). The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of *Xenopus* oocytes. EMBO J. *17*, 5427-5437.

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11: 194-200

Lagnado CA, Brown CY, Goodall GJ (1994) AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: The functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol. Cell. Biol. 14: 7984-7995

Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. Plant Cell 5: 1749-1759

Malter JS, Hong Y (1991) A redox switch and phosphorylation are involved in the post-transcriptional up-regulation of the adenosine-uridine binding factors by phorbol ester and ionophore. J. Biol. Chem. 266: 3167-3171

Maquat LE (1995) When cells stop making sense: Effects of nonsense codons on RNA metabolism in vertebrate cells. RNA 1: 453-465

Mattaj IW (1993) RNA recognition: A family matter? Cell 73: 837-840

Matzke MA, Matzke AJM, Eggleston WE (1996) Paramutation and transgene silencing: a common response to invasive DNA? Trends Plant Sci. 1: 382-388

McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ (1989) Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. Plant Cell 1: 229-239

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenlyated RNAs. Plant Mol. Biol. 9: 611-623

McClure BA, Guilfoyle TJ (1989) Rapid redistribution of auxin-regulated RNAs during gravitropism. Science 243: 91-93

Meins F, Jr. and Kunz C (1995) Gene Silencing in Transgenic Plants: A Heuristic Autoregulation Model. *In* P Meyer, ed, Gene Silencing in Higher Plants and Related Phenomena in Other Eukaryotes, Vol 197. Springer-Verlag, Berlin, pp 105-120

Muhlrad D, Decker CJ, Parker R (1994) Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. 8: 855-866

Muhlrad D, Decker CJ, Parker R (1995) Turnover mechanisms of the stable yeast PGK1 mRNA. Mol. Cell. Biol. 15: 2145-2156

Muller,E.W. and Kuhn,L.C. (1988). A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. Cell *53*, 815-825.

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*.. Plant Cell 2: 279-289

Nellen W, Lichtenstein C (1993) What makes an mRNA anti-sense-itive? Trends Biochem. Sci. 18: 419-423

Newman TC, Ohme-Takagi M, Taylor CB, Green PJ (1993) DST sequences, highly conserved among plant *SAUR* genes, target reporter transcripts for rapid decay in tobacco. Plant Cell 5: 701-714

Oda K, Yamato K, Ohta E, Nakamura Y, Takemura M, Nozato N, Akashi K, Kanegae T, Ogura Y, Kohchi T, Ohyama K (1992) Gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* mitochondrial DNA. J. Mol. Biol. 223: 1-7

Ohme-Takagi M, Taylor CB, Newman TC, Green PJ (1993) The effect of sequences with high AU content on mRNA stability in tobacco. Proc. Natl. Acad. Sci. USA 90: 11811-11815

Okamuro JK and Goldberg RB (1989) Regulation of plant gene expression: General Principles. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 15. pp 1-82

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.

Ross J (1995) mRNA stability in mammalian cells. Microbiol. Rev. 59: 423-450

Ross J (1996) Control of messenger RNA stability in higher eukaryotes. Trends Genet. 12: 171-175

Rouault TA, Hentze MW, Haile DJ, Harford JB, Klausner RD (1989) The iron-responsive element binding protein: A method for the affinity purification of a regulatory RNA-binding protein. Proc. Natl. Acad. Sci. USA 86: 5768-5772

Russell PJ, Hambidge SJ, Kirkegaard K (1991) Direct introduction and transient expression of capped and non-capped RNA in *Saccharomyces cerevisiae*. Nucleic Acids Res. 19: 4949-4953

Schiebel W, Haas B, Marinkovic S, Klanner A, Sanger HL (1993a) RNA-Directed RNA Polymerase from Tomato Leaves: II Catalytic in vitro properties. J. Biol. Chem. 263: 11858-11867

Schiebel W, Haas B, Marinkovic S, Klanner A, Sanger HL (1993b) RNA-Directed RNA Polymerase from Tomato Leaves: I Purification and physical properties. J. Biol. Chem. 268: 11851-11857

Schiebel,W., Pelissier,T., Riedel,L., Thalmeir,S., Schiebel,R., Kempe,D., Lottspeich,F., Sanger,H.L., and Wassenegger,M. (1998). Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell *10*, 2087-2101.

Seeley KA, Byrne DH, Colbert JT (1992) Red light-independent instability of oat mRNA in-vivo. Plant Cell 4: 29-38

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

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.

Shobuike,T., Sugano,S., Yamashita,T., and Ikeda,H. (1995). Characterization of cDNA encoding a mouse homolog of fission yeast dhp1 gene: structural and functional characterization. Nucleic Acids Res. *23*, 357-361.

Shyu A-B, Belasco JG, Greenberg ME (1991) Two distinct destabilizing elements in the c-*fos* message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. 5: 221-231

Siflow CD, Key JL (1979) Stability of polysome-associated polyadenylated RNA from soybean suspension culture cells. Biochemistry 18: 1013-1018

Smith CJS, Watson CF, Bird CR, Ray J, Schuch W, Grierson D (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol. Gen. Genet. 224: 477-481

Stevens A (1993) Eukaryotic nucleases and mRNA turnover. *In* JG Belasco, G Brawerman, eds, Control of Messenger RNA Stability, Academic Press, Inc. San Diego, CA, pp 449-471

Stewart CN, Adang MJ, All JN, Boerma HR, Cardineau G, Tucker D, Parrott WA (1996) Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis* cryIAc gene. Plant Physiol. 112: 121-129

Sullivan ML, Green PJ (1993) Post-transcriptional regulation of nuclear-encoded genes in higher plants: the roles of mRNA stability and translation. Plant Mol. Biol. 23: 1091-1104

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

Tanzer MM, Meagher RB (1994) Faithful degradation of soybean rbcS mRNA in vitro. Mol. Cell. Biol. 14: 2640-2650

Tanzer MM, Meagher RB (1995) Degradation of the soybean ribulose-1,5-bisphosphate carboxylase small-subunit mRNA, SRS4, initiates with endonucleolytic cleavage. Mol. Cell. Biol. 15: 6641-6652

Taylor CB, Green PJ (1995) Identification and characterization of genes with unstable transcripts (*GUT*s) in tobacco. Plant Mol. Biol. 28: 27-38

Thompson DM, Tanzer MM, Meagher RB (1992) Degradation products of the mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean and transgenic petunia. Plant Cell 4: 47-58

Till,D.D., Linz,B., Seago,J.E., Elgar,S.J., Marujo,P.E., Elias,M.D., Arraiano,C.M., McClellan,J.A., McCarthy,J.E.G., and Newbury,S.F. (1998). Identification and developmental expression of a 5'-3' exoribonuclease from *Drosophila melanogaster* . Mech. Dev. *79*, 51-55.

Vakalopoulou E, Schaack J, Shenk T (1991) A 32-kilodalton protein binds to AU-rich domains in the 3' untranslated regions of rapidly degraded mRNAs. Mol. Cell. Biol. 11: 3355-3364

Van Blokland R, Van der Geest N, Mol JNM, Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. Plant J. 6: 861-877

van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (1990) Flavonoid genes in petunia.: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2: 291-299

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

van Hoof A and Green PJ (1997) Control of mRNA decay in plants. *In* DR Orris, JB Harford, eds, Post-transcriptional Gene Regulation, Wiley and Sons, New York,

Voelker TA, Staswick P, Chrispeels MJ (1986) Molecular analysis of two phytohemagglutinin genes and their expression in *Phaseolus vulgaris* cv. Pinto, a lectin-deficient cultivar of the bean. EMBO J. 5: 3075-3082

Vongs A, Kakutani T, Martienssen RA, Richards EJ (1993) *Arabidopsis thaliana* DNA methylation mutants. Science 260: 1926-1928

Vreken P, Raué HA (1992) The rate-limiting step in yeast *PGK1* mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region. Mol. Cell. Biol. 12: 2986-2996

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

Wessenegger M, Heimes S, Riedel L, Sanger HL (1994) RNA-Directed *De Novo* Methylation of Genomic Sequences in Plants. Cell 76: 567-576

Yamamoto KT, Mori H, Imaseki H (1992) cDNA cloning of indole-3-acetic acid-regulated genes: Aux22 and SAUR from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol. 33: 93-97

Yu,S.-M., Kuo,Y.-H., Sheu,G., Sheu,J.-J., and Liu,L.F. (1991). Metabolic derepression of α-amylase gene expression in suspension-cultured cells of rice. J. Biol. Chem. *266*, 21131-21137.

Zhang S, Sheng J, Liu Y, Mehdy MC (1993) Fungal elicitor-induced bean proline-rich protein mRNA down-regulation is due to destabilization that is transcription and translation dependent. Plant Cell 5: 1089-1099

Zhang S, Ruiz-Echevarria MJ, Quan Y, Peltz SW (1995) Identification and characterization of a sequence motif involved in nonsense-mediated mRNA decay. Mol. Cell. Biol. 15: 2231-2244

Zhang S, Mehdy MC (1994) Binding of a 50-kD protein to a U-rich sequence in an mRNA encoding a proline-rich protein that is destabilized by fungal elicitor. Plant Cell 6: 135-145

Zubiaga AM, Belasco JG, Greenberg ME (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol. Cell. Biol. 15: 2219-2230

CHAPTER 1-1

A PROCEDURE TO SELECT MUTANTS OF ARABIDOPSIS DEFECTIVE IN SEQUENCE-SPECIFIC mRNA DEGRADATION

INTRODUCTION

Rates of messenger RNA (mRNA) degradation are highly variable in eukaryotic cells (Reviewed in Ross, 1995; Caponigro and Parker, 1996; Johnson et al., 1998, also see the introduction to this thesis). Some messages are degraded rapidly (minutes), while others persist in the cell for much longer (days). These differences allow for precise control of gene expression. It is important to know how the cell discriminates between long and short-lived mRNAs if we are to fully understand how gene expression is regulated.

Cis-regulatory elements have been identified that act as either instability or stability determinants (for example, Holcik and Liebhaber, 1997; Chen and Shyu, 1995). These elements have been defined by inserting them into messages of average stability (half-life on the order of hours) and then measuring the half-life of the resulting chimeric transcripts (Ross, 1995). Instability sequences target these otherwise stable reporters for rapid degradation while stability elements stabilize the reporter mRNA. Our studies have focused on messages that are very unstable because in many cases these messages encode important regulatory proteins. This is logical because short-lived messages allow the cell to achieve optimum control over gene expression in time and space, characteristics that are important for the expression of key regulatory genes. Several sequences that target transcripts for rapid turnover in plants have been identified. Among these are the DST element (Newman et al., 1993), the AUUUA repeat (Ohme-Takagi, 1993), and premature stop-codons (van Hoof and Green, 1996). We are interested in understanding the

mechanisms by which the plant cell recognizes these sequences and targets the transcripts that contain them for rapid degradation.

Biochemical approaches have resulted in the isolation of sequence-specific RNA binding proteins that are involved in modulating mRNA stability. The best example is the iron-responsive element (IRE) which is bound by the IRE-binding protein (IRE-BP) under iron-limiting conditions. Binding by the IRE-BP protects the transferrin mRNA from degradation. The IRE-BP was purified from human liver by preparing biotinylated-IRE RNA that was transcribed in vitro. A specific RNA binding protein was eluted from biotin-agarose at high salt (Roualt et al., 1989). This led to the cloning of a cDNA for the IRE-BP (Roualt et al., 1990). In addition, many RNA binding proteins have been isolated that interact with AU-rich elements (ARE) in vitro. AREs are potent instability determinants that result in rapid turnover of several mammalian proto-oncogenes and often contain AUUUA repeats (Chen and Shyu, 1995). These experiments have relied on UV-crosslinking and electrophoretic mobility shift assays to show that incubation of the protein with a radiolabeled RNA probe results in a specific interaction. The challenge has been to understand the contribution of these ARE-binding proteins to ARE-mediated mRNA degradation in vivo.

Isolation of sequence-specific RNA-binding proteins from plant cells has not been as successful perhaps because it is difficult to prepare cytoplasmic protein extracts that are free of non-specific ribonucleases (Feldbrugge et al., unpublished data). This may be due to the prominence of the plant vacuole, which is known to contain many non-specific ribonucleases and other hydrolytic enzymes (Boller and Kende, 1979; Abel and Glund, 1987). Despite these difficulties, there is at least one example of a sequence-specific

RNA-binding-protein that may be involved in mRNA turnover in plants. The PRP-BP binds specifically to a sequence in the 3' untranslated region (UTR) of the *Phaseolus vulgaris* proline rich protein (*PvPRP*) mRNA which is destabilized in response to fungal ellicitor treatment of bean cell cultures (Zhang and Mehdy, 1994; Zhang et al., 1993). The gene that encodes this protein and how it affects *PvPRP* mRNA stability are still unknown.

An alternative to purification of sequence-specific RNA binding proteins would be to isolate mutant plants that have lost the ability to recognize mRNA instability determinants. This approach allows several important advantages. First, genes identified in a mutant selection would be very likely to play a role in mRNA degradation in vivo. Second, basic information about the mechanisms of mRNA degradation may be obtained by studying mutants. Third, other cellular factors that might be involved in sequence-specific interactions, such as RNA molecules, may be identified as long as they are encoded by the nuclear genome. Finally, complications such as the presence of non-specific RNA degrading activities in protein extracts are eliminated.

Threfore, parallel methods were developed to isolate mutants defective in DST- and AUUUA-mediated mRNA degradation. There were several reasons to focus on these instability determinants. The DST element has been well characterized and is likely to play an important role in regulating the expression of many plant genes (Newman et al., 1993; Sullivan and Green, 1996; Gil and Green, 1996). The AUUUA repeat contains 11 copies of the core sequence of the AREs, the most widely studied instability determinant (Chen and Shyu, 1995, Lagnado et al., 1994; Zubinga et al., 1995). The capacity to apply genetic analysis to ARE-mediated mRNA decay is limited in

mammalian cell cultures and this element is not known to function in model genetic systems such as yeast. Therefore, studies in Arabidopsis may provide a unique opportunity to study ARE-mediated mRNA degradation using mutants.

The selection strategy presented here is the first one designed to select mutants that are defective in rapid mRNA degradation mediated by a specific sequence element. This approach has resulted in the isolation of mutants defective in rapid mRNA degradation mediated by the DST element (see Chapter 1-2). This procedure was based on a selectable phenotype that we engineered using transgenic Arabidopsis plants and is theoretically adaptable to any *cis*-regulatory element that results in rapid mRNA degradation. Utilization of this method should lead to an increase in our understanding of the basic mechanisms that are responsible for rapid degradation of mRNA in higher plants.

RESULTS

Premise of the mutant selection strategy

In order to isolate mutants of Arabidopsis defective in a sequence-specific mRNA degradation pathway we first needed to engineer plants that would display a mRNA abundance phenotype that could be readily screened for and/or selected. This mutant selection strategy relied on two reporter genes that each contained the DST or AUUUA instability determinants in their 3' UTR (Figure 1-1-1). In wild-type (wt) plants, the instability determinant functions effectively and the abundance of the reporter mRNA is

maintained at a low level. Mutants that are defective in rapid mRNA degradation mediated by the instability determinant would be expected to have an increased abundance of both reporter mRNAs. If transgenes are chosen that encode readily selectable and/or screenable enzymatic activities, it should be possible to isolate mutants by virtue of an increase in reporter gene expression.

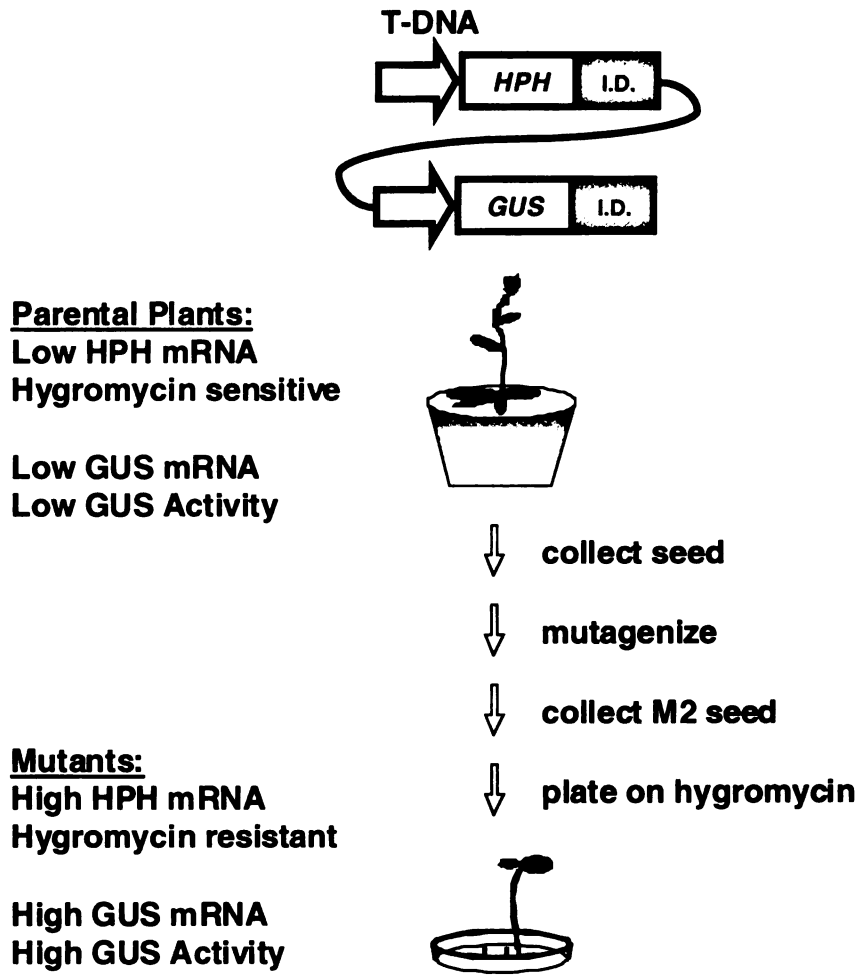

Figure 1-1-1. A strategy for selecting mutants defective in a sequence-specific mRNA decay pathway. Transgenic plants are generated using a construct such as the one represented above. Arrows represent promotor sequences. Hygromycin phosphotransferase (*HPH*) and β-glucuronidase (*GUS*) are examples of reporter genes that confer selectable and screenable phenotypes, respectively. I.D. represents the instability determinant being studied, in this case either DST or AUUUA.

HPH and *GUS* are chosen as reporter genes

We initially considered hygromycin phosphotransferase (*HPH*), dihydrofolate reductase (*DHFR*), and β-glucuronidase (*GUS*) as potential transgenes for this strategy. *HPH* and *DHFR* confer resistance to hygromycin and methotrexate, respectively, and there are many commercially available substrates that facilitate detection of *GUS* gene expression (Nunberg et al., 1980; Gritz and Davies, 1983; Jefferson et al., 1987). The impact of mRNA destabilization upon each of these selectable marker and reporter genes was an important consideration. A large difference in mRNA abundance between plants expressing a destabilized reporter versus a non-destabilized reporter would be optimal for the isolation of mutants with a wider range of mRNA abundance phenotypes.

Previous experiments with transgenic tobacco plants showed that abundance of the human *β-Globin* (*Globin*) transcript was reduced by ~14 fold when the AUUUA repeat was inserted into its 3'UTR or by ~7.5 fold when a dimer of the DST element (DSTx2) was inserted (Ohme-Takagi et al., 1993; Newman et al., 1993). In order to assess the effect of AUUUA repeats and the DST element on the abundance of *GUS*, *HPH*, and *DHFR* transcripts in Arabidopsis, T-DNA constructs were generated that placed either the AUUUA repeat, DSTx2 or a tetramer of the DST element (DSTx4) into the 3'UTR of each selectable marker or reporter gene. Similar constructs using the *Globin* coding region were also analyzed so that the results in Arabidopsis could be compared with those already obtained in tobacco. These constructs were designed to have a reference gene within the same T-DNA to serve as a loading control for Northern blot analysis. A representative construct is depicted in Figure 1-1-2.

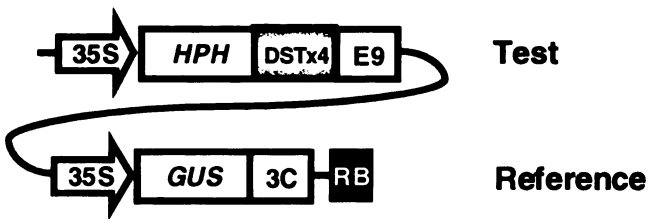

Figure 1-1-2. The basic structure of transgenes used to assess various reporters and instability determinants in transgenic Arabidopsis calli. The cauliflower mosaic virus 35S promoter (35S) was used to control the expression of the *DHFR, HPH, GUS*, and *Globin* reporter genes. Polyadenlytion signals derived from the pea ribulose 1,5-bisphosphate carboxylase small subunit E9 (*rbcS-E9,* E9) gene were used as mRNA processing signals for *DHFR, HPH*, and *Globin*. The 3' UTR of the of the *rbcS-3C* (3C) gene was used as a polyadenylation signal for *GUS*. The reporters were paired in test: reference combinations as follows: *DHFR* with *GUS*, *HPH* with *GUS* (shown), *GUS-AUUUA* with *Globin*, *GUS-DSTx2* with *DHFR*, *GUS-DSTx4* with *HPH*, and *Globin* with *GUS*. The right border (RB) of the transfer-DNA (T-DNA) has been indicated by a black rectangle. The T-DNA also includes the neomycin phosphotranserase cassette so that kanamycin resistant transgenic tissue could be selected.

These constructs were used to generate transgenic Arabidopsis calli by co-cultivating *Agrobacterium* strains harboring the relevant constructs with root explants of Arabidopsis seedlings (Valveken et al., 1988). Pools of at least 100 independent transgenic calli were gathered for each construct and the mRNA abundance of each construct was analyzed by Northern blot analysis. Radiolabelled probes complementary to the *DHFR, HPH, GUS*, and *Globin* coding regions were used to determine the abundance of these reporter genes on blots that were loaded with equal amounts of total RNA from pooled calli. Signals corresponding to reference and test transcripts were quantitated by PhosphorImager. The test signal for each construct was normalized using the reference signal and the average normalized value was plotted relative to non-destabilized control constructs (Figure 1-1-3).

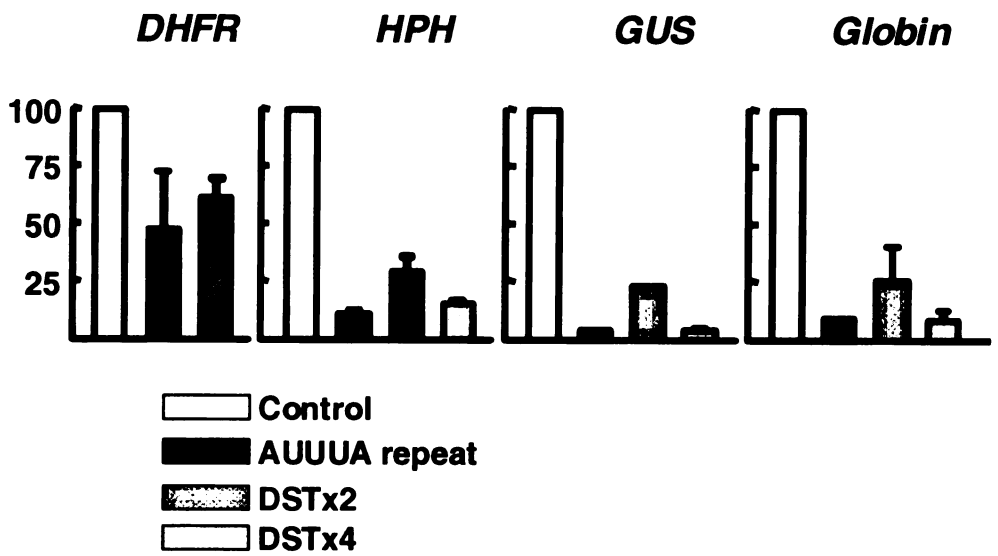

Figure 1-1-3. Accumulation of reporter transcripts in Arabidopsis. This histogram depicts the normalized abundance of reporter transcripts in transgenic Arabidopsis calli. Data are presented as a percentage of the non-destabilized controls; where multiple experiments were done error bars have been plotted (standard deviation).

One important observation from this experiment was that neither a dimer of the DST element (DSTx2) nor the AUUUA repeat destabilized the *DHFR* transcript to the same extent as they did *HPH*, *GUS*, or *Globin*. It should be noted that the absolute abundance of the *DHFR* transcript was lower than that of *HPH, GUS*, or *Globin*, indicating that perhaps the DHFR transcript was relatively unstable to begin with (data not shown). This might explain why insertion of instability sequences into this transcript did not result in the same reduction in abundance that was observed for *HPH, GUS*, and *Globin*. These results indicated that *DHFR* would not be an appropriate selectable marker for our mutant selection strategy because an increase in mRNA abundance in a putative mutant may not have been detectable above the already high relative abundance of the transcript in wt plants. On the other hand, the observed effect of the AUUUA repeat and the DST element on *HPH* and *GUS* mRNA abundance was consistent with

previously obtained *Globin* data and indicated that using these reporters would provide a wider range in which to select new mRNA abundance phenotypes in mutants.

Selection of transgenic plants to serve as the parental lines for mutagenesis

HPH and *GUS* were chosen as the reporter genes for our selection strategy (Figure 1-1-1). DSTx4 was used instead of DSTx2 because of the larger decrease in mRNA abundance it caused. New constructs were made and then used to generate transgenic Arabidopsis plants harboring both the selectable marker and reporter transcripts destabilized by AUUUA (p1514) or DSTx4 (p1519). In addition, a positive control construct was made that carried the same genes without instability determinants (Figure 1-1-4). Since it was important to isolate plants with single inserts of the T-DNA and that expressed the reporter genes to the appropriate level, several independent transgenic lines were generated with each construct and these were examined to choose the best lines for mutagenesis. Transgenic lines in which the reporter gene expression reflected the average mRNA abundance found in the experiments shown in Figure 1-1-3, became candidates to serve as the parental line for mutagenesis.

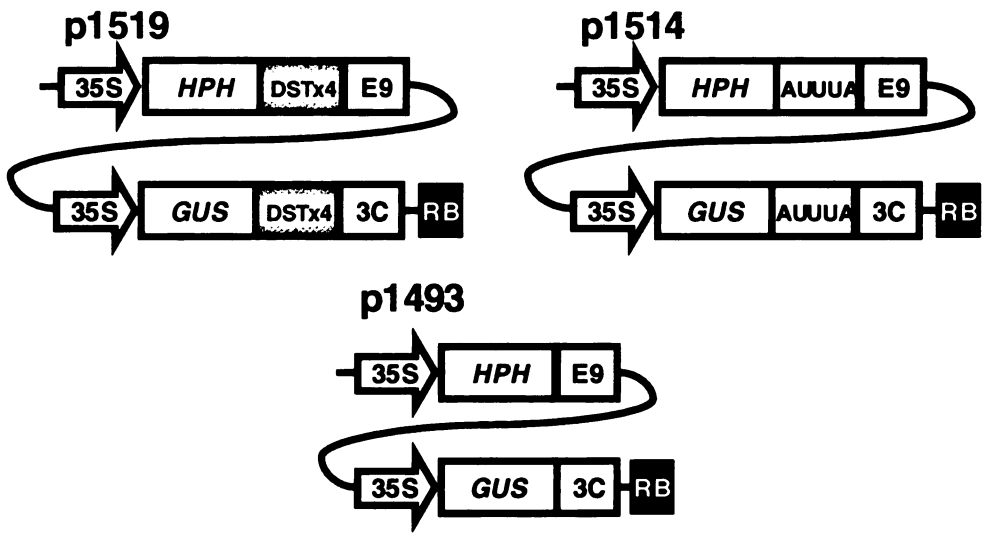

Figure 1-1-4. T-DNA constructs used in mutant selection strategy. The T-DNAs also include the neomycin phosphotranserase cassette so that kanamycin resistant transgenic tissue could be selected. Also not shown is the nopaline synthase (*nos*) gene which contains a BamHI restriction enzyme site and is located between the *GUS-3C* gene and the right border (RB).

In order to identify lines with one insert of the T-DNA, T_2 (progeny of the self-fertilization of the primary transformant) seed were plated on primary seed selection medium containing 50 μg/ml kanamycin. Three p1519 (DSTx4) and three p1514 (AUUUA) lines that showed a ratio of three resistant to one sensitive seedling, indicating inheritance of a single, dominant locus were chosen. Single inserts were confirmed by Southern blot analysis (Figure 1-1-5).

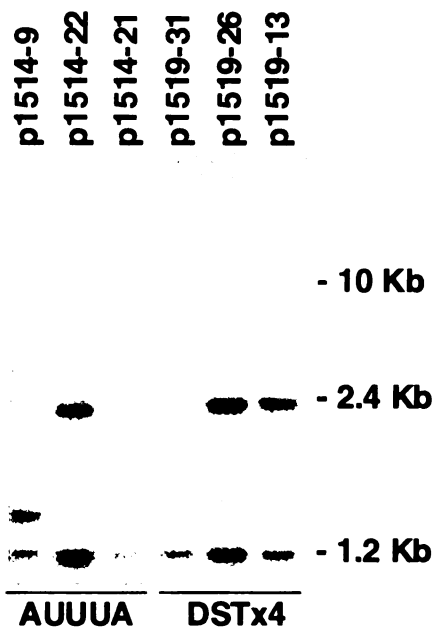

Figure 1-1-5. Southern blot analysis of candidate lines for mutagenesis. Genomic DNA was extracted from transgenic plants, digested with BamHI, separated on a 1% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with a radiolabeled probe complementary to the nopaline synthase (*nos*) coding region; adjacent to the right border of the T-DNA. A 1.2 kb fragment that is part of the T-DNA is present in all of the lanes. Additional bands represent the distance to BamHI sites in the genome. The number of additional bands is indicative of the number of copies of the T-DNA.

p1514-9 and p1519-31 had simple banding patterns indicating that these lines had single inserts. Northern blot analysis confirmed that each of these transgenic lines expressed the *HPH* and *GUS* reporter transcripts to levels that were expected based on previous experiments (Figure 1-1-6). Insertion of the AUUUA repeat into the 3'UTR of the *HPH* or *GUS* transcripts resulted in an increase in mRNA size of 60 nucleotides. Insertion of DSTx4 resulted in an increase of 180 nucleotides. Based on the results of this experiment and those previously described, p1514-9 and p1519-31 were chosen as the parental lines for mutagenesis. The higher molecular weight *GUS* transcript (indicated by (•) in Figure 1-1-6) that is present in the p1519-31 lane is a consistent characteristic of

this line. We chose p1519-31 for mutagenesis despite this because this line had the simplest T-DNA insertion and because the additional band did not seem to alter *GUS* activity (data not shown). We measured the abundance of the lower molecular weight band, which is the expected size, throughout these experiments.

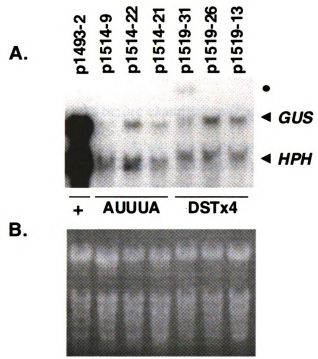

Figure 1-1-6. Northern blot analysis of candidate lines for mutagenesis. 20 μg of total RNA from 12-day-old seedlings was separated on a 1% agarose gel and transferred to a nylon membrane. A. The membrane was simultaneously hybridized with radiolabeled probes complimentary to the *GUS* and *HPH* coding sequences. B. A photograph of the ethidium-bromide stained gel is shown indicating approximately equal loading of total RNA.

Lines p1514-9 and p1519-31 showed decreased hygromycin resistance

In order for our selection strategy to be successful, the decrease in mRNA abundance that resulting from the insertion of the AUUUA repeat or DSTx4 inserted into the *HPH* transcript would have to result in decreased hygromycin resistance. In order to test this, p1514-9, p1519-31, and p1493-2 seeds were plated on Arabidopsis germination media containing 500, 750 and 100 μg/ml hygromycin. Seedlings were allowed to germinate and grow for four weeks before examination (Figure 1-1-7).

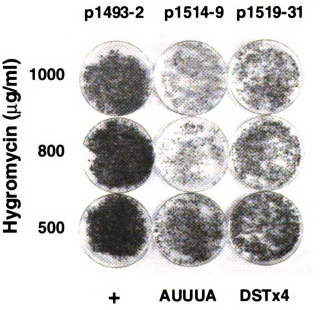

Figure 1-1-7. p1514-9 and p1519-31 show increased hygromycin sensitivity. Approximately 500 seeds were surface sterilized and plated on primary seed selection medium containing 50 μg/ml kanamycin plus the indicated concentration of hygromycin. The photograph was taken after four weeks.

This experiment indicated that decreases in mRNA abundance due to insertion of instability sequences into the *HPH* transcript caused increased hygromycin sensitivity in

p1514-9 and p1519-31 seedlings. This was especially clear at high concentrations of hygromycin (1000 μg/ml) where very few seedlings escape this selection.

Mutagenesis of lines p1514-9 and p1519-31

Plants homozygous at the T-DNA locus present in lines p1514-9 and p1519-31 were obtained by plating T_3 families on kanamycin and selecting those that segregated 100% resistance. Seed from multiple T_3 plants were pooled so that adequate T_4 seed would be available for ethylmethane sulfonate (EMS) mutagenesis (Rédei and Koncz, 1992, Lightner and Caspar, 1998). EMS is a standard mutagen for genetic analysis of Arabidopsis. It is thought to cause point mutations primarily due to GC to AT transitions that result from ethylation of DNA (Feldman et al., 1994). Standard EMS treatments result in multiple, hemizygous mutations in each plant derived from mutagenized seed (M_1) such that only about 2000 M_2 plants (self-fertilized progeny of M_1) need be analyzed to find a homozygous mutation in any one of the ~ 20,000 Arabidopsis genes (Haughn and Somerville, 1986). We followed standard previously described EMS mutagenesis protocols (Redei and Koncz, 1992). Table 1-1-1 describes the mutagenesis of p1514-9 and p1519-31.

Table 1-1-1. EMS mutagenesis of transgenic Arabidopsis lines.

p1514-9 AUUUA			
Exp.	# of M_1 Seed	Group	# of M_1/Group
Pilot	5000	Pilot	5000
1	100,000	1-20	5000
2	100,000	21-40	5000
3	50,000	41-52	4000
Total	255,000		4800

p1519-31 DSTx4			
Exp.	# of M_1 Seed	Group	# of M_1/Group
1	100,000	1-20	5000
2	100,000	21-40	5000
3	25,000	41-46	4000
Total	225,000		4900

M_1, first generation of mutagenized seed.

Following mutagenesis, M_1 seeds were planted in groups of ~5000 plants and M_2 seed were collected from each group. M_2 seeds were collected in groups in order to obtain an initial approximation of the number of independent mutations, as mutants selected from different groups were likely to represent independent mutations. The effectiveness of EMS mutagenesis was evaluated by scoring the occurrence of albinism and embryo lethality in M_1 plants, as previously described (Redei and Koncz, 1992).

Selection of putative mutants

Putative mutants were selected by plating ~4000 M_2 seeds on primary seed selection plates containing 1000 µg/ml hygromycin and 50 µg/ml kanamycin. After 3-4 weeks putative mutants, defined as seedlings that had developed at least two true leaves, were chosen from the primary plates and moved to plates lacking hygromycin.

Development of two true leaves was consistently absent in non-mutagenized p1519-31 and p1514-9 seedlings. After one to two weeks on plates lacking hygromycin, putative mutants were transferred to soil. Table 1-1-2 summarizes the results of the selection experiments and the number of putative mutants that were obtained.

Table 1-1-2. Hygromycin selection experiments

p1514-9 AUUUA		
M_2 group	# M_2 plated	# putative mutants
Pilot	4000	4
1-20	40,000	2
21-40	127,000	126
41-52	668,000	71
Total	**839,000**	**203**

p1519-31 DSTx4		
M_2 group	# M_2 plated	# putative mutants
1-20	90,000	14
21-40	444,000	263
41-52	200,000	46
Total	**730,000**	**323**

#, number; M_2, second generation after mutagenisis

The abundance of the *HPH* and *GUS* reporter mRNAs was determined by Northern blot analysis of leaf RNA extracted from M_2 plants selected on hygromycin. These experiments allowed rapid scoring of the key mutant phenotype. The abundance of the *HPH* and *GUS* transcripts was normalized using the translation initiation factor 4-A (*eIF4-A*) transcript abundance; these were then compared to the normalized *HPH* and *GUS* mRNA abundance of parental samples that had been grown in parallel. Where this was not possible, for example if the plant was too small to obtain an adequate RNA sample, mRNA abundance was assayed in M_3 plants. In addition, M_3 seeds were plated

on hygromycin to determine whether the resistance phenotype was inherited. Hygromycin resistance was found to be heritable in approximately 20% of the M_3 families tested, suggesting a high rate of false positives.

323 putative mutants derived from mutagenesis of line p1519-31 (DSTx4) and 203 derived from p1514-9 (AUUUA) were analyzed by these criteria. mRNA abundance was analyzed by Northern blot analysis for all putative mutants. Only three putative mutants met the criteria of heritable increases in *HPH* and *GUS* mRNA abundance. These were DST-M_2#64 (group 21), DST-M_2#114 (group 31), and DST-M_2#290 (group 30). No mutants were isolated from the AUUUA population even though over 800,000 p1514-9 M_2 seeds were plated. DST-M_2#114 and DST-M_2#64 were isolated relatively early in the selection process and were characterized in detail. These mutations were found to be in independent genes and have been renamed *dst1* (DST-M_2#114) and *dst2* (DST-M_2#64) to reflect their defects in DST-mediated mRNA degradation. A detailed characterization of these mutants is the subject of the next chapter of this thesis. DST-M_2#290 is currently being analyzed. The immediate priorities are to establish the mode of inheritance of the mutation and to determine whether this mutant complements either *dst1* or *dst2*.

DISCUSSION

Genetic selection strategies such as the one presented here have been called second generation screens or 'targeted genetics' because they rely on engineered phenotypes rather than those intrinsic to the plant (for example, see Hooley, 1998).

Targeted genetics can provide a novel class of mutants to an area where classical screens have already been saturated. The recently isolated *age* (auxin-responsive gene expression) mutants provide an excellent example (Oono et al., 1998). Using a fusion of an auxin-responsive promoter element to the *GUS* reporter gene, Oono et al., were able to isolate auxin response mutants that misexpressed the reporter. These mutants provide a useful complement to the many auxin-response mutants that have been identified by traditional means.

Perhaps more importantly, targeted genetics can provide an entry into areas that have been inaccessible to genetic analyses because obvious, intrinsic phenotypes are lacking. This was the case for sequence-specific mRNA degradation mediated by AUUUA repeats or the DST element. No targets of the AUUUA-mediated mRNA degradation pathway have been definitively identified in plants. Therefore, it is difficult to imagine an intrinsic phenotype that would result from the disruption of this pathway. The DST element is likely to be involved in the constitutive instability of several Arabidopsis *SAUR* transcripts. Therefore, it may be possible to devise a direct screen for mutants based on the abundance of these transcripts. However, this would be extremely labor-intensive and many of the mutants would be defective in the transcriptional regulation of these genes by auxin. In fact, auxin-response mutants have been isolated that result in misexpression from the *SAUR-AC1* promoter (Leyser et al. 1996). It is for these reasons that a targeted genetic approach was necessary in order to explore the genetic basis for sequence-specific mRNA degradation in plants.

The mutants isolated in this study were very rare. Three were isolated from a total of approximately 1.5×10^6 mutagenized seeds that were plated during the course of

the DST and AUUUA mutant selections. One of the possible explanations for this observation is that genes involved in AUUUA- and DST-mediated mRNA degradation are essential. Since it is likely that mRNA degradation pathways limit the expression of key regulatory genes, it is possible that if transcripts targeted by these pathways are overexpressed or misexpressed during early development of the plant due to a mutation, the result would be embryo lethality. A relevant and interesting observation is that overexpression of AUUUA-containing oncogene messages due to mutations that delete AREs have resulted in neoplastic transformation in mammalian cells (Piechaczyk et al., 1985). This finding highlights the potential consequences that can result when mRNA degradation pathways are rendered ineffective by mutations.

If these pathways are essential for development of plants, one might also expect redundancy to have evolved in these systems. This is another reason why mutants in the AUUUA pathway may not have been isolated. As is described in the next chapter of this thesis, *dst1* and *dst2* were partially dominant mutations. If the DST-mediated mRNA degradation pathway is redundant, then one might expect to only uncover dominant alleles that interact with and negate redundant members of the pathway. Mutations in the ethylene receptor illustrate how dominant negative alleles can provide access to redundant genes (reviewed by Theologis, 1998). The original ethylene receptor mutations were all dominant. After a receptor gene was isolated by map based cloning, it was found that ethylene receptors constitute a gene family in Arabidopsis. Loss-of-function mutant phenotypes were not observed until triple and quadruple mutants were constructed (Hua and Meyerowitcz, 1998).

There are also technical explanations for the observed rarity of sequence-specific mRNA degradation mutants. Approximately 80% of the putative mutants that were chosen for further study were found to be false positives. This may have been due to overzealous picking of putative mutants. However, this may have been important because the mutants that were recovered, *dst1* and *dst2*, showed only slight increases in *HPH* and *GUS* mRNA abundance and may have been missed if only the healthiest plants were rescued from selection plates. In any case, this rate of false-positives, decreased the efficiency of this selection.

Targeted genetics approaches have been used previously to isolate mutations that affect the function of specific promoter sequences (Susek et al., 1993; Bowling et al., 1994; Jackson et al., 1995; Martin et al., 1997; Oono et al., 1998). Here, the utility of such a procedure has been extended to *cis*-regulatory elements located in the 3'UTR. Therefore, the mutants isolated using this procedure have the potential to provide insight into rapid sequence-specific mRNA degradation in plants and eukaryotes in general and the cloning of *dst* genes may aid in the ellucidation of the molecular machinery that catalyzes the degradation of DST-containing transcripts. These studies have the potential to answer many fundamental questions about mRNA turnover in plants. For example, do DST-recognition components recruit the basal mRNA decay machinery more rapidly to DST-containing transcripts? One may find that *dst* gene products interact with poly(A) binding-proteins and/or poly(A) ribonucleases so that the poly(A) tails of DST-containing messages are removed more rapidly. The basic mechanisms of mRNA degradation in plants are still largely unknown. Any insight into factors that interact with *dst* gene

products may reveal clues that will lead to the basic components responsible for this process.

MATERIALS AND METHODS

Plasmid construction and plant transformation

Standard molecular cloning procedures were used (Sambrook et al., 1989). Construction of the chimeric genes described in Figure 1-1-2 and Figure 1-1-4 was as described by Newman et al., 1993. Chimeric genes were constructed in pBluescript SKII+ (Stratagene) or pUC (New England Biolabs) and were then transferred to derivatives of pMON505 which is a binary vector that facilitates transformation of plants via *Agrobacterium tumefaciens* (*Agrobacterium*)(Rogers et al., 1987, Fang et al., 1989). Chimeric genes had the basic structure: SacI-35S-BglII-XbaI-Coding region-BamHI-poly(A) sequence-ClaI. DST and AUUUA sequences were inserted at the BamHI site between the coding sequence and the poly(A) signal. The source of the *Globin* and *GUS* coding regions has been described (Newman et al., 1993). The *HPH* coding region was obtained as a BamHI fragment of pLG90 (Gritz and Davies, 1983). The *DHFR* coding sequence was described by Nunberg et al., (1980). The DST sequence used in these experiments is the same as that found in the soybean *SAUR-15A* gene and was synthesized as described by Newman et al, 1993. The construction of the AUUUA repeat was described by Ohme-Takagi et al., 1993. The use of the 35S promoter and

polyadenylation signals from the 3' ends of the E9 and 3C genes was as described by Newman et al., 1993.

Transgenic Arabidopsis calli were generated by co-cultivation of *Agrobacterium* with root explants (Valveken et al., 1988). *Agrobacterium* strain LBA4404 was transformed with pMON505 derivatives by electroporation using the Gene-Pulser (BioRad) according to the manufacturer's guidelines. Generation and selection of transgenic Arabidopsis plants using the vacuum infiltration method of *Agrobacterium*-mediated transformation has been described (N. Bechtold et al.,1993 and Web site: http://www.bch.msu.edu/pamgreen/vac.htm). *Agrobacterium* strain GV3101 C58C1 Rif^r (pMP90) (Koncz and Schell, 1986) was transformed with pMON505 derivatives by electroporation using the Gene-Pulser (BioRad) according to the manufacturer's guidelines.

Plant material and growth conditions

The Columbia (Col-O) accession of *Arabidopsis thaliana* was used in all transformations except for p1514 and p1519. To avoid any hygromycin resistant contamination of p1514-9 and p1519-31 during large-scale selection experiments, these constructs were introduced into a Columbia mutant that lacks trichomes (*gl1*, Col-PRL). Arabidopsis calli were maintained on Gamborg's B5 (An, 1985) plates in an incubator set at 16 hours light (125 $\mu E/m^2$) /8 hours dark and 21°C. Arabidopsis plants were grown in standard Arabidopsis soil in controlled environment growth chambers set at 16 hours light (125 $\mu E/m^2$) /8 hours dark, 21°C.

EMS mutagenesis and selection of hygromycin resistant plants

Seeds homozygous at the p1514-9 and p1519-31 loci (fourth generation of self-fertilized progeny following transformation) were soaked for 16 hours in a 0.3% (v/v, water) solution of EMS. Following mutagenesis, seeds were washed extensively with water over a 12-hour period and then sown on soil. A thorough protocol is given in Lightner and Caspar, (1998). The effectiveness of EMS mutagenesis was evaluated by scoring the occurrence of albinism and embryo lethality in M_1 plants as described by Redei and C. Koncz (1992); and Lightner and Caspar, (1998).

Seed selection media consisted of 4.3 g/L Murashige and Skoog salt mixture (GibcoBRL), B5 vitamin mixture (100 mg/l *myo*-inositol, 10 mg/l thiamine hydrochloride, 1 mg/l nicotinic acid, 1 mg/l pyriodoxine), 1% sucrose, 0.5 g/L MES at pH 5.7, 0.8% phytagar, 1000μg/ml hygromycin and 50 μg/ml kanamycin. Hygromycin was omitted when selecting transgenic plants or scoring inheritance of the T-DNA.

RNA analysis

RNA was isolated from transgenic calli (Figure 1-1-3) and pooled seedlings (Figure 1-1-6) using the method described by Puissant and Houdebine (1990) with modifications described by Newman et al., (1993). For analysis of mRNA abundance in individual plants, two rosette leaves were harvested from mature plants and RNA was extracted using the method described by Verwoerd et al. (1989). Northern blot analysis was as described by Newman et al. (1993). Hybridization probes corresponding to the

DHFR, eIF4-A, Globin, GUS, and *HPH* coding regions were labeled using ^{32}P dCTP by the random-primed method of Feinberg and Vogelstein (1983). The translation initiation factor, *eIF4-A*, was used as normalization standard for northern blots as described by Taylor et al., (1993). mRNA abundance was quantitated by measuring the intensity of radioactive bands on Northern blots using a Molecular Dynamics PhosphorImager.

DNA analysis

Southern blotting was performed as described in Sambrook et al., 1989. DNA was extracted from dark-grown seedlings using the method described by Saghai-Maroof et al. (1984). 15μg of DNA was digested with BamHI and loaded on a 1% agarose gel which was transferred to a nylon membrane. Prehybridization, hybridization, and washing conditions were the same as those for Northern blots (Newman et al., 1993). Hybridization probes corresponding to the nos coding region were labeled using ^{32}P dCTP by the random-primed method of Feinberg and Vogelstein (1983).

ACKNOWLEDGEMENTS

I am grateful to Dr. Michael L. Sullivan who constructed the chimeric genes used in this study and got me started on this project. Dr. Sullivan and Debrah M. Thompson generated the data presented in Figure 1-1-3. The selection of putative mutants in the AUUUA pathway was mainly the work of Miguel A. Pérez-Amador. I would also like to thank Linda Danhof and Jonathan Vogel for tireless technical assistance. This work was

funded by grants from the United States Department of Energy, the United States Department of Agriculture, and the McKnight Foundation to Pamela J. Green.

REFERENCES

Abel,S. and Glund,K. (1987). Ribonuclease in plant vacuoles: purification and molecular properties of the enzyme from cultured tomato cells. Planta *172*, 71-78.

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

Bechtold,N., Ellis,J., and Pelletier,G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris *316*, 1194-1199.

Boller,T. and Kende,H. (1979). Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiol. *63*, 1123-1132.

Bowling,S.A., Guo,A., Cao,H., Gordon,A.S., Klessig,D.F., and Dong,X. (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell *6*, 1845-1857.

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 mRNA degradation. Trends Biochem. Sci. *20*, 465-470.

Fang,R.-X., Nagy,F., Sivasubramaniam,S., and Chua,N.-H. (1989). Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell *1*, 141-150.

Feinberg,A.P. and Vogelstein,B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6-13.

Feldmann,K.A., Malmgren,C., and Dean,C. (1999). Mutagenesis in Arabidopsis. In ARABIDOPSIS, E.M.Meyerowitz and C.R.Somerville, eds. (Plainview, New York: Cold Spring Harbor Laboratory Press), pp. 137-172.

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.

Gritz,L. and Davies,J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in Escherichia coli and Saccharomyces cerevisiae. Gene *25*, 179-188.

Haughn,G. and Somerville,C.R. (1987). Selection for herbicide resistance at the whole-plant level. In Biotechnology in Agricultural Chemistry, H.M.LeBaron, R.O.Mumma,

R.C.Honeycutt, and J.H.Duesing, eds. (Washington, D.C.: American Chemical Society), pp. 98-107.

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.

Hooley,R. (1998). Auxin signaling: Homing in with targeted genetics. Plant Cell *10*, 1581-1583.

Hua,J. and Meyerowitz,E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell *94*, 261-271.

Jackson,J.A., Fuglevand,G., Brown,B.A., Shaw,M.J., and Jenkins,G.I. (1995). Isolation of Arabidopsis mutants altered in the light-regulation of chalcone synthase gene expression using a transgenic screening approach. Plant J. *8*, 369-380.

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

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.

Koncz,C. and Schell,J. (1986). The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. *204*, 383-396.

Lagnado,C.A., Brown,C.Y., and Goodall,G.J. (1994). AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: The functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol. Cell. Biol. *14*, 7984-7995.

Leyser,H.M.O., Pickett,F.B., Dharmasiri,S., and Estelle,M. (1996). Mutations in the *AXR3* gene of *Arabidopsis* result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. Plant J. *10*, 403-413.

Lightner,J. and Caspar,T. (1998). Seed mutagenesis of Arabidopsis. Methods Mol. Biol. *82*, 91-103.

Martin,T., Hellmann,H., Schmidt,R., Willmitzer,L., and Frommer,W.B. (1997). Identification of mutants in metabolically regulated gene expression. Plant J. *11*, 53-62.

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.

Nunberg,J.H., Kaufman,R.J., Chang,A.C.Y., Cohen,S.N., and Schimke,R.T. (1980). Structure and genomic organization of the mouse dihydrofolate reductase gene. Cell *19*, 355-364.

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.

Oono,Y., Chen,Q.G., Overvoorde,P.J., Kohler,C., and Theologis,A. (1998). age Mutants of Arabidopsis exhibit altered auxin-regulated gene expression. Plant Cell *10*, 1649-1662.

Piechaczyk,M., Yang,J.Q., Blanchard,J.M., Jeanteur,P., and Marcu,K.B. (1985). Posttranscriptional mechanisms are responsible for accumulation of truncated c-myc RNAs in murine plasma cell tumors. Cell *42*, 589-597.

Puissant,C. and Houdebine,L.-M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. BioTechniques *8*, 148-149.

Redei,G.P. and Koncz,C. (1992). Classical mutagenesis. In Methods in Arabidopsis Research, C.Koncz, N.-H.Chua, and J.Schell, eds. (Singapore: World Scientific), pp. 16-82.

Rogers,S.G., Klee,H.J., Horsch,R.B., and Fraley,R.T. (1987). Improved vectors for plant transformation: expression cassette vectors and new selectable markers. Methods Enzymol. *153*, 253-277.

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

Rouault,T.A., Hentze,M.W., Haile,D.J., Harford,J.B., and Klausner,R.D. (1989). The iron-responsive element binding protein: A method for the affinity purification of a regulatory RNA-binding protein. Proc. Natl. Acad. Sci. USA *86*, 5768-5772.

Rouault,T.A., Tang,C.K., Kaptain,S., Burgess,W.H., Haile,D.J., Samaniego,F., McBride,O.W., Harford,J.B., and Klausner,R.D. (1990). Cloning of the cDNA encoding an RNA regulatory protein--the human iron-responsive element-binding protein. Proc. Natl. Acad. Sci. USA *87*, 7958-7962.

Saghai Maroof,M.A., Biyashev,R.M., Yang,G.P., Zhang,Q., and Allard,R.W. (1994). Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. Proc. Natl. Acad. Sci. U. S. A *91*, 5466-5470.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

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.

Susek,R.E., Ausubel,F.M., and Chory,J. (1993). Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. Cell *74*, 787-799.

Taylor,C.B., Bariola,P.A., DelCardayré,S.B., Raines,R.T., and Green,P.J. (1993). RNS2: A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. Proc. Natl. Acad. Sci. USA *90*, 5118-5122.

Theologis,A. (1998). Ethylene signalling: redundant receptors all have their say. Curr. Biol. *8*, R875-R878.

Valveken,D., Van Montagu,M., and Van Lijsebettens,M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA *85*, 5536-5540.

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.

Verwoerd,T.C., Dekker,B.M., and Hoekema,A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res *17*, 2362.

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

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

Zubiaga,A.M., Belasco,J.G., and Greenberg,M.E. (1995). The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Mol. Cell. Biol. *15*, 2219-2230.

CHAPTER 1-2

MUTANTS OF ARABIDOPSIS DEFECTIVE IN A SEQUENCE-SPECIFIC mRNA DEGRADATION PATHWAY

This manuscript has been submitted for publication (Mark A. Johnson, Miguel A. Pérez-Amador, and Pamela J. Green).

ABSTRACT

One of the ways a cell can rapidly and tightly regulate gene expression is to target specific mRNAs for rapid decay. A number of mRNA instability sequences that mediate rapid mRNA decay have been identified in various eukaryotes, but the nature of the cellular components that play critical roles in sequence-specific decay in vivo has been more difficult to establish. Here, we used a novel genetic strategy to isolate rare mutants of Arabidopsis that selectively elevate the abundance of mRNAs that contain the plant instability sequence DST. Analysis of these mutants identified two loci that are important for DST-mediated mRNA decay and provided a powerful inroad into the mRNA decay machinery. Similar strategies should be applicable to the in vivo analysis of sequence-specific mRNA decay in other eukaryotes.

The small-auxin-up-RNA (*SAUR*) transcripts are among the most unstable mRNAs in plants, having half-lives of 10-50 minutes (1). The *SAUR* genes have been implicated in auxin-induced cell elongation and their transcripts appear to be constitutively unstable so that their abundance can be rapidly altered in response to transcriptional control by the plant hormone auxin. All unstable *SAUR* transcripts contain conserved DST sequences, so called because of their location downstream of the coding region (1). A dimer of the DST element (2) or the 3' end of the Arabidopsis *SAUR-AC1* gene, which contains a DST element (3), are sufficient to destabilize reporter transcripts in plant cells. The DST sequence is approximately 45 nucleotides in length and is comprised of three highly conserved domains separated by two variable regions. Mutagenesis studies have demonstrated that residues within two of the conserved domains are necessary for instability function (4). Our detailed understanding of the DST element, its robust effect on mRNA levels, and its potential biological importance make it an attractive model for understanding how mRNA instability sequences function in plants. Although genetic strategies have proven critical for the identification of components of the general decay machinery in yeast (5), they have rarely been applied to the study of rapid mRNA decay mediated by specific sequences. Most in vivo studies of the latter have been limited to overexpression of RNA binding proteins (6) or correlations between target mRNA levels and the presence of various RNA binding proteins (7). However, it was recently demonstrated that mice made deficient for tristetraprolin by gene disruption were defective in rapid degradation of tumor necrosis factor-α mRNA mediated by an AU-rich element (ARE). It was later shown that tristetraprolin binds AREs in vitro (8). AREs are perhaps the most well studied class of mRNA instability

sequence in multi-cellular eukaryotes (9). Therefore, these results highlight how a single mutant can provide important insights about sequence-specific mRNA decay mechanisms in eukaryotes.

We have devised an approach to isolate mutants of *Arabidopsis thaliana* that are defective in the sequence-specific mRNA decay pathway mediated by the DST element. Because this strategy facilitated the isolation of rare mutations, it provided us with a unique opportunity to study a sequence-specific mRNA degradation pathway in vivo. Our approach was based on a phenotype that we engineered using two DST-containing reporter genes that were introduced into Arabidopsis via *Agrobacterium tumefaciens* mediated transformation (10). These reporter genes were designed to allow selection of mutants that elevate expression of these genes due to defects in DST-mediated mRNA degradation. Plasmid 1519 (p1519), used to generate the parental line for mutagenesis, contained a tetramer of the DST element inserted into the 3' UTR of the hygromycin phosphotransferase (*HPH*) and β-glucuronidase (*GUS*) genes (Figure 1-2-1-A). *HPH* confers resistance to hygromycin and *GUS* expression is readily detected by incubation of plant tissue with substrates that are cleaved to colorimetric or fluorescent products (11). We used a tetramer of the DST element because previous experiments had indicated that a greater decrease in reporter mRNA abundance was achieved relative to a dimer (12). This was beneficial because it provided a greater range of potential elevated mRNA abundance mutant phenotypes. p1493 lacks the DST sequence and p1493-2 plants served as a non-destabilized control throughout our experiments (Figure 1-2-1-A). In addition to the *HPH* and *GUS* reporter genes, the T-DNA contained the *nptII* (neomycin phosphotransferase) cassette so that kanamycin resistant transgenic plants could be selected. Transgenic line

p1519-31 was chosen for mutagenesis because segregation of kanamycin resistance and Southern blot analysis indicated that it harbored a single insert of the T-DNA (13). In addition, the abundance of the *HPH-DST* and *GUS-DST* messages in this line reflected the average abundance of these transcripts in a population of p1519 transformants (13). The location of the T-DNA insertion in p1519-31 was mapped to chromosome II near position 51 (14).

The DST tetramer resulted in an approximately 10 fold decrease in steady-state *HPH* mRNA abundance in plants harboring p1519 compared to those transformed with p1493 (13). The half-life of the *HPH* transcript was reduced by approximately 3 fold in p1519-31 plants relative to p1493-2 as measured in an actinomycinD time-course conducted on 12-day-old seedlings grown in liquid culture (Figure 1-2-1-B and 1-C). The disparity between the effect of the DST sequence on the steady-state abundance and the half-life of the *HPH* transcript (10 fold vs. 3 fold) is most likely due to dampening that is commonly observed when general inhibitors of transcription are used to measure mRNA decay rates (15).

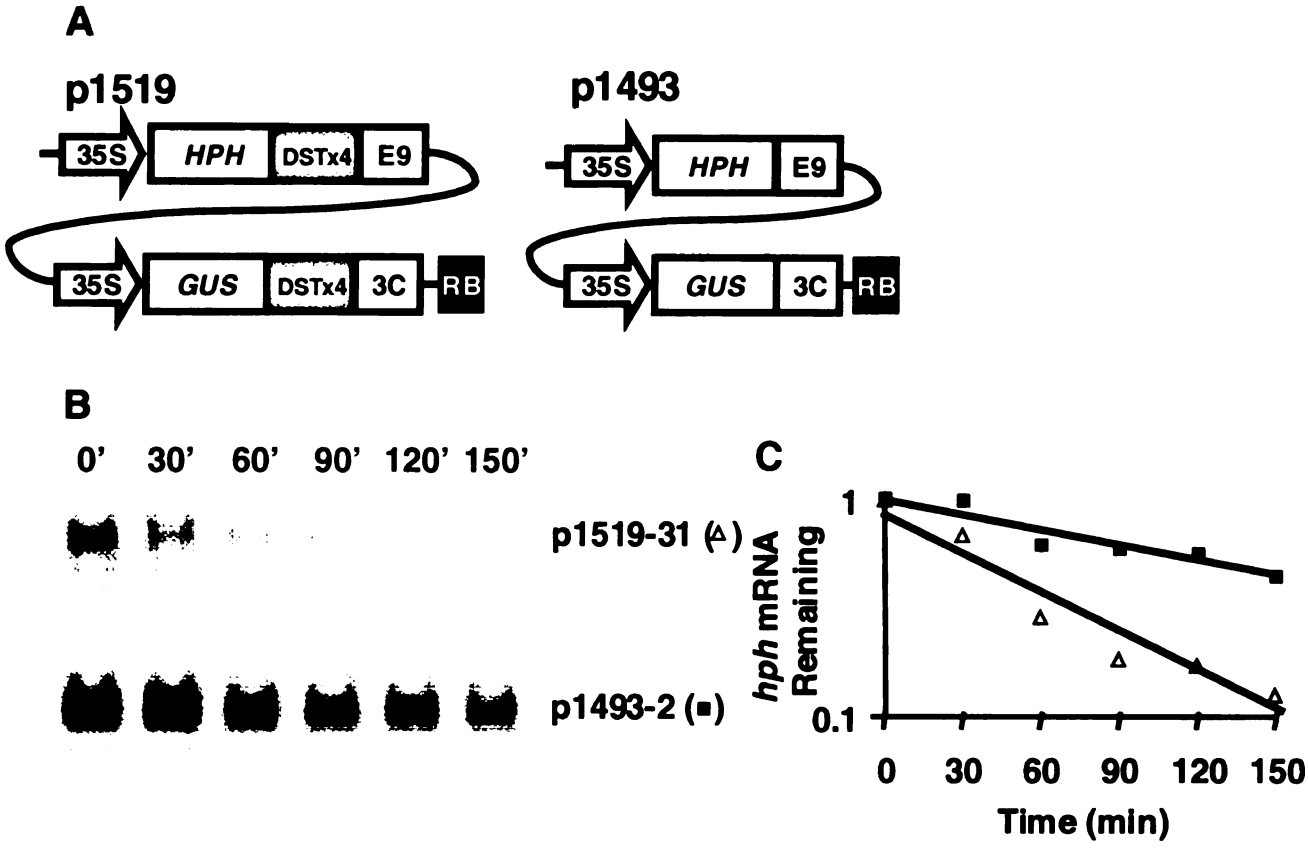

Figure 1-2-1. Structure of transgenes and kinetics of degradation of reporter transcripts used for *dst* mutant selection. (A) Diagrams representing the plant transformation vectors, p1519 and p1493 are shown. p1519 was used to generate transgenic *Arabidopsis* plants that served as the parental line for the *dst* mutant selection. The cauliflower mosaic virus 35S promoter was used to control the expression of the *HPH* and *GUS* genes (28). Polyadenylation signals were derived from the 3' ends of the pea ribulose 1,5-bisphosphate carboxylase small subunit E9 (*rbcS-E9,* E9) and *rbcS-3C* (3C) genes. The *HPH* and *GUS* transcripts encoded by p1519 have been destabilized by a tetramer of the DST sequence (DSTx4). p1493 was used to generate transgenic plants that served as a non-destabilized control during the mutant selection and in subsequent experiments. (B) Comparison of the stability of *HPH* transcripts in parental seedlings (p1519-31) and non-destabilized, control seedlings (p1493-2). Seedlings were grown in liquid culture for 12 days. ActinomycinD (75 μg/ml) was added, samples were removed from the cultures, and frozen in liquid nitrogen at 30 minute intervals. Northern blot analysis was performed with 10 μg of total RNA extracted from each sample (29). The blot was hybridized with a radiolabeled probe complementary to the *HPH* transcript. The blot displaying the p1519-31 time course has been overexposed relative to the p1493-2 blot so that the signal at all time points would be visible. (C) Signals obtained from Northern blots were quantitated by PhosphorImager (Molecular Dynamics) and plotted on a semi-log scale. Linear regression analysis is shown for each set of points.

Destabilization of the *HPH* transcript by the DST element resulted in reduced resistance to hygromycin in p1519-31 seedlings relative to p1493-2 seedlings, particularly at higher concentrations of the antibiotic (Figure 1-2-2-A). We hypothesized that mutants defective in DST-mediated mRNA degradation would have increased abundance of the *HPH-DST* transcript and would therefore have increased resistance to hygromycin relative to the parental line. A preconstruction of the mutant selection revealed that seedlings expressing the non-destabilized *HPH* message could be easily identified among a lawn of seedlings expressing the destabilized *HPH* transcript (Figure 1-2-2-B). Because we were interested in mutations affecting the DST pathway in *trans*, our goal was to identify mutants with increases in both *HPH-DST* and *GUS-DST* mRNA abundance.

p1519-31 seeds were subjected to ethylmethane sulfonate (EMS) mutagenesis, sown on soil and the M_1 plants were allowed to self-fertilize (Figure 1-2-2-C) (16). M_2 seeds were collected from groups of M_1 plants and were plated on seed selection media containing 1000 μg/ml hygromycin (17). Out of ~730,000 M_2 seeds that were plated, 323 seedlings that appeared to exhibit increased resistance to hygromycin were transferred to soil for propagation. By plating M_3 progeny from these plants on selective media, we found that approximately 20% of the original putative mutants displayed heritable hygromycin resistance. In most of these lines, resistance was not attributed to an increase in *HPH-DST* mRNA abundance. Three mutants, selected from independent M_1 groups, showed a heritable increase in *HPH-DST* mRNA abundance; these three also showed an increase in *GUS-DST* mRNA abundance. Two of these mutants, *dst1* and *dst2* that were isolated first, have been characterized in greater detail.

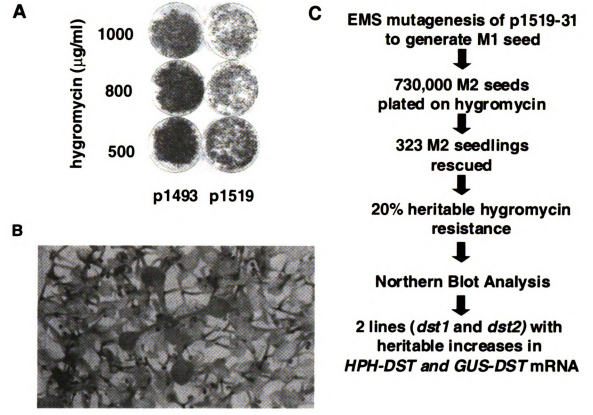

Figure 1-2-2. The *dst* selection strategy. (A) A titration of hygromycin resistance was performed on p1493-2 and p1519-31 seedlings. Approximately 500 seeds were surface sterilized and plated on primary seed selection media containing 500, 800, or 1000 µg/ml hygromycin (17). (B) In order to preconstruct the mutant selection, five p1493-2 seeds were mixed with 500 p1519-31 seeds and plated on primary seed selection media containing 1000 µg/ml hygromycin. All five resistant seedlings were recovered, one of which is shown. (C) Steps in the *dst* selection.

The abundance of the *HPH-DST* and *GUS-DST* transcripts was 3 to 5 fold higher in *dst1* and *dst2* compared to p1519-31 plants (Figure 1-2-3-A). In contrast, the abundance of the *eIF4-A* transcript, used as a non DST-containing control, was equivalent in mutants and p1519-31 (Figure 1-2-3-B). In addition, two other mRNAs, encoding potential messenger ribonucleases, that do not contain DST elements were equally abundant in p1519-31 and the mutants (13). Increased *HPH-DST* and *GUS-DST* mRNA abundance is a consistent phenotype that has been observed in the progeny of all backcrosses to p1519-31 (13).

Because both the *HPH-DST* and *GUS-DST* transcripts were elevated in these mutants, it was highly unlikely that the phenotype was due to a mutation in one or more of the DST elements present in the 3'UTR of the *HPH* transcript. To examine this possibility directly, the DST tetramer was amplified by polymerase chain reaction from genomic DNA extracted from *dst1* and *dst2* and was sequenced (18). The DST tetramers in both mutants were found to be unaltered.

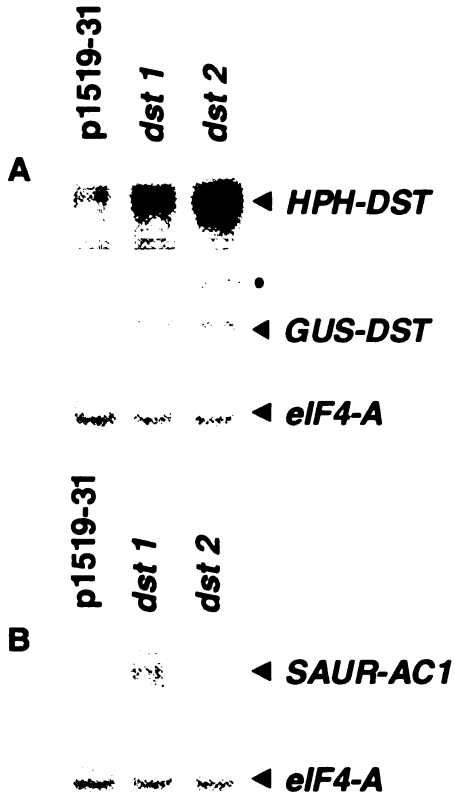

Figure 1-2-3. mRNA abundance of selected transcripts in *dst1* and *dst2*. Total RNA was prepared from leaves of 60-day-old plants and analyzed by Northern blot hybridization (29). *dst1* and *dst2* plants derived from the first backcross to p1519-31 were used as the source of material in this experiment. Radiolabeled probes were prepared against the indicated transcripts and were used in sequential hybridizations of the same Northern blot. (A) The abundance of DST-containing reporter transcripts, *HPH-DST* and *GUS-DST*, are elevated in *dst1* and *dst2*. The higher molecular weight *GUS* transcript (•) is a consistent characteristic of p1519-31. It is most likely the result of a partial recognition of a cryptic downstream polyadenylation signal that is activated by incomplete integration of the T-DNA. We have measured the abundance of the lower molecular weight band (the expected size) throughout these experiments, although the two bands are coordinately regulated in the mutants. (B) The abundance of an endogenous DST-containing message, *SAUR-AC1*, is elevated in *dst1* and *dst2*, whereas the abundance of the *eIF4A* (27) transcript, which does not contain a DST element, is equivalent in the mutants and p1519-31.

Genetic analysis indicated that *dst1* and *dst2* are partially dominant mutations in independent single genes. To arrive at this conclusion, we examined the inheritance of the *dst* mutations by measuring *HPH-DST* mRNA abundance in the rosette leaves of mature

plants derived from crosses of *dst1* and *dst2* to p1519-31 by Northern blot hybridization. We used Northern blots rather than relying on reporter protein activity because direct measurements were much more sensitive to the relatively small differences in mRNA abundance observed in the mutants. Progeny of the second backcross to p1519-31 (F_1) and the progeny of self-fertilizations of these plants (F_2) were used in these studies. Ten F_1 plants were examined for each mutant. *HPH-DST* mRNA abundance was on average 2.1 ± 0.22 fold higher in *dst1* F_1 and 2.8 ± 0.23 fold higher in *dst2* F_1 plants compared to p1519-31 (19). These levels of *HPH-DST* mRNA abundance are intermediate between p1519-31 and mutant levels as would be expected if heterozygotes showed a partial mutant phenotype. Segregation of increased *HPH-DST* mRNA abundance in the F_2 populations was also consistent with partial dominance. Of 46 F_2 plants segregating *dst1*, nine were observed with high *HPH-DST* mRNA abundance (3.1 ± 0.17 fold p1519-31), 23 had intermediate *HPH-DST* mRNA abundance (1.7 ± 0.07) and 14 showed *HPH-DST* mRNA abundance that was similar to p1519-31 (0.9 ± 0.04). Segregation of the *dst2* mutant phenotype was similar to that of *dst1*. Of 53 F_2 plants analyzed, 13 fell into the mutant class (4.0 ± 0.16), 28 fell into the intermediate class (2.2 ± 0.11), and 12 were similar to p1519-31 (1.0 ± 0.04). Segregation in *dst1* and *dst2* F_2 populations was most easily explained by a ratio of 1:2:1 (wild-type(wt):intermediate:mutant) as would be expected for inheritance of a partially dominant single gene (*dst1* $\chi^2 = 1.1$; *dst2* $\chi^2 = 0.2$; $p > 0.5$).

Crossing *dst1* with *dst2* yielded F_1 plants with intermediate *HPH-DST* mRNA abundance, indicating that *dst1* and *dst2* are not allelic. When the F_2 plants from this cross were analyzed, 10/51 showed *HPH-DST* mRNA abundance similar to p1519-31. Had the *dst* mutations been in the same gene, or if they were tightly linked, we would not have

expected to observe any F_2 plants with *HPH-DST* mRNA abundance similar to p1519-31. No class of F_2 plants was observed with an additive effect on *HPH-DST* mRNA abundance, as might be expected if *dst1* and *dst2* function independently to destabilize DST-containing messages (20). In addition, the finding that F_1 plants from the *dst1* x *dst2* cross showed an intermediate mRNA abundance phenotype indicated a lack of additive interaction between these loci because the individual mutants crossed to p1519-31 showed the same phenotype in the F_1.

In order to begin the characterization of the molecular basis of these mutant phenotypes, the *dst1* locus was mapped using molecular markers. This analysis showed that *dst1* is located near position 68 on chromosome 5 (14).

If *dst1* and *dst2* are indeed mutations that disrupt the DST-mediated mRNA degradation pathway *in trans*, one would expect the abundance of endogenous DST-containing messages to be elevated as well. We analyzed the abundance of the *SAUR-AC1* transcript, the only DST-containing message in Arabidopsis that has been examined at the level of mRNA stability (3). The abundance of this message is elevated in *dst1* and *dst2*, indicating that these mutations may elevate the abundance of any message destabilized by DST sequences (Figure 1-2-3-B). The abundance of messages that lack DST sequences was unchanged in these mutants; for example, the abundance of the *eIF4-A* transcript is equivalent in the mutants and p1519-31 (Figure 1-2-3-B). In addition, initial DNA microarray experiments indicated, as expected, that most transcripts were not elevated in *dst1* compared to p1519-31; however, a new *SAUR* mRNA, containing a potential DST element, was among those that were more abundant in *dst1* (21). Taken together, these

observations suggest that the *dst* mutations specifically affect the abundance of transcripts containing DST instability sequences.

The simplest explanation for the elevation of DST-containing transcripts in *dst1* and *dst2* is a defect that results in a decreased rate of DST-mediated mRNA degradation. This is likely because the DST sequence is the only regulatory element shared by the four transcripts that were elevated in the mutants. To test this hypothesis, we measured the kinetics of *HPH-DST* mRNA degradation in p1519-31, *dst1*, and *dst2* plants. We anticipated that the measurable differences in *HPH-DST* mRNA decay rates would be minimal because the difference in *HPH-DST* abundance between *dst1* or *dst2* and p1519-31 was only about three fold (Figure 1-2-3-A) and such differences are often dampened by time course analysis using general transcription inhibitors, as discussed above (15). Figure 1-2-4-A shows representative Northern blots from these experiments with the average, normalized *HPH-DST* mRNA abundance at each time point plotted on a semi-log scale in Figure 1-2-4-B. The average half-life of the *HPH-DST* transcript was found to be 24 minutes for *dst1*, 23 minutes for *dst2*, and 18 minutes for p1519-31 (Figure 1-2-4-B). The differences between mutant and p1519-31 mRNA decay curves are particularly evident at later time points (Figure 1-2-4-B). We conclude that *dst1* and *dst2* cause an increase in message stability that results in increased abundance of DST-containing mRNAs.

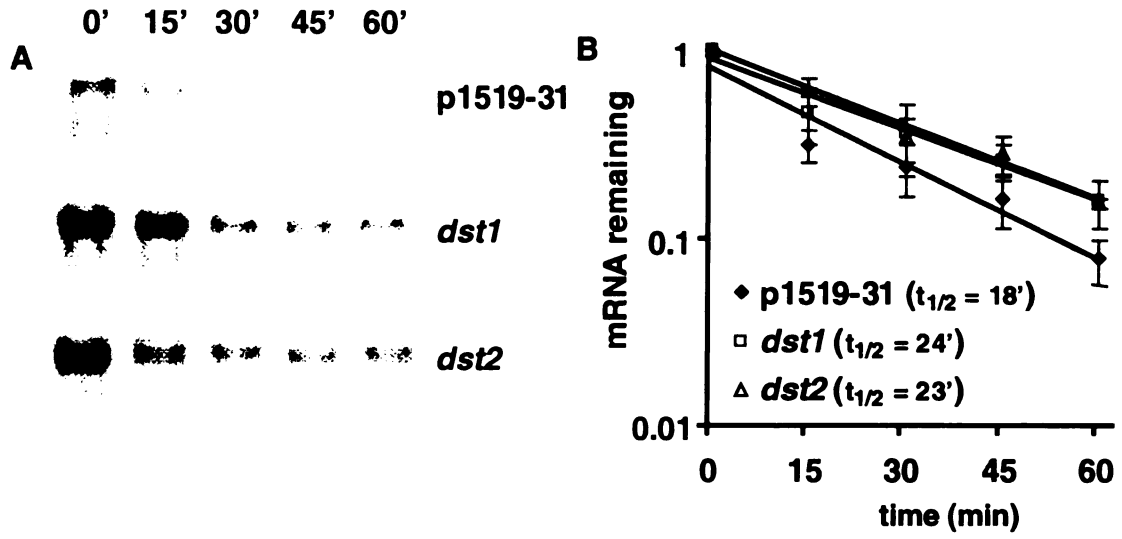

Figure 1-2-4. Analysis of *HPH-DST* mRNA stability in leaves from p1519-31, *dst1*, and *dst2* plants. Rosette leaves from 12 F_3 plants derived from the second backcross of *dst1* and *dst2* to p1519-31 were harvested. p1519-31 rosette leaves were harvested from 12 plants. Leaves were incubated in buffer for 30 minutes before addition of 150 µg/ml cordycepin (30). Four leaves were harvested every 15 minutes for 60 minutes and frozen in liquid nitrogen. Total RNA was extracted from each sample and analyzed by Northern blot hybridization (29). Blots were hybridized with a radiolabeled probe complementary to the *HPH-DST* transcript and radioactive signals were quantititated by PhosphorImager (Molecular Dynamics). *HPH-DST* signals were normalized by rehybridizing the blots with a probe complementary to the *eIF4A* transcript (27). (A) Representative Northern blots showing the *HPH-DST* signal over the 60-minute time-course in p1519-31, *dst1*, and *dst2*. (B) Average normalized *HPH-DST* signals (± standard error of the mean) from the three time-courses are plotted on a semi-log scale. The average half-life of the *HPH-DST* transcript is given on the graph. Results are expressed as the relative amount of *HPH-DST* mRNA remaining at each time point after normalization with the *eIF4A* signal compared to the first time point.

Neither of the *dst* mutants had any obvious defects in development or morphology. Because expression of an auxin responsive gene, *SAUR-AC1*, was altered in these mutants, we analyzed phenotypes associated with this hormone. *dst1* and *dst2* displayed normal root gravitropism as indicated by the wavy root assay or by rotating vertical plates following germination of seedlings (22). Additionally, there was no loss or enhancement of apical

dominance in these plants, nor were there alterations in root length or morphology, other phenotypes that have been associated with auxin response mutants (23). The *SAUR* gene family is large in Arabidopsis and, interestingly, there are several family members that are highly similar in the coding region to *SAUR-AC1* yet lack DST elements (24). Therefore, it is not surprising that a modest increase in mRNA abundance of one or a few of these genes does not cause any obvious altered phenotypes. Although the number of endogenous targets of the DST-mediated decay pathway is unknown, the *dst* mutants should be powerful tools to address this question. For example, DNA microarray analysis should allow additional targets of the pathway to be identified by revealing mRNAs that accumulate preferentially in either *dst1* or *dst2*.

The *dst* mutant selection had two interesting characteristics. First, these mutants were extremely rare having been isolated at a frequency of less than 1/200,000. In Arabidopsis, null mutations in nonessential genes are typically expected at frequencies of ~1/2000 in EMS populations (25). Second, the *dst* mutations did not fully restore the abundance of the *HPH* or *GUS* transcripts to the levels found in plants with non-destabilized transcripts. These observations indicate that *dst1* and *dst2* may be relatively weak alleles that allow partial function of the DST-mediated mRNA decay pathway. If proper regulation of DST-containing mRNAs is an essential function for Arabidopsis then this may have precluded the isolation of stronger alleles. Alternatively, functional redundancy among components that recognize instability determinants may also contribute to the low frequency of sequence-specific decay mutants. Perhaps it is by necessity rather than coincidence that both *dst* mutants are partially dominant. Whether the corresponding genes encode DST-binding proteins, DST-specific ribonucleases or other effectors,

dominant negative interactions between mutant derivatives and redundant proteins or other components of the decay machinery are easy to imagine. Similar obstacles may explain why successful selections for sequence-specific mRNA decay mutants have yet to be reported in other eukaryotic systems.

Based on our work, selections for mutants defective in rapid mRNA degradation mediated by specific sequence elements should be feasible in other eukaryotic model organisms if selectable marker genes that facilitate detection of small expression differences can be engineered. The potential impact of this approach is demonstrated by a series of very informative mutants of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* that are defective in a general mRNA decay pathway responsible for the degradation of transcripts with premature stop codons (26). Finally, by providing the means to clone *dst* genes, this work opens a new avenue to elucidate the molecular machinery responsible for rapid sequence-specific mRNA degradation in plants and will likely provide novel information applicable to other eukaryotes.

REFERENCES AND NOTES

1. B. A. McClure and T. J. Guilfoyle, *Science* **243**, 91(1989); C. S. Brown, M. A. Gee, T. J. Guilfoyle, *Plant Cell* **1**, 229 (1989); A. R. Franco, M. A. Gee, T. J. Guilfoyle, *J.Biol.Chem.* **265**, 15845 (1990); B. A. McClure, G. Hagen,; K. T. Yamamoto, H. Mori, H. Imaseki, *Plant Cell Physiol.* **33**, 93 (1992); P. Gil et al., *Plant Physiol.* **104**, 777 (1994).

2. T. C. Newman, M. Ohme-Takagi, C. B. Taylor, P. J. Green, *Plant Cell* **5**, 701 (1993).

3. P. Gil and P. J. Green, *EMBO J.* **15**, 1678 (1996).

4. M. L. Sullivan and P. J. Green, *RNA* **2**, 308 (1996).

5. G. Caponigro and R. Parker, *Microbiol.Rev.* **60**, 233 (1996).

6. R. G. Jain, L. G. Andrews, K. M. McGowan, P. H. Pekala, J. D. Keene, *Mol.Cell.Biol.* **17**, 954 (1997); X. C. Fan and J. A. Steitz, *EMBO J.* **17**, 3448 (1998); S. S. Peng, C. Y. Chen, N. Xu, A. B. Shyu, *EMBO J.* **17**, 3461 (1998).

7. P. Loflin, C. Y. A. Chen, A. B. Shyu, *Genes and Development* **13**, 1884 (1999); P. R. Bohjanen, B. Petryniak, C. H. June, C. B. Thompson, T. Lindsten, *Mol.Cell Biol.* **11**, 3288 (1991).

8. W. S. Lai et al., *Mol. Cell. Biol.* **19**, 4311 (1999); E. Carballo, W. S. Lai, P. J. Blackshear, *Science*, **281**, 1001 (1998); G. A. Taylor et al., *Immunity*, **4**, 445 (1996).

9. C.-Y. A. Chen and A.-B. Shyu, *Trends Biochem.Sci.* **20**, 465 (1995).

10. Constructs were introduced into Arabidopsis using the vacuum infiltration method of *Agrobacterium tumefaciens* mediated transformation [N. Bechtold, J. Ellis, G. Pelletier, *C.R.Acad.Sci.Paris* **316**, 1194 (1993) and Web site: http://www.bch.msu.edu/pamgreen/vac.htm]. To avoid any hygromycin resistant contamination of p1519-31 during large-scale selection experiments, this construct was introduced into a mutant that lacks trichomes (Col-PRL, *gl1*). p1493 and other constructs that confer hygromycin resistance were introduced into wt Columbia plants (Col-0).

11. L. Gritz and J. Davies, *Gene* **25**, 179 (1983); R. A. Jefferson, T. A. Kavanagh, M. A. Bevan, *EMBO J.* **6**, 3901 (1987).

12. M. L. Sullivan, D. M. Thompson, P. J. Green, unpublished data.

13. M. A. Johnson and P. J. Green, unpublished data.

14. Mapping of the p1519-31 transgene locus and the *dst1* locus were carried out using SSLP markers [C. J. Bell and J. R. Ecker, Genomics **19**, 137 (1994) and at the Web site

http://genome.bio.upenn.edu/SSLP_info/SSLP.html]. F3 families derived from a cross between p1519-31 and Landsberg erecta (*gl1-1*) [M. Koorneef, L.W. Dellaert, J.H. van der Veen, *Mutation Research/*, **93,** 109 (1982)] were scored for kanamycin resistance. 15 families were identified that showed 100% kanamycin resistance. These were used to map the transgene locus and were scored for 7 markers spread across the genome. Linkage was detected only to markers on chromosome II, the closest marker being nga1126 (position 51, 0 recombinants out of 30 chromosomes analyzed). To map the *dst1* locus, leaves of F_2 plants from a cross of *dst1* with Landsberg erecta (*gl1-1*) were scored for 3 fold or higher *HPH-DST* mRNA abundance compared to p1519-31 by Northern blot analysis (19). 30 F_2 plants were identified and scored for 18 markers spread across the genome. Linkage was again detected with nga1126 (0 recombinants out of 16 chromosomes analyzed) due to the necessity of the p1519 transgene for scoring the mutant phenotype. In addition, linkage was detected to nga139 (map position 51, 2 recombinants out of 56 chromosomes analyzed) and nga76 (map position 68, 0 recombinants out of 56 chromosomes analyzed), defining the location of *dst1* on chromosome 5.

15. A.-B. Shyu, M. E. Greenberg, J. G. Belasco, *Genes Dev.* **3,** 60 (1989); J. G. Belasco and G. Brawerman, in *Control of messenger RNA stability*, J. Belasco and G. Brawerman, Eds. (Academic Press, San Diego,1993), ch. 18; S. Zhang, J. Sheng, Y. Liu, M. C. Mehdy, *Plant Cell* **5,** 1089 (1993); G. Caponigro and R. Parker, *Microbiol.Rev.* **60,** 233 (1996); H. Holtorf, H. Schöb, C. Kunz, R. Waldvogel, F. Meins, Jr., *Plant Cell* **11,** 471 (1999).

16. Seeds homozygous at the p1519-31 locus (fourth generation of self-fertilized progeny following transformation) were soaked for 16 hours in a 0.3% (v/v, water) solution of EMS. Following mutagenesis, seeds were washed extensively with water over a 12 hour period and then sown on soil. The effectiveness of EMS mutagenesis was evaluated by scoring the occurrence of albinism and embryo lethality in M_1 plants as described in G. P. Redei and C. Koncz, in *Methods in Arabidopsis Research*, C. Koncz, N.-H. Chua, J. Shell, Eds. (World Scientific Publishing, Singapore, 1992) pp.16-82.

17. Seed selection media consisted of 4.3 g/L Murashige and Skoog salt mixture (GibcoBRL), B5 vitamin mixture (100 mg/l *myo*-inositol, 10 mg/l thiamine hydrochloride, 1 mg/l nicotinic acid, 1 mg/l pyriodoxine), 1% sucrose, 0.5 g/L MES at pH 5.7, 0.8% phytagar, 1000μg/ml hygromycin and 50 μg/ml kanamycin.

18. Genomic DNA was prepared from *dst1* and *dst2* F_2 plants from the first backcross to p1519-31 as described in M. A. Saghai-Maroof, K. M. Soliman, R. A. Jorgensen, R. W. Allard, *Proc. Natl. Acad. Sci.* **81,** 8014 (1984). PCR primers complementary to the 3' end of the *HPH* coding region and the 5' end of the E9 3'UTR were used to amplify the DST elements with a polymerase with proof-reading capability (Pfu, Stratagene) under standard PCR cycling conditions. Products were ligated into a derivative of pBluescript SKII(-) and multiple individual clones were sequenced for each reaction.

19. Signals from Northern blots were quantitated with a PhosphorImager (Molecular Dynamics). The *HPH-DST* signal was normalized using the *eIF4A* (27) signal. p1519-31 samples were analyzed in parallel and the average fold increase of segregants compared to p1519-31 *HPH-DST* mRNA abundance is reported ± the standard error of the mean. RNA was extracted from two rosette leaves of individual plants as described by T. C. Verwoerd, B. M. Dekker, A. Hoekema, *Nucleic Acids Res* **17**, 2362 (1989). Northern blots were processed as described previously (2).

20. In the F_2 of a cross between two partially dominant mutations with no additive effect on one another, such as is proposed here, one would expect a ratio of 1:8:7 (wt:Intermediate:Mutant) or 1:8:6 if double mutants are not viable. We observed 10 wt, 26 intermediate, and 15 mutant plants out of 51 F_2 plants that were tested. This gives a ratio of ~3:8:5, which does not precisely fit simple models of inheritance. Because it is clear that *dst1* and *dst2* are partially dominant mutations in single genes, this may indicate subtle interactions between these loci. For example, *dst1* and *dst2* may partially suppress one another, explaining the higher than expected proportion of wt segregants.

21. M. A. Pérez-Amador, M. A. Johnson, J. Landgraf, E. Wisman, P. J. Green, unpublished data.

22. C. J. Bell and E. P. Maher, *MGG* **220**, 289 (1990); K. Okada and Y. Shimura, *Aust.J.Plant Physiol.* **19**, 439 (1992).

23. W. Boerjan et al., *Plant Cell* **7**, 1405 (1995); L. Hobbie and M. Estelle, *Plant Cell Environ.* **17**, 525 (1994); H. M. O. Leyser, F. B. Pickett, S. Dharmasiri, M. Estelle, *Plant J.* **10**, 403 (1996).

24. T. J. Guilfoyle, personal communication; M. A. Johnson and P. J. Green, unpublished sequence database search results.

25. G. Haughn and C. R. Somerville, in *Agricultural Chemistry*, H. M. LeBaron, R. O. Mumma, R. C. Honeycutt, J. H. Duesing, Eds. (American Chemical Society, Washington, D.C., 1987) p. 98.

26. P. Leeds, S. W. Peltz, A. Jacobson, M. R. Culbertson, *Genes Dev.* **5**, 2303 (1991); R. Pulak and P. Anderson, *Genes Dev.* **7**, 1885 (1993); Y. Cui, K. W. Hagan, S. Zhang, S. W. Peltz, *Genes Dev.* **9**, 423 (1995); F. He and A. Jacobson, *Genes Dev.* **9**, 437 (1995); B. S. Lee and M. R. Culbertson, *Proc.Natl.Acad.Sci.USA* **92**, 10354 (1995); B. M. Cali, S. L. Kuchma, J. Latham, P. Anderson, *Genetics* **151**, 605 (1999).

27. The eukaryotic translation initiation factor 4A (*eIF4-A*) gene was cloned from Arabidopsis by C. B. Taylor, P. A. Bariola, S. B. DelCardayré, R. T. Raines, P. J. Green, *Proc.Natl.Acad.Sci.USA* **90**, 5118 (1993).

28. p1519 and p1493 are derivatives of pMON505-70 [R.-X. Fang, F. Nagy, S. Sivasubramaniam, N.-H. Chua, *Plant Cell* **1**, 141 (1989)]. The *HPH* coding region was obtained as a BamHI fragment of pLG90 [L. Gritz and J. Davies, *Gene* **25**, 179 (1983)].

Use of the 35S promoter, polyadenylation signals from the 3' ends of the E9 and 3C genes, and synthesis and insertion of the DST tetramer were as described previously (2).

29. RNA extraction and Northern blot analysis was performed essentially as described by T. C. Newman, M. Ohme-Takagi, C. B. Taylor, P. J. Green, *Plant Cell* **5**, 701 (1993).

30. Cordycepin (150 μg/ml) was used to inhibit transcription in mature leaves because actinomycinD was less effective in this tissue (13). This protocol, including the leaf incubation medium (1mM Pipes, pH 6.26; 1mM sodium citrate; 1mM KCl; 15 mM sucrose) was adapted from previous reports [K. A. Seeley, D. H. Byrne, J. T. Colbert, *Plant Cell* **4**, 29 (1992); H. Holtorf, H. Schöb, C. Kunz, R. Waldvogel, F. Meins, Jr., *Plant Cell* **11**, 471 (1999)].

31. We thank Dr. Ambro van Hoof, Dr. Michael Thomashow and James Kastenmayer for reading the manuscript and Dr. Joanne Chory for providing pLG90. We also thank Dr. Michael Sullivan and Debrah Thompson for construction of p1519 and p1493; and Jonathan Vogel and Linda Danhof for technical assistance. This work was funded by grants from the United States Department of Energy, the United States Department of Agriculture, and the McKnight Foundation to P.J.G. M.A. P-A. received postdoctoral fellowships from NATO-Spain and from the Ministerio de Educación y Ciencia, Spain.

CHAPTER 1-3

ANALYSIS OF THE DST ELEMENT AND DST-LIKE SUBDOMAINS WITHIN THE *SAUR-AC1* 3' UNTRANSLATED REGION

INTRODUCTION

The DST element is perhaps the best-studied instability determinant that is plant-specific. The demonstration that this sequence is sufficient to destabilize messenger RNA (mRNA) came when Newman et al. showed that a dimer of the element, when inserted into the 3' untranslated region (UTR), targeted otherwise stable reporter RNAs for rapid turnover (1993). The DST element was originally found in the 3' UTR of the unstable small-auxin-up-RNA (*SAUR*) genes and has been found in the 3'UTR of several *SAURs* from diverse plant species (McClure et al., 1989; Yamamoto et al., 1992; Gil et al., 1994). The element consists of three conserved subdomains separated by two variable regions. These subdomains are referred to as GGA, ATAGAT, and GTA after the DNA sequence that comprises the core of each conserved region. Sullivan and Green showed that substitution mutations in either the ATAGAT or GTA subdomains abolished the effect of the DST dimer (1996), demonstrating the necessity of these sequences for DST function.

The experiments described above explored the function of a synthetic DST sequence derived from the soybean *SAUR-15A* gene (McClure et al., 1989). In order to study a DST element in its native context, Gil and Green cloned the *SAUR-AC1* gene from Arabidopsis (Gil et al, 1994). The 3'UTR of this gene is sufficient to target reporter transcripts for rapid turnover, reducing the half-life of the human *β-globin* (*Globin*) transcript to a similar extent as did a dimer of the DST element (Newman et al., 1993; Gil and Green, 1996). While these two experiments are not directly comparable because the half-life measurements were done using different methods, this result raises some

interesting questions because the *SAUR-AC1* 3' UTR contains only one canonical DST element. Previous experiments indicated that a monomer of the DST element was not sufficient to cause rapid turnover of reporter transcripts (Newman et al., 1993) and experiments reported in Chapter 1-1 of this thesis indicate that a DST tetramer is more effective as an instability determinant than a dimer. These results would support the hypothesis that DST elements serve as recognition sites for the mRNA degradation machinery and become more and more effective as additional copies are added, with a minimum of two copies required for function. The function of the *SAUR-AC1* 3'UTR as an instability determinant, which contains one DST element, would appear to contradict this assertion. To test the contribution of the DST element to *SAUR-AC1* 3'UTR instability function, Gil and Green made substitution mutations in the ATAGAT and GTA subdomains (unpublished data). These mutations, tested individually, reduced the abundance of reporter transcripts to the same extent as did the wild type *SAUR-AC1* 3'UTR, indicating that individually, they are not required for *SAUR-AC1* 3'UTR instability function. Interestingly, upon analysis of the sequence of the *SAUR-AC1* 3'UTR upstream of the DST element, several potentially redundant ATAGAT- and GTA-like subdomains were noted. This raised the possibility that perhaps these redundant elements were contributing to the instability function of the *SAUR-AC1* 3'UTR and were providing the additional DST sequences that experiments with synthetic elements had shown to be necessary.

The purpose of the experiments outlined in this chapter was to analyze the contribution of these ATAGAT and GTA-like subdomains and to enhance our understanding of DST function by studying the element in its native context. Two

approaches were taken. First, all DST and DST-like subdomains were replaced with the same substitutions that had been shown to abolish the function of the DST dimer (Sullivan and Green, 1996). In the second approach, the *SAUR-AC1* 3'UTR was divided into three regions and each was tested for instability function. Both of these approaches were somewhat risky because the DST element overlaps polyadenylation signals within the *SAUR-AC1* 3'UTR. We expected that chimeric transcripts might be aberrantly polyadenylated, however, because so little is known about polyadenylation signals in plants, there was no way to know for certain until we analyzed the constructs in transgenic plants. In some cases, this limitation has made the results of these experiments difficult to interpret. However, this study suggests a possible role for upstream redundant ATAGAT and GTA-like subdomains in the instability function of the *SAUR-AC1* 3' UTR and provides some interesting tools for future analysis of DST-mediated mRNA degradation.

RESULTS

Mutagenesis of all DST and DST-like subdomains

The 3'UTR of the *SAUR-AC1* transcript contains one DST element along with other DST-like subdomains that are iterated upstream. In Figure 1-3-1, the DST element, ATAGAT-, and GTA-like subdomains have been highlighted. The ATAGAT subdomain consists of the sequence ATAGAT and the GTA subdomain consists of the sequence

CAATGCGTA. ATAGAT-like subdomains were defined as having one mismatch and GTA-like subdomains were defined as having one or two mismatches.

In order to assess the contribution of the DST element and ATAGAT and GTA-like subdomains to *SAUR-AC1* instability function, all of the residues that are highlighted in Figure 1-3-1 were mutated. ATAGAT and ATAGAT-like subdomains were replaced with GCATGC, changing 5 of the six conserved residues. GTA subdomains (CAATGCGTA) were replaced with CATAGGCCT which changes the GTA residues, conserved in all *SAUR* DST elements, and three residues upstream. Either of these mutations is known to abolish the function of the synthetic DST dimer (Sullivan and Green, 1996).

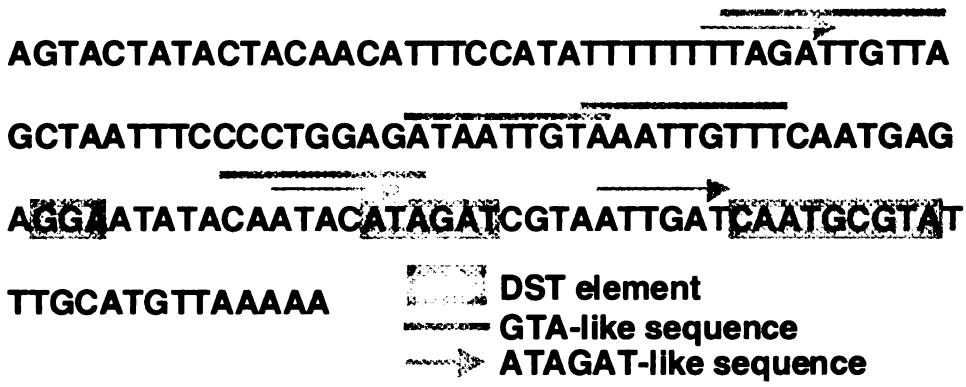

Figure 1-3-1. The *SAUR-AC1* 3'UTR contains one DST-element and DST-like subdomains. The sequence of the *SAUR-AC1* 3'UTR immediately 3' of the stop codon to the beginning of the poly(A) tail is shown (Gil et al., 1994). The DST subdomains ("GGA", "ATAGAT", and "GTA", are highlighted by gray boxes. DST-like subdomains are highlighted by bars and arrows as described in the figure.

The fully mutated *SAUR-AC1* 3' UTR was constructed by polymerase chain reaction using four overlapping oligonucleotides. These mutations altered 29% of the nucleotides in the *SAUR-AC1* 3'UTR, changing the GC content from 29% to 45%. The fully mutated 3'UTR was inserted 3' of the *Globin* coding sequence and the resulting

chimeric gene was introduced into a binary vector containing a *GUS* reference gene so that the effect on message stability could be assessed in transgenic plants (Figure 1-3-2). Control constructs containing the wild type *SAUR-AC1* 3'UTR and the E9 3'UTR, were also generated.

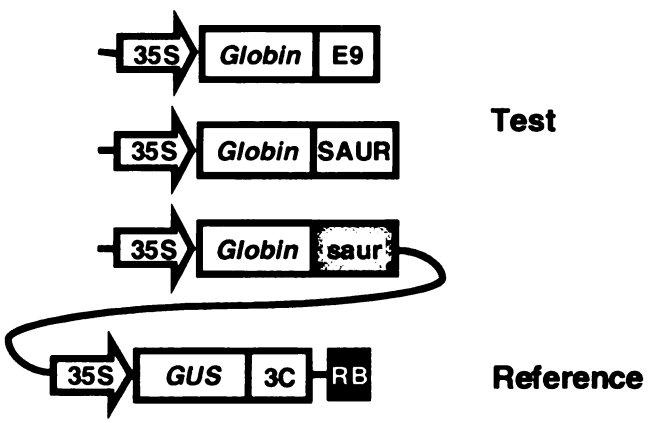

Figure 1-3-2. The structure of chimeric genes used to test mutations in the *SAUR-AC1* 3'UTR. The cauliflower mosaic virus 35S promoter (35S) was used to control the transcription of test and reference genes. The reference gene, β-glucuronidase (GUS), was included in the same binary vector as the test gene to facilitate normalization of mRNA abundance data. The 3'UTRs of the pea ribulose 1,5-bisphosphate carboxylase small subunit E9 (E9) and 3C (3C) genes were used as a negative control (lacking instability activity) and as a 3'UTR for the reference gene, respectively. *SAUR* represents the wild type *SAUR-AC1* 3'UTR, while *saur* (shaded) represents the fully mutated version. The right border (RB) of the transfer-DNA (T-DNA) has been indicated by a black rectangle. The T-DNA also includes the neomycin phosphotransferase cassette so that kanamycin resistant transgenic plants could be selected.

These constructs were introduced into Arabidopsis via the vacuum infiltration method of *Agrobacterium tumefaciens* mediated transformation (Bechtold et al., 1994). Approximately 100 independent, 12-day-old primary transformants (T_1) from each construct were pooled and harvested for Northern blot analysis (Figure 1-3-3).

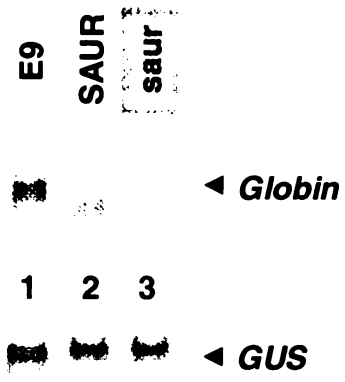

Figure 1-3-3. Mutations in the *SAUR-AC1* 3' UTR affect polyadenylation of the *Globin* reporter transcript. A Northern blot containing 10 μg of total RNA from pools of T_1 seedlings was hybridized sequentially with radiolabelled *Globin* and *GUS* probes. The 3'UTR of each *Globin* reporter is given above each lane (E9, wild type *SAUR-AC1*, fully mutated *SAUR-AC1*).

The top panel of this Northern blot shows the abundance of *Globin* transcripts containing the three different 3'UTRs that were tested. The *Globin-SAUR* transcript was 2.1 fold less abundant than the *Globin-E9* transcript after each signal was normalized with the reference signal (*GUS*) (compare lanes 1 and 2). This difference was less than that observed by Gil and Green (4 fold, 1996), but still indicates that the wild type *SAUR-AC1* 3'UTR functioned as an instability determinant. As one can see on examination of lane 3, the fully mutated *SAUR-AC1* 3'UTR results in larger *Globin* transcripts. This was most likely due to the inadvertent mutation of polyadenylation signals within the *SAUR-AC1* 3'UTR. The larger transcripts were probably produced when downstream polyadenylation sites within the T-DNA were utilized, this will be discussed further below.

T_1 plants were allowed to self-fertilize and the progeny were collected so that T_2 plants could be analyzed. Approximately 100 T_2 seedlings from 10 independent transformants were plated and harvested for Northern blot analysis. As expected, the results with these pools of seedlings were similar to those shown in Figure 1-3-3 (data not shown). Because aberrant transcripts were produced from these constructs, it was not possible to address the contribution of DST and DST-like subdomains to the

accumulation of the *Globin* transcript using this approach. In order to overcome this obstacle, a second approach was undertaken in which the *SAUR-AC1* 3'UTR was divided into sections and each was tested for instability function.

Gain of function analysis of DST and DST-like subdomains

Another way to test the contribution of the DST-like subdomains present in the 3' UTR of *SAUR-AC1* was to test whether they have stand-alone instability function. This approach has been used successfully in our laboratotory to show that the DST element and AUUUA repeats are instability determinants. One simply inserts the sequence to be tested between the coding region of a reporter gene and a 3'UTR that provides polyadenylation signals. The *SAUR-AC1* 3'UTR was divided into three sections that contained no DST-like subdomain (N), redundant DST-like subdomains (R), and the DST element (D). The N region consisted of the first 31 nucleotides of the *SAUR-AC1* 3'UTR, ending at the first arrow shown in Figure 1-3-1. The R region was defined as the next 48 residues, containing one ATAGAT-like subdomain and three GTA-like subdomains, ending five nucleotides upstream of the boxed DST element shown in Figure 1-3-1. The D region comprised the next 52 nucleotides and ended 8 nucleotides downstream of the GTA subdomain shown in Figure 1-3-1. Sections of the 3'UTR were constructed using overlapping oligonucleotides and were inserted between the *Globin* coding region and the E9 polyadenylation signal, as depicted in Figure 1-3-4. The same test and reference gene strategy described above was used to construct binary vectors to be introduced into Arabidopsis.

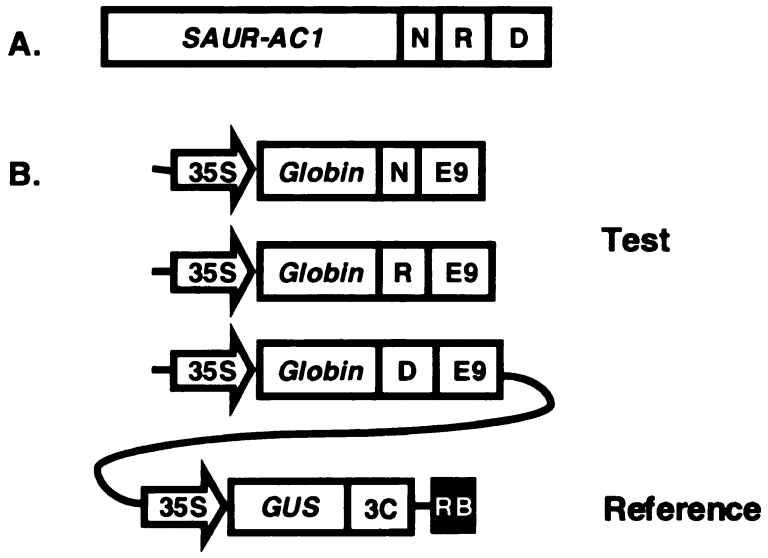

Figure 1-3-4. Structure of chimeric genes used to test individual subdomains within the *SAUR-AC1* 3'UTR for instability function. A. The *SAUR-AC1* gene structure is depicted with the 3'UTR divided into sections called N, R, and D as described in the text. B. Constructs used in this study were derivatives of those described in Figure 1-3-2.

Transgenic plants expressing these constructs were tested for instability function using the same method described above. Figure 1-3-5 shows the results of Northern blot analysis of RNA isolated from pools of T_1 seedlings. Pools of seedlings expressing the *Globin* reporter with the E9 3'UTR (*Globin-E9*) alone were included in the analysis as a negative control. Insertion of the N, R, and D regions resulted in an increase of the *Globin* transcript size that was expected for each (Figure 1-3-5-A). By comparing the ratios of *Globin/GUS* mRNA accumulation presented in Figure 1-3-5-B, it was found that the N region, had almost no effect on message accumulation in transgenic seedlings. In contrast, the D region had a mild effect (71% of E9) and the R region had a large effect on the accumulation of the *Globin* reporter mRNA, reducing the abundance to 37% compared to plants expressing *Globin* with the E9 3'UTR alone.

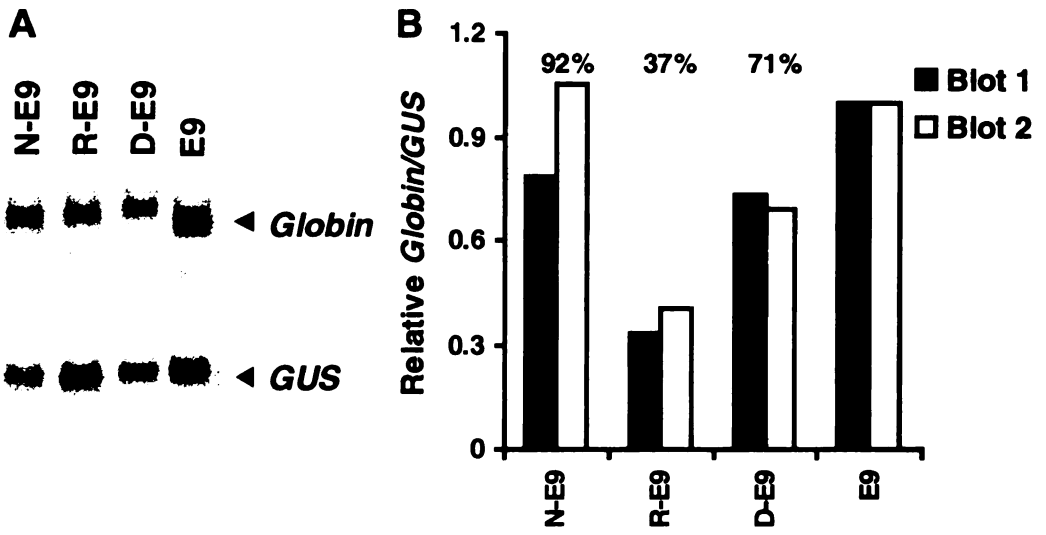

Figure 1-3-5. Analysis of the N, R, and D regions of the *SAUR-AC1* 3' UTR. A. A Northern blot containing 10 μg of total RNA from a pool of T_1 seedlings was hybridized sequentially with radiolabeled *Globin* and *GUS* probes. The 3'UTR of each construct is given above each lane. B. *Globin* and *GUS* transcript abundance was quantitated using a PhosphorImager. *Globin* transcript abundance was normalized using the *GUS* abundance and these values were plotted relative to the E9 control. The values obtained from independent Northern blots are shown. The average *Globin/GUS* ratio from the two blots is given above each bar and is expressed as a percentage of the *Globin-E9* value.

These results pointed to a possible role for the R region in the instability function of the *SAUR-AC1* 3'UTR. In addition, it is interesting to note that the D region was not sufficient to reduce the abundance of the *Globin* reporter. This result is compatible with those of previous experiments that showed that mutating the ATAGAT or GTA subdomains within the DST element had no effect on the function of the *SAUR-AC1* 3'UTR as an instability determinant (P. Gil and P.J. Green, unpublished). These results are also in agreement with the finding that a monomer of the DST element from the soybean *SAUR-15A* gene is insufficient to destabilize *Globin* transcripts (Newman et al., 1993). These observations indicated that perhaps the R and D regions of the *SAUR-AC1* 3'UTR function together to achieve full instability function. In order to test this hypothesis, *Globin* constructs containing combinations of the N,R, and D (NR, RD, NN,

RR, and DD) regions were introduced into Arabidopsis. These constructs had the same structure as those shown in Figure 1-3-4 and were analyzed in the same way, using pools of T_1 seedlings.

The results of this experiment are presented in Figure 1-3-6. When the R region was introduced into the *Globin* transcript as a dimer (RR) or in combination with the D region (RD), smaller transcripts were produced. This is clear in lanes 2 and 4 of Figure 1-3-6-A. These smaller transcripts are most likely the result of cleavage and polyadenylation at sites within the introduced sequences. This conjecture is reasonable considering that the *SAUR-AC1* polyadenylation signals are likely located within the N, R, and D regions. The larger transcripts were thought to be full length and would therefore contain the introduced putative instability sequences and sequences from the E9 3'UTR. In order to verify that the larger transcripts were full-length, the blot shown in Figure 1-3-6-A was stripped and rehybridized with a probe complementary to the E9 3'UTR (Figure 1-3-6-B). Only the larger transcripts were detected with the E9 probe.

Polyadenylation within the introduced dimers of N, R, and D regions confounded analysis of their impact on message stability based on mRNA abundance. The smaller transcripts present in lanes 2 and 4 of Figure 1-3-6-A probably do not contain the entire RD or RR sequences and may therefore be relatively stable following cleavage and polyadenylation. In addition, it is difficult to make conclusions about the instability function of these sequences based solely on the abundance of the full-length-transcript because the abundance of this species is dependent upon the rate of cleavage and polyadenylation at sites within the introduced sequences.

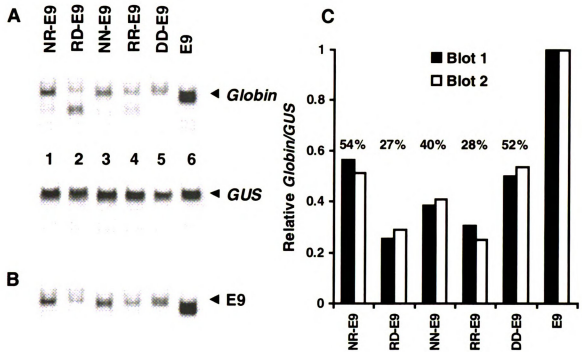

Figure 1-3-6. Analysis of combinations of N, R, and D regions. A. A Northern blot containing 10 μg of total RNA from pools of T_1 seedlings was hybridized sequentially with radiolabeled *Globin* and *GUS* probes. The 3'UTR of each construct is given above each lane. B. The blot was stripped and hybridized with a radiolabeled probe complementary to the E9 3'UTR. C. Full-length *Globin* and *GUS* transcript abundance was quantitated using a PhosphorImager. *Globin* transcript abundance was normalized using the *GUS* abundance and these values were plotted relative to the abundance of the *Globin-E9* control transcript. The values obtained from independent Northern blots are shown. The average *Globin/GUS* ratio from the two blots is given above each bar and is expressed as a percentage of the *Globin-E9* value.

For the sake of discussion, the abundance of each full-length *Globin* transcript was quantitated and is plotted relative to the *Globin-E9* transcript in Figure 1-3-6-C. From these data, it would appear that the R region has a greater effect on *Globin* message abundance when it is paired with the D (RD, 27% of E9) region then when paired with the N region (NR, 54% of E9). However, this may simply reflect the obvious difference in internal polyadenylation between these two constructs. Likewise, the RR dimer had the largest impact on the abundance of the *Globin* message (28% of E9) compared to the

NN dimer (40% of E9) or the DD dimer (52% of E9). Again, these data must be considered with caution because the RR dimer served as a site for internal cleavage and polyadenylation.

DISCUSSION

The *SAUR-AC1* 3'UTR, which contains one DST element and several DST-like subdomains, is a mRNA instability determinant that contributes to the constitutive instability of the *SAUR-AC1* message (Gil and Green, 1996). The experiments described in this chapter were designed to assess the contribution of the DST element and DST-like subdomains to the overall instability function of the *SAUR-AC1* 3'UTR. The goal of this work was to gain a better understanding of how the DST element in particular, and instability determinants in general, function within their native context. Two approaches were taken, one in which all of the DST and DST-like subdomains were mutated and a second where the *SAUR-AC1* 3'UTR was divided into three regions and each was tested for instability function.

Mutating all of the DST and DST-like subdomains apparently abolished the polyadenylation signals within the *SAUR-AC1* 3'UTR and resulted in aberrant, large reporter transcripts when expressed in transgenic plants (Figure 1-3-3). This made it impossible to pursue this approach further. It was expected that these mutations would have resulted in stabilization of the reporter transcript. The function of individual subdomains was to be addressed by adding back wild-type sequences and analyzing the effect on reporter transcript abundance. We were aware of the potential for problems due

to incorrect polyadenylation of these chimeric transcripts, so we devised an alternative strategy.

The second approach was more informative. In these experiments, the *SAUR-AC1* 3'UTR was divided into three regions called N (no DST-like subdomain), R (redundant DST-like subdomains), and D (DST element). These sequences were introduced into the 3'UTR of a *Globin-E9* reporter message and were studied using Northern blot analysis of RNA extracted from transgenic Arabidopsis plants. These experiments indicated that the R region may have instability function. The introduction of this sequence into the *Globin-E9* 3'UTR caused an almost three-fold decrease in message abundance compared to *Globin-E9* (Figure 1-3-5). Interestingly, the R region was more effective at reducing reporter transcript abundance than was the D region. The instability function of a dimer of the DST element derived from the soybean *SAUR-15A* gene is well established (Newman et al., 1993; Sullivan and Green, 1996), however, a monomer of the element is much less effective (Newman et al., 1993). Therefore, the finding that a monomer of the D region of the *SAUR-AC1* 3'UTR did not greatly reduce *Globin* mRNA abundance was perhaps not surprising. Nonetheless, these results raise some intriguing questions: Is a dimer of the DST element necessary because recognition factors that bind this sequence only function as dimers? Do the redundant DST-like subdomains present within the R region of the *SAUR-AC1* 3'UTR serve as an alternative binding site(s) for DST recognition factor(s)? Do the R and D regions function synergistically to achieve instability function?

To begin to address these types of questions, homo- and heterodimers of the N, R, and D regions were analyzed (Figure 1-3-6). One important question was whether RD

would reduce mRNA abundance of the *Globin* reporter to a greater extent then ND. Unfortunately, when the RD region was introduced into the *Globin-E9* 3'UTR, two transcripts were apparent on Northern blots hybridized with *Globin* probes. The simplest explanation for the presence of the smaller band was that the RD sequence had served as an internal polyadenylation signal. This conjecture was supported when only the larger band hybridized to a probe complementary to the E9 3'UTR, located downstream of the RD insertion. A smaller than expected transcript was also produced when a homodimer of the R region was analyzed. This complication was unexpected because the 3' end of the *SAUR-AC1* transcript has been identified and is known to be downstream of the N, R, and D regions (Gil et al., 1994). When the R region was introduced into the *Globin* 3'UTR as a homodimer or as a heterodimer with the D region, unexpected polyadenylation signals may have been created. Plant polyadenylation signals are poorly defined and they generally do not contain the AAUAAA sequence that marks the polyadenylation site in almost all mammalian transcripts (reviewed by Hunt, 1994). Indeed, prediction of polyadenylation signals within individual plant genes seems to be nebulous at best. The lack of sophisticated knowledge of poly(A) site selection in plants makes it difficult to determine if the R region contains partial polyadenylation signals that may have been activated upon dimerization or when it was combined with the D region.

In any case, this complexity precluded analysis of the RD and RR sequences in terms of their instability function by simply assaying mRNA accumulation. The data obtained by analyzing the abundance of the full-length transcript were interesting because they provided support for the hypothesis that the R and D regions function together. The

Globin/GUS ratio was lowest in transgenic plants with the RD and RR sequences inserted into the *Globin* 3'UTR. However, conclusions cannot be drawn from these results because the contribution of internal polyadenylation to the decreased accumulation of the full-length transcript in these plants cannot be estimated.

A method that uses the general transcription inhibitor, cordycepin, to measure mRNA stability in Arabidopsis plants has been developed and is described in Chapter 1-2 of this thesis. This method may now be used to directly measure the effect of the R region on message stability using the transgenic plants generated for this study. The demonstration that the R region results in rapid turnover of the *Globin* reporter would solidify the hypothesis that this region plays an important role in rapid mRNA decay mediated by the *SAUR-AC1* 3'UTR. In addition, if one assumes that transcripts detected on Northern blots are largely mature, cytoplasmic mRNAs, then it may be possible to address the effect of the RD and RR dimers on message stability by conducting half-life analysis on the full-length transcript. These experiments would allow the stability of the full-length transcripts to be addressed without regard for the steady-state abundance, which is probably influenced by internal polyadenylation due to insertion of the RR and RD sequences.

In Chapter 1-2 of this thesis, two mutants defective in DST-mediated mRNA degradation were described (*dst1* and *dst2*). One of the interesting features of these mutants was that they both had elevated *SAUR-AC1* message abundance. Interestingly, this effect was greater in *dst1* compared to *dst2*. The abundance of the hygromycin phosphotransferase (*HPH*) mRNA that was destabilized by a DST tetramer (*HPH-DST*) was also elevated in both mutants, but is higher in *dst2* (Figure 1-2-3). These subtle

differences in the phenotype of these mutants may point to differences in recognition of independent DST and DST-like subdomains. For example, perhaps the *dst1* gene product is involved in the recognition of GTA subdomains and the *dst2* gene product is involved in the recognition of ATAGAT subdomains. The tetramer of the DST element contains an equal ratio of ATAGAT to GTA subdomains, the two subdomains important for DST function (Sullivan and Green, 1996). The proportion of GTA-like subdomains is higher in the *SAUR-AC1* 3'UTR, it contains four ATAGAT-like and five GTA-like subdomains (Figure 1-3-1). Because the *SAUR-AC1* 3' UTR has a higher proportion of GTA-like subdomains and the *dst1* mutant shows hyperelevation of the *SAUR-AC1* transcript relative to *dst2*, one might speculate that the *dst1* gene product is involved in recognition of GTA subdomains. Likewise, if the *dst2* gene product was responsible for recognition of the ATAGAT subdomain, one might expect the defect to be more obvious on the *HPH-DST* message because it has a higher proportion of ATAGAT subdomains.

The R region, implicated in *SAUR-AC1* function by this study, has one ATAGAT-like subdomain and three GTA-like subdomains. If the above speculation is correct, one might expect that the *dst1* mutant would show hyperelevation of the *Globin-R-E9* reporter transcript relative to the *dst2* mutant. Therefore, the *Globin* reporter transcripts and transgenic plants generated for this study might allow us to learn more about the function of the *dst1* and *dst2* gene products by introducing these constructs into the mutant backgrounds and determining whether the mutants differentially recognize independent regions of the *SAUR-AC1* 3'UTR.

Future studies into DST-mediated mRNA degradation will be enhanced by combining the reporter constructs described in this chapter with further analysis of the

mutants described in the previous chapter. It seems probable that recognition of synthetic DST elements and those residing in native transcripts, such as *SAUR-AC1*, is a modular process. It will be fascinating to uncover the cellular factors that comprise recognition modules and to understand how they interact with the basal mRNA degradation machinery to achieve rapid turn over of DST-containing messages.

MATERIALS AND METHODS

Plasmid construction and plant transformation

Standard molecular cloning procedures were used to construct the chimeric genes described in Figure 1-3-2 and Figure 1-3-4 (Sambrook et al., 1989). The fully mutated *SAUR-AC1* 3'UTR (Figure 1-3-2) was synthesized using four oligonucleotides. Two 98 base oligonucleotides that overlapped by 24 nucleotides were synthesized that incorporated all of the mutations in DST and DST-like subdomains. These were annealed and the overhanging ends were filled by 8 cycles of polymerase chain reaction (PCR). The double stranded product of this PCR was then subjected to a second round of PCR (25 cycles) using two smaller primers (24 and 26 bases) complementary to each end. The oligonucleotides were designed such that the 5' end of the final double-stranded product was a BamHI site and the 3' end was a KpnI site. This facilitated introduction of the mutated portion of the *SAUR-AC1* 3'UTR into a version of the entire *SAUR-AC1* 3'UTR that contained a BamHI site four base pairs 3' the stop codon and a KpnI site 15 base pairs 3' of the end of the *SAUR-AC1* transcript. Only one insertion and

one substitution were required to introduce these restriction sites using mutagenic oligonucleotides and the Kunkel method of site-directed mutagenesis (Kunkel et al., 1987). The fully mutated *SAUR-AC1* 3'UTR, and the *SAUR-AC1* 3'UTR with the BamHI and KpnI sites (wt) were introduced into a pUC derivative with the structure: 35S:*Globin*:E9 (p1630) by replacing the E9 3'UTR with the fully mutated *SAUR-AC1* 3'UTR (p1658) or the wt *SAUR-AC1* 3'UTR (p1657). Three test genes 35S:*Globin*:E9, 35S:*Globin*:wt *SAUR* (with BamHI and KpnI sites) and 35S:*Globin*:fully mutated *SAUR* were used in this study. The 35S promoter in these three constructs was a shorter version (-417 to +8; Feldbrügge et al., 1994) than that used by Newman et al., (1993) (–940 to +9) which was used for all of the other constructs.

The N, R, D, NR, RD, NN, RR, and DD regions of the *SAUR-AC1* 3'UTR were constructed by synthesizing overlapping, complementary oligonucleotides with a BglII site on the 5' end and a BamHI site on the 3'end. Oligonucleotides were annealed and introduced at the BamHI site of a pUC derivative containing a test gene with the structure: SacI-35S-BglII-XbaI-*Globin* coding region-BamHI-E9-ClaI (Newman et al., 1993). Homodimers were constructed by screening for clones with two insertions of the annealed oligonucleotides.

Test genes, described above, were transferred to a derivative of pMON505 which is a binary vector that facilitates transformation of plants via *Agrobacterium tumefaciens* (*Agrobacterium*) (Rogers et al., 1987, Fang et al., 1989). The transfer DNA also contains a reference gene with the structure 35S-GUS-3C. This test and reference strategy and the source of the *Globin* and *GUS* coding regions was described by Newman et al., (1993).

Generation and selection of transgenic Arabidopsis plants using the vacuum infiltration method of *Agrobacterium*-mediated transformation has been described (N. Bechtold et al., 1993 and Web site: http://www.bch.msu.edu/pamgreen/vac.htm). *Agrobacterium* strain GV3101 C58C1 Rif^r (pMP90) (Koncz and Schell, 1986) was transformed with pMON505 derivatives by electroporation using the Gene-Pulser (BioRad) according to the manufacturer's guidelines.

Plant material and growth conditions

The Columbia (Col-O) accession of *Arabidopsis thaliana* was used in all transformations. Arabidopsis seedlings were grown on seed selection media which consisted of 4.3 g/L Murashige and Skoog salt mixture (GibcoBRL), B5 vitamin mixture (100 mg/l *myo*-inositol, 10 mg/l thiamine hydrochloride, 1 mg/l nicotinic acid, 1 mg/l pyriodoxine), 1% sucrose, 0.5 g/L MES at pH 5.7, 0.8% phytagar, and 50 μg/ml kanamycin. Plates were kept in incubators set at 16 hours light (125 μE/m^2) /8 hours dark, 21°C. Arabidopsis plants were grown in standard Arabidopsis soil in controlled environment growth chambers set at 16 hours light (125 μE/m^2) /8 hours dark, 21°C.

RNA analysis

RNA was isolated from pooled seedlings using the method described by Puissant and Houdebine (1990) with modifications described by Newman et al., (1993). T_1 pools consisted of ~200 individual seedlings, all of which were likely to be from independent

transformation events (Ye et al., 1999). *T2* pools consisted of ~100 individual seedlings from 9-10 independent transformants. Northern blot analysis was as described in Newman et al. (1993). Hybridization probes corresponding to the *Globin* and *GUS* coding regions were labeled using α-^{32}P dCTP by the random-primed method of Feinberg and Vogelstein (1983). The hybridization probe corresponding to the E9 3'UTR was generated by transcribing the non-coding strand of this sequence in vitro using T3 RNA polymerase in the presence of α-^{32}P UTP, as described by Diehn et al., 1998. Hybridization and washing conditions for the E9 RNA probe were also as described in Diehn et al., 1998.

mRNA abundance was quantitated by measuring the intensity of radioactive bands on Northern blots using a Molecular Dynamics PhosphorImager.

AKNOWLEDGMENTS

I would like to thank Dr. Ambro van Hoof for identifying DST-like subdomains in the *SAUR*-AC1 3'UTR.

REFERENCES

Bechtold,N., Ellis,J., and Pelletier,G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris *316*, 1194-1199.

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.

Fang,R.-X., Nagy,F., Sivasubramaniam,S., and Chua,N.-H. (1989). Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell *1*, 141-150.

Feinberg,A.P. and Vogelstein,B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6-13.

Feldbrügge,M., Sprenger,M., Dinkelbach,M., Yazaki,K., Harter,K., and Weisshaar,B. (1994). Functional analysis of a light-responsive plant bZIP transcriptional regulator. Plant Cell *6*, 1607-1621.

Gil,P., Liu,Y., Orbovic,V., Verkamp,E., Poff,K.L., and Green,P.J. (1994). Characterization of the auxin-inducible *SAUR-AC1* gene for use as a molecular genetic tool in *Arabidopsis* . Plant Physiol. *104*, 777-784.

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.

Hunt,A.G. (1994). Messenger RNA 3' end formation in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. *45*, 47-60.

Koncz,C. and Schell,J. (1986). The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. *204*, 383-396.

Kunkel,T.A., Roberts,J.D., and Zakour,R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. *154*, 367-382.

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.

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.

Puissant,C. and Houdebine,L.-M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. BioTechniques *8*, 148-149.

Rogers,S.G., Klee,H.J., Horsch,R.B., and Fraley,R.T. (1987). Improved vectors for plant transformation: expression cassette vectors and new selectable markers. Methods Enzymol. *153*, 253-277.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

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.

Yamamoto,K.T., Mori,H., and Imaseki,H. (1992). cDNA cloning of indole-3-acetic acid-regulated genes: Aux22 and SAUR from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol. *33*, 93-97.

Ye,G.N., Stone,D., Pang,S.Z., Creely,W., Gonzalez,K., and Hinchee,M. (1999). Arabidopsis ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. Plant J. *19*, 249-257.

CHAPTER 2-1

USE OF POLY(G) INSERTIONS TO GAIN INSIGHT INTO THE STEPS INVOLVED IN PLANT mRNA DEGRADATION

INTRODUCTION

Understanding the nature of the nucleolytic events that result in messenger RNA (mRNA) degradation is a fundamental goal of mRNA turnover research. mRNAs have two features that suggest the potential for multiple steps in the degradation process. These features, the 7-methyl guanine cap and the poly(A) tail, are thought to protect the 5' and 3' ends of the message from degradation. One might suspect that specific enzymes or groups of enzymes have evolved that are responsible for removing these distinct features. On the other hand, mRNA degradation may be a more stochastic process, involving a series of endonucleases that digest RNA at non-specific sites. Unfortunately, ascertaining the steps involved in the degradation of individual transcripts has not been possible in most cases because once the process begins it is apparently completed very rapidly. This supposition is verified by the common observation that only full-length transcripts are detected on Northern blots. Therefore, the ability to analyze intermediates in the mRNA decay process, which would provide a great deal of information about the nucleolytic events involved in the process, has been limited.

There are a few cases of naturally occurring mRNA decay intermediates. For example, intermediates in gro α and transferrin receptor mRNA degradation have been analyzed in mammalian cells (Stoeckle, 1992; Binder et al., 1994). In the case of gro α, an intermediate corresponding to the first 70% of the 5' portion of the message accumulates, but no 3' intermediate is found. The 5' intermediate could be caused by an endoribonuclease or by a 3' to 5' exoribonuclease that stops at a particular site. Two degradation products are observed for the transferrin receptor, suggesting an

endonucleolytic cleavage event. Interestingly, the 3' intermediate has been found to be polyadenylated, indicating that the endonuclease activity precedes deadenylation.

In plant cells, there are two cases of naturally occurring mRNA decay intermediates that have been thoroughly studied. The ribulose-1,5-bisphosphate carboxylase small subunit RNA (*SRS4*) from soybean is degraded into a series of distinct fragments (Thompson et al., 1992). The analysis of these fragments indicated that the *SRS4* transcript is degraded by a complex combination of endo- and exonucleolyitc events. Degradation of the oat phytochrome A (*PHYA*) transcript also results in fragments that were displayed on Northern blots. Analysis of these fragments led to the proposal that while approximately 25% of the *PHYA* transcripts are deadenylated prior to further degradation, most of the transcripts are degraded without prior deadenylation (Higgs and Colbert, 1994). It is still unclear how the body of the *PHYA* transcript is degraded and models involving endo- or exoribonucleases or combinations of both have been proposed. These experiments have provided information about the degradation of the *SRS4* and *PHYA* transcripts, but information about the mechanisms responsible for the turnover of other plant mRNAs is lacking.

A very fruitful alternative to analysis of naturally occurring mRNA decay intermediates has been to trap intermediates by introducing stable secondary structures into mRNAs. This approach was first used in *Saccharomyces cerevisiae* and has contributed tremendously to the elucidation of a general pathway of mRNA degradation in yeast (Vreken and Raué, 1992; reviewed in Caponigro and Parker, 1996). The most common insertion has been an 18 nucleotide poly-guanosine [poly(G)] tract, which is capable of forming a stable secondary structure and is thought to block the progress of 5'

to 3' or 3' to 5' exoribonucleases. By introducing poly(G) tracts into reporter genes, it was found that in most cases, a deadenylated intermediate 5' of the poly(G) tract was stabilized. This provided strong evidence for a pathway that utilized a 5' to 3' exoribonuclease that degraded the body of the message after deadenylation. These results were further strengthened by analyzing the accumulation of intermediates in yeast mutants defective for exoribonucleases. For example, in strains with a deletion in the *XRN1* gene, which encodes the predominant 5' to 3' exoribonuclease, this intermediate does not accumulate. The major pathway of yeast mRNA degradation and the use of poly(G) insertions to provide experimental evidence for this pathway are summarized in Figure 2-1-1.

To get a first approximation of the kinds of mRNA degradation pathways that might be involved in degrading plant messages, we designed reporter gene constructs that contained poly(G) tracts and introduced them into plant cells from two plant species. Poly(G) tracts were inserted into reporter messages that might be degraded by distinct mechanisms so that potentially diverse means of mRNA degradation could be analyzed. The results of these experiments and their implications for the mechanisms of mRNA degradation in plants will be described in this chapter.

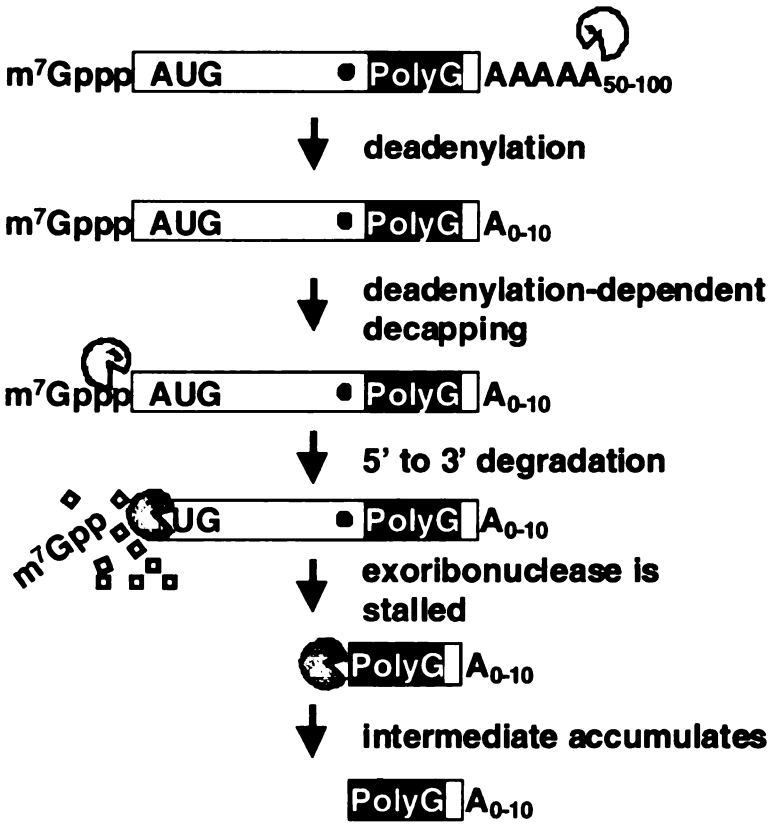

Figure 2-1-1. The effect of a poly(G) insertion on the major yeast mRNA degradation pathway. "Pacmen" represent components of the yeast decay machinery as follows: white, proposed deadenylating nuclease; light gray, decapping enzyme [Dcp1p, Dcp2p (Beelman et al., 1996; Legrandeur and Parker, 1998; Dunckley and Parker, 1999)]; dark gray, 5' to 3' exoribonuclease [Xrn1p (Stevens, 1978; Muhlrad et al., 1994)]. The intermediate that accumulates is trimmed to its 5' end and contains an oligo(A) tail. In strains with a deleted *XRN1* gene, this intermediate fails to accumulate. A version of this figure appears in Caponigro and Parker (1996).

RESULTS

Poly(G) tracts were inserted into reporter constructs that had previously been utilized to test mRNA sequences for their function as instability determinants in plant cells (Newman et al., 1993; Ohme-Takagi et al., 1993; Gil and Green, 1996). Figure 2-1-2 shows the basic structure of these reporters. These constructs provided the potential to test whether rapid mRNA turnover mediated by a dimer of the DST element (DSTx2),

the AUUUA repeat, or the SAUR-AC1 3' UTR would cause accumulation of different poly(G) stabilized intermediates. These constructs (test genes) were introduced into *Agrobacterium* transfer DNAs (T-DNA) along with a β-glucuronidase (GUS) reference gene.

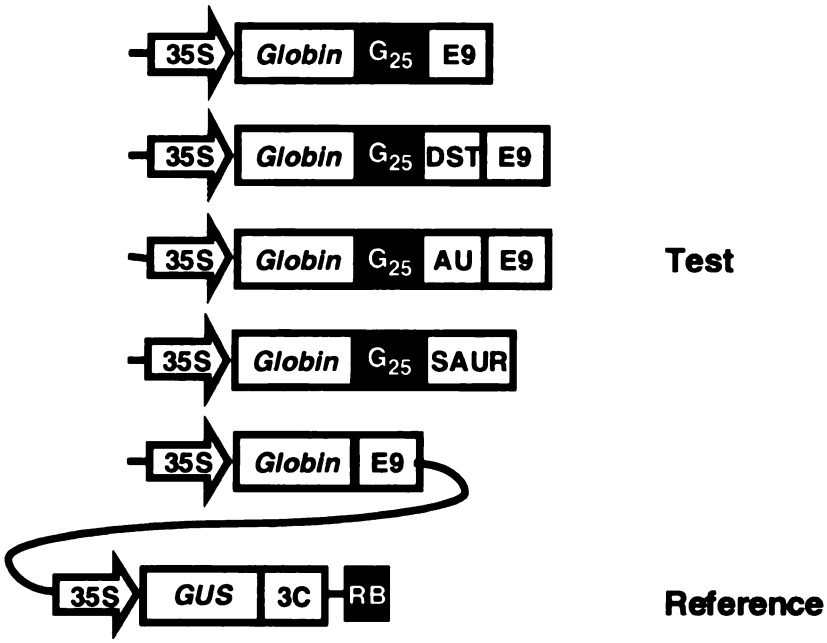

Figure 2-1-2. Poly(G) tracts were inserted into a variety of plant reporter transcripts. The cauliflower mosaic virus 35S promoter (35S) was used to control the transcription of test and reference genes. The human β-Globin (*Globin*) coding sequence was used as the test reporter transcript in these studies. The reference gene, β-glucuronidase (GUS), was included in the same binary vector with each of the test genes to facilitate normalization of mRNA abundance data. The 3'UTRs of the pea ribulose 1,5-bisphosphate carboxylase small subunit E9 (E9) and 3C (3C) genes were used as 3'UTRs for the test and reference genes, respectively. G_{25} represents the 25 nucleotide poly(G) insertion; DST represents a dimer of the DST element; AU represents the AUUUA repeat; *SAUR* represents the *SAUR-AC1* 3'UTR. The right border (RB) of the T-DNA has been indicated by a black rectangle. The T-DNA also includes the neomycin phosphotransferase cassette so that kanamycin resistant transgenic plants or stably transformed tobacco cells could be selected.

These constructs were used to generate stably transformed tobacco cell lines and transgenic Arabidopsis plants. RNA extracted from both systems was analyzed using polyacrylamide and agarose/formaldehyde gel blots. No detectable intermediates were

stabilized by the insertion of poly(G) tracts into any of these reporter transcripts in either tobacco cells or Arabidopsis seedlings.

In all cases, only full-length *Globin* messages were detected. Hybridization probes corresponding to the 5' (*Globin* coding sequence) or the 3' (E9 3'UTR) portions of these reporters detected only full-length *Globin* transcripts. An example of one such experiment is shown in Figure 2-1-3. One possible explanation for the failure to detect poly(G) intermediates is that our gel systems were not sufficient to detect the small fragments that may have been stabilized. To address this concern, fragments of the *Globin* reporter were generated in vitro by ribonuclease H (RNase H) digestion. RNase H digests RNA at RNA:DNA duplexes such that one can produce specific RNA fragments by incubating RNA with oligonucleotides complementary to sequences within the transcript. This approach was used to generate fragments of the *Globin* reporter that would correspond to poly(G) stabilized intermediates that accumulated 5' or 3' of the insertion. In addition, a fragment was made in vitro that corresponded to an intermediate that would have accumulated in vivo if the poly(G) tract blocked exonucleases that attacked the transcript from the 5' and 3' ends simultaneously (Figure 2-1-3-A). As can be seen in lanes 1, 2, and 3 of the Northern blot shown in Figure 2-1-3-B, fragments generated in vitro corresponding to the possible poly(G) stabilized intermediates were observed in the gel system. In contrast, only the full-length Globin transcript was observed when the RNA sample was analyzed without prior RNase H treatment (lane 4). This experiment indicated that although the gel system was adequate to detect poly(G) stabilized intermediates, these intermediates did not accumulate in vivo.

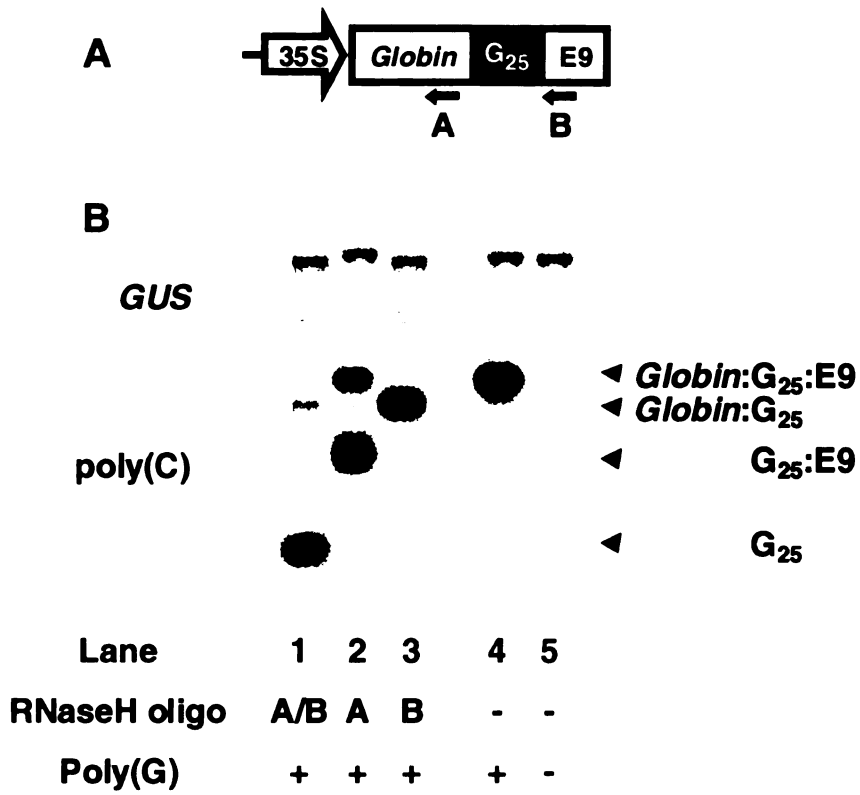

Figure 2-1-3. Poly(G) stabilized intermediates do not accumulate in Arabidopsis. A. A schematic diagram of the *Globin* reporter under analysis. Oligonucleotides for RNase H digestion are indicated with arrows. B. Northern blot analysis. The top panel shows the result of hybridization with radiolabeled *GUS* (reference gene) probe. The bottom panel shows the result of hybridization with radiolabeled oligo(C) probe that detects all transcripts containing the poly(G) tract. All lanes contain 20 μg of total RNA extracted from pools of 10-day-old Arabidopsis seedlings. Lanes 1-3 of the bottom panel show the results of RNase H digestion of the *Globin* reporter. The oligonucleotide(s) used in each experiment is indicated in the key below the panel. The predicted identity of each species observed on the Northern blot is given on the right of the lower panel. RNA samples in lanes 1-4 are from plants expressing the transcript shown in A. The RNA sample in lane 5 is from plants expressing a similar reporter that lacks the poly(G) tract, indicating that the hybridizing RNA species in lanes 1-4 were derived from the poly(G)-containing transgene.

DISCUSSION

Insertion of poly(G) tracts into reporter transcripts was a potentially powerful and rather obvious approach given the success of similar studies conducted in yeast (reviewed

in Caponigro and Parker, 1996). It is perhaps not surprising that similar constructs have been introduced into a diverse array of organisms. However, successful stabilization of poly(G) intermediates has only been reported thus far in yeast (for example, Vreken and Raue, 1992) and *Chlamydomonas reinhardtii* (Gera and Baker, 1998; Drager et al., 1998 and 1999); poly(G) intermediates have not been detected in a multi-cellular organism. At least three laboratories working on mammalian systems have attempted these experiments with similar results as those reported here for plants (L. Maquat, personal communication; A.-B. Shyu, personal communication; G. Goodall, personal communication). This lack of poly(G) intermediates may be indicative of differences in mRNA decay mechanisms between simple and more complex eukaryotes.

There are several technical and biological explanations for the lack of poly(G) intermediates in plant cells. For example, the poly(G) insertion may not form a strong secondary structure when introduced into the cell. The use of an oligo(C) hybridization probe in the experiment shown in Figure 2-1-3, indicated that the poly(G) insertion was present in the reporter transcripts, but that does not mean that the poly(G) insertion formed a stable structure that could block exoribonucleases. It is possible that there are RNA binding proteins in the plant cell that do not allow this structure to form. Alternatively, the plant cell may have mRNA helicases that can unwind the poly(G) structure allowing ribonucleases to proceed through it. An additional possibility is that exoribonucleases homologous to the ones in yeast that cause poly(G) intermediates exist in plant cells, but they are capable of degrading the poly(G) structure. Of course it is also possible that such 5' to 3' exoribonucleases are not important for plant mRNA degradation.

Recently, sequences similar to the yeast *XRN1* gene have been deposited in the Arabidopsis genome sequencing database. To begin to characterize the function of these Arabidopsis *XRN*-like genes, cDNAs were cloned and introduced into yeast, all of them led to the accumulation of poly(G) intermediates in yeast strains harboring an *XRN1* deletion (James P. Kastenmayer and Pamela J. Green, unpublished). The role of these genes in mRNA degradation in the plant cell has yet to be determined, however, these results indicate that mRNases that can be stalled by poly(G) structures are present in Arabidopsis.

The studies outlined in this chapter did not provide access to the steps involved in degrading plant mRNAs. However, they point to a potential difference between mRNA decay mechanisms between yeast and multi-cellular eukaryotes such as plants. The few examples of mRNA decay intermediates that have been analyzed in plant and mammalian cells also point to unique mechanisms for mRNA decay mechanisms in these systems. In particular, the degradation of the gro α, transferrin receptor, *SRS4*, and *PHYA* transcripts suggest a role for endonucleases in mRNA degradation. Endonucleases have not been implicated in yeast mRNA degradation. One possibility is that poly(G) insertions fail to block exoribonucleases in plants and animal cells because endoribonucleases, which may be capable of degrading poly(G) tracts, are important for mRNA degradation in these systems.

The presence of several sequences in the Arabidopsis sequence database that bear similarity to components of the yeast mRNA decay machinery argues against vast differences between yeast and plant mRNA decay mechanisms. In addition to the *XRN*-like genes already mentioned, there are potential homologues of two members of the

decapping complex and of a human gene encoding a poly(A) ribonuclease. Deadenylation and decapping are thought to be the first and rate-limiting steps, respectively, of the major yeast mRNA decay pathway (Caponigro and Parker, 1996). The role of these genes in mRNA degradation in plants has yet to be established, however, the presence of these sequences in the Arabidopsis genome is intriguing and points to the potential for some level of conservation among these pathways between yeast and plants.

The constructs and transgenic plants generated for the work presented in this chapter may be useful in analyzing the role of the mRNases. One of the goals of our laboratory is to isolate mutants of Arabidopsis with T-DNA insertions in each of the potential mRNases that have been identified through database searches. It is quite possible that novel poly(G) intermediates will become apparent when one or more of these genes are disrupted.

MATERIAL AND METHODS

Plasmid construction and plant transformation

Standard molecular cloning procedures were used to construct the chimeric genes described in Figure 2-1-2. (Sambrook et al., 1989). The poly(G) tract was synthesized using two oligonucleotides:
5'GATCTTCTAGAGGGGGGGGGGGGGGGGGGGGG-3'
5'GATCCCCCCCCCCCCCCCCCCCCCCCCCCTCTAGAA-3'. When annealed, these

oligonucleotides have a BamHI site on the 5' end and a BglII site on the 3' end. This facilitated the introduction of the poly(G) tract into the unique BamHI site present in pUC derivatives with the structure: SacI-35S-BglII-*Globin*-BamHI-E9-ClaI or SacI-35S-BglII-*Globin*-DSTx2-BamHI-E9 when an instability determinant was present. The BamHI site in SacI-35S-BglII-*Globin*-poly(G)-BamHI-E9-ClaI was filled in using T4 DNA polymerase so that the SAUR-AC1 3'UTR could replace the E9 3'UTR following digestion with ClaI and blunt-end ligation.

These test genes were transferred to a derivative of pMON505 which is a binary vector that facilitates transformation of plant cells via *Agrobacterium tumefaciens* (Rogers et al., 1987, Fang et al., 1989). The transfer DNA also contains a reference gene with the structure 35S-GUS-3C. This test and reference strategy and the source of the *Globin* and *GUS* coding regions were described by Newman et al., (1993).

Generation of and selection of transgenic Arabidopsis plants using the vacuum infiltration method of *Agrobacterium*-mediated transformation has been described (N. Bechtold et al.,1993 and Web site: http://www.bch.msu.edu/pamgreen/vac.htm). *Agrobacterium* strain GV3101 C58C1 Rif^r (pMP90) (Koncz and Schell, 1986) was transformed with pMON505 derivatives by electroporation using the Gene-Pulser (BioRad) according to the manufacturer's guidelines.

Generation of and selection of stably transformed tobacco cells (*Nicotiana tabaccum* cv Bright Yellow 2 [NT-1; An, 1985]) was as described previously by Newman et al. (1993). *Agrobacterium* strain LBA4404 was used in these experiments and was transformed with pMON505 derivatives by electroporation.

RNA analysis

RNA was extracted from pools of transgenic tobacco calli, liquid tobacco cultures, and pools of transgenic Arabidopsis seedlings using the method described by Puissant and Houdebine (1990) with modifications described by Newman et al., (1993).

Northern blot analysis was as described in Newman et al., 1993 with the following exceptions. Standard Northern blotting procedures were enhanced in order to resolve potentially small poly(G) stabilized intermediates. 2.2% NuSieve 3:1 Agarose (FMC, Inc.) / 2% formaldehyde gels were used to separate RNA. These gels were blotted using standard methods. In some experiments polyacrylamide gels (6% acrylamide, 7M urea) were used to achieve enhanced resolution. These gels were run and transferred to blots as described in Gera and Baker (1998).

RNA samples for the experiment shown in Figure 2-1-3 were extracted from pools of 10-day-old Arabidopsis seedlings. These pools consisted of a total of ~100 T_2 (self-fertilization progeny of primary transformants) seedlings from at least 10 independent transformants. T_2 seedlings were grown on seed selection media which consisted of 4.3 g/L Murashige and Skoog salt mixture (GibcoBRL), B5 vitamin mixture (100 mg/l *myo*-inositol, 10 mg/l thiamine hydrochloride, 1 mg/l nicotinic acid, 1 mg/l pyriodoxine), 1% sucrose, 0.5 g/L MES at pH 5.7, 0.8% phytagar, and 50 µg/ml kanamycin. Plates were kept in incubators set at 16 hours light (125 $\mu E/m^2$) /8 hours dark, 21°C.

RNase H treatment

Oligonucleotides complementary to the 3' end of the Globin coding sequence (pg-244) or the 5' end of the E9 3' UTR (pg-316) were used to digest the reporter transcript at specific sites in vitro as described in Diehn et al. (1998). Briefly, 20 μg of total RNA was incubated with 2 μg of each oligonucleotide at 65°C for 30 minutes in a 400 ml water bath. To anneal the oligonucleotides to their cognate mRNA, the water bath was allowed to cool to 30°C. The reactions were then placed at room temperature for 5 minutes. RNase H digestions were done at 37°C in a total volume of 50 μls that included 2 units of RNase H and RNase H reaction buffer (4 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM KCl, 1 mM DTT) (GibcoBRL).

ACKNOWLEDGEMENTS

I started this project during my rotation in the Green Laboratory under the careful tutelage of Dr. Michael Sullivan. I'd like to thank Mike for his incredible patience and for all he taught me during that ten-week period.

REFERENCES

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

Bechtold,N., Ellis,J., and Pelletier,G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris *316*, 1194-1199.

Beelman,C.A., Stevens,A., Caponigro,G., LaGrandeur,T.E., Hatfield,L., Fortner,D.M., and Parker,R. (1996). An essential component of the decapping enzyme required for normal rates of mRNA turnover. Nature *382*, 642-646.

Binder,R., Horowitz,J.A., Basilion,J.P., Koeller,D.M., Klausner,R.D., and Harford,J.B. (1994). Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. EMBO J. *13*, 1969-1980.

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

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.

Drager,R.G., Girard-Bascou,J., Choquet,Y., Kindle,K.L., and Stern,D.B. (1998). *In vivo* evidence for 5'-->3' exoribonuclease degradation of an unstable chloroplast mRNA. Plant J. *13*, 85-96.

Drager,R.G., Higgs,D.C., Kindle,K.L., and Stern,D.B. (1999). 5' to 3' exoribonucleolytic activity is a normal component of chloroplast mRNA decay pathways. Plant Journal *19*, 521-531.

Dunckley,T. and Parker,R. (1999). The DCP2 protein is required for mRNA decapping in saccharomyces cerevisiae and contains a functional MutT motif. EMBO J. *18*, 5411-5422.

Fang,R.-X., Nagy,F., Sivasubramaniam,S., and Chua,N.-H. (1989). Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell *1*, 141-150.

Gera,J.F. and Baker,E.J. (1998). Deadenylation-dependent and -independent decay pathways for α1-tubulin mRNA in *Chlamydomonas reinhardtii*. Mol. Cell. Biol. *18*, 1498-1505.

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.

Higgs,D.C. and Colbert,J.T. (1994). Oat phytochrome A mRNA degradation appears to occur via two distinct pathways. Plant Cell *6*, 1007-1019.

Koncz,C. and Schell,J. (1986). The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. *204*, 383-396.

LaGrandeur,T.E. and Parker,R. (1998). Isolation and characterization of Dcp1p, the yeast mRNA decapping enzyme. EMBO J. *17*, 1487-1496.

Muhlrad,D., Decker,C.J., and Parker,R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. *8*, 855-866.

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.

Puissant,C. and Houdebine,L.-M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. BioTechniques *8*, 148-149.

Rogers,S.G., Klee,H.J., Horsch,R.B., and Fraley,R.T. (1987). Improved vectors for plant transformation: expression cassette vectors and new selectable markers. Methods Enzymol. *153*, 253-277.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Stevens,A. (1978). An exoribonuclease from Saccharomyces cerevisiae: effect of modifications of 5' end groups on the hydrolysis of substrates to 5' mononucleotides. Biochem. Biophys. Res Commun. *81*, 656-661.

Stoeckle,M.Y. (1992). Removal of a 3' non-coding sequence is an initial step in degradation of *groα* mRNA and is regulated by interleukin- 1. Nucleic Acids Res. *20*, 1123-1127.

Thompson,D.M., Tanzer,M.M., and Meagher,R.B. (1992). Degradation products of the mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase in soybean and transgenic petunia. Plant Cell *4*, 47-58.

Vreken,P. and Raué,H.A. (1992). The rate-limiting step in yeast *PGK1* mRNA degradation is an endonucleolytic cleavage in the 3'-terminal part of the coding region. Mol. Cell. Biol. *12*, 2986-2996.

CHAPTER 2-2

CLONING AND INITIAL CHARACTERIZATION OF A POLY(A) RIBONUCLEASE FROM ARABIDOPSIS

INTRODUCTION

With few exceptions, the poly(A) tail is common to all eukaryotic messenger RNA (mRNA) molecules. There is also recent evidence that this structure is present on many bacterial RNAs (reviewed in Carpousis et al., 1999). The poly(A) tail appears to be involved in controlling mRNA stability in prokaryotes and eukaryotes; in prokaryotes it is thought to be a signal for rapid mRNA degradation while in eukaryotes it protects mRNA from degradation (reviewed in Jacobson, 1996). The poly(A) tail is also an enhancer of translation in eukaryotes (reviewed in Gallie, 1998). The role of the poly(A) tail in controlling mRNA function has made it a target for developmental regulation. For example, in *Xenopus*, poly(A) tails are added, or removed to promote or inhibit expression of certain genes during oocyte maturation (Fox and Wickens, 1990; Varnum et al., 1992; Sheets et al., 1995). The poly(A) tail is also a point of post-transcriptional control of gene expression in general, and in yeast it has been found that deadenylation triggers the degradation of the majority of cytoplasmic messages (reviewed in Caponigro and Parker, 1996).

The major yeast mRNA degradation pathway is thought to begin with deadenylation which is followed by cleavage of the of the 7-methyl guanosine cap (decapping) and eventual degradation of the body of the mRNA by a 5' to 3' exoribonuclease. These three steps are dependent upon one another such that decapping cannot occur without deadenylation and 5' to 3' degradation will not occur without prior decapping (reviewed in Caponigro and Parker, 1996). Nucleases that are responsible for decapping and degradation of the body of the transcript have been cloned and their role in

the major yeast mRNA degradation pathway has been demonstrated genetically and biochemically (Muhlrad et al., 1994; Beelman et al., 1996; Legrandeur and Parker, 1998; Dunckley and Parker, 1999). In contrast, the enzyme(s) responsible for complete degradation of the poly(A) tail has not been identified. Two candidates for this activity have been studied in yeast. First, a poly(A) binding-protein (PAB)-dependent poly(A) nuclease was cloned, however, recently it was shown that this activity is most likely responsible for trimming poly(A) tails to their mature length (Brown and Sachs, 1998). The exosome, an additional candidate, is a recently described complex of ~10 3' to 5' exoribonucleases involved in degradation of yeast mRNA, but the substrate for this complex is thought to be deadenylated mRNA (reviewed in van Hoof and Parker, 1999). Therefore the deadenylating nuclease remains unknown in yeast.

A 3' to 5' exoribonuclease that preferentially degrades poly(A) was purified from calf thymus (Korner and Wahle, 1997). This enzyme, was shown to have characteristics that identify is a candidate poly(A) ribonuclease and was called PARN. In vitro, PARN is Mg2+-dependent, and at low salt concentrations was dependent on spermidine or PAB. At physiological salt concentrations, spermidine activation was not as strong and PAB was inhibitory (Korner and Wahle, 1997). Incubation of PARN plus PAB with radiolabeled capped and polyadenylated mRNA species results in phased degradation products that are consistent with protection of the poly(A) tail by PAB (Korner and Wahle, 1997). A cDNA clone corresponding the human homologue was subsequently obtained from the human genome sequencing project and is referred to as HuPARN (Korner et al., 1998). The protein encoded by the *HuPARN* cDNA was expressed in *Escherichia coli* and purified and was found to have similar properties to the enzyme

originally purified from calf thymus. Experiments conducted in *Xenopus* oocytes implicate HuPARN as a potential deadenylating nuclease in vivo (Korner et al., 1998).

A sequence similar to *HuPARN* was identified in the Arabidopsis genomic sequence by Korner et al. (1998). To determine if *AtPARN* (for *Arabidopsis thaliana* PARN-like gene) is involved in mRNA degradation in plants, a cDNA corresponding to this gene was cloned and we have begun to characterize its function. These studies have the potential to answer many interesting questions about PARN in particular and mRNA degradation in general. For example, it is not known what role if any this type of protein may play in general mRNA degradation mechanisms. Is PARN responsible for triggering general mRNA degradation by removal of poly(A) tails? Is AtPARN important for post-transcriptional control of gene expression in plants? Does conservation of PARN indicate deadenylation plays an important role in plant mRNA decay pathways as it does in yeast? By applying the molecular genetic techniques available in Arabidopsis to the study of *AtPARN*, we may answer some of these important questions.

RESULTS

The RNaseD family and cloning of AtPARN

Sequence analysis revealed that *HuPARN* was a member of an ancient gene family of RNases named after it's founding member, RNaseD (Korner et al., 1998; Mian, 1997; Moser et al., 1997). RNaseD is involved in processing of tRNAs in *E. coli* (reviewed in Deutscher, 1993). This family includes *PAN2* from yeast which is a subunit

of the PAB-dependent poly(A) trimming activity described above. The feature that unites this gene family are three 3' to 5' exoribonuclease (Exo) domains which contain acidic residues involved in the coordination of metal ions necessary for nuclease activity (Barnad et al., 1989; Joyce and Steitz, 1994). This catalytic domain is also common to the proofreading domain of *E. coli* DNA polymerase I. Mian used a hidden Markof model to find RNaseD family members and to characterize important amino acids in these proteins (1997). This algorithm allows one to find specific domains within unrelated polypeptides; outside of the Exo domains many of the RNaseD family members are not similar. For example, there are several open reading frames within the yeast genome that lack Exo domains, but are more similar to *HuPARN* than is *PAN2*, an RNaseD family member (Korner et al., 1998).

A region of the Arabidopsis genome was identified that bore significant similarity to the *HuPARN* sequence. Among the conserved residues were the three Exo domains, the hallmark of the RNaseD family. The genomic sequence was used to design primers for rapid amplification of cDNA ends (RACE) by polymerase chain reaction (PCR). The predicted amino acid sequence of the resulting clone is shown in an alignment with the HuPARN amino acid sequence in Figure 2-2-1. AtPARN and HuPARN are 21% identical at the amino acid level. Interestingly, AtPARN has a 38 amino acid N-terminal extension. Eleven out of 38 residues in this N-terminal sequence are serine or threonine, amino acids that are often over-represented in N-terminal chloroplast targeting signals. However, the AtPARN polypeptide failed to enter chloroplasts in a targeting assay (John Froehlich, data not shown).

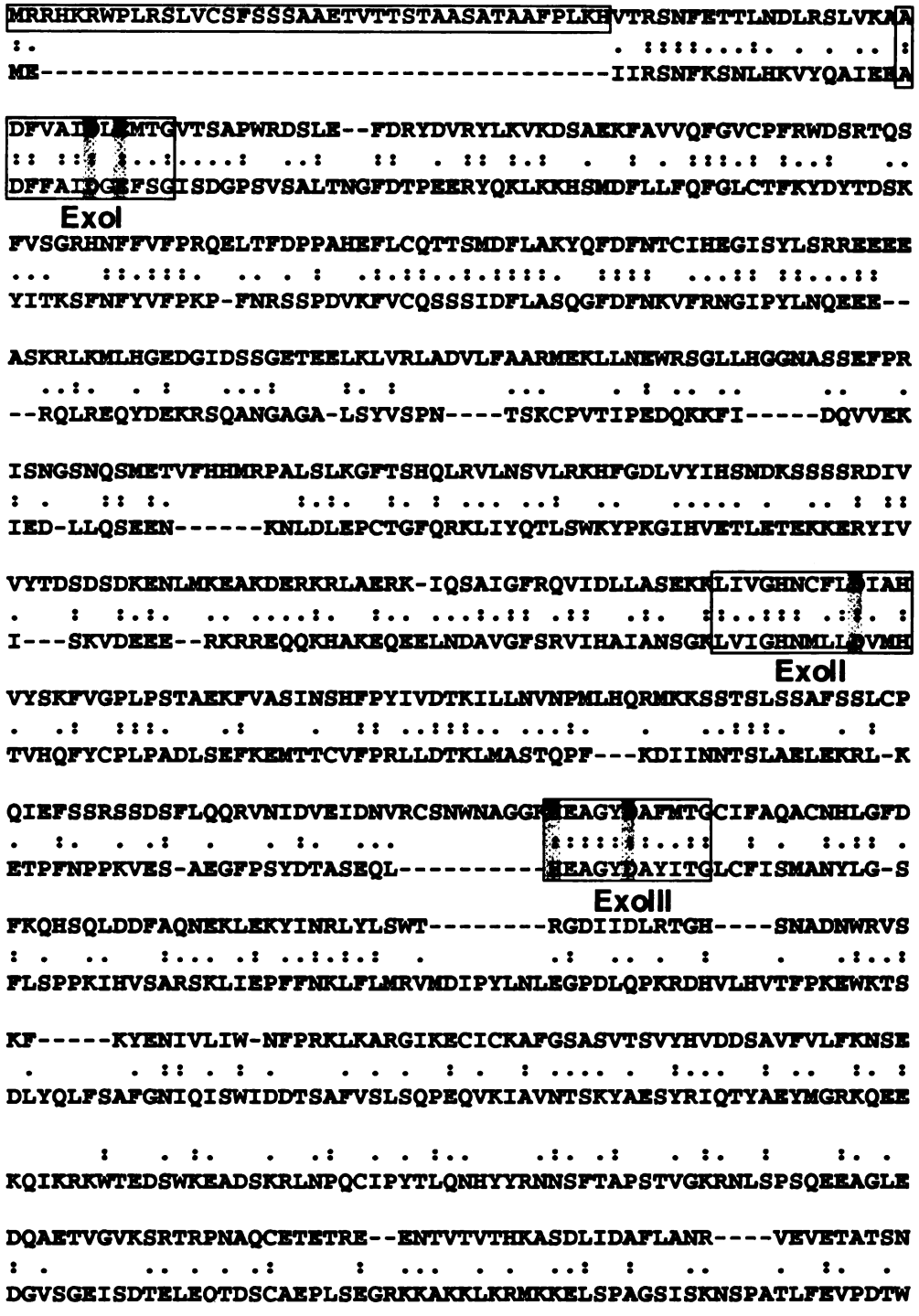

Figure 2-2-1. Sequence alignment of the AtPARN and HuPARN amino acid sequences. The AtPARN polypeptide, listed as the first line, is predicted to have 689 amino acids; HuPARN consists of 639 amino acids. The N-terminal extension found in AtPARN is boxed. Three Exo domains are also boxed and key residues are shaded. Sequence identity is indicated by a :, chemically similar residues (.) are also indicated.

RnaseD family members have diverse functions (Deutscher, 1993, Moser et al., 1997). Therefore it may be important to analyze the relative sequence similarity between different family members in order to make functional approximations. AtPARN and HuPARN share similarity outside of the Exo domains and are more similar to each other than they are two other members of the RNaseD family. BLAST (basic local alignment search tool) searches using the predicted AtPARN amino acid sequence find only HuPARN and a gene from *Caenorhabditis elegans* (K10C81, also described in Korner et al., 1998). AtPARN is not significantly similar with other members of the RNaseD family, including PAN2, in these searches. The close relationship between HuPARN and AtPARN relative to other members of the RNaseD family may indicate functional similarity between these proteins.

AtPARN is expressed throughout the plant

To determine the expression pattern of *AtPARN* in Arabidopsis plants, Northern blot analysis was performed on total RNA isolated from roots, stems, leaves, and flowers. As Figure 2-2-2 indicates, the *AtPARN* mRNA was found in all of these plant organs.

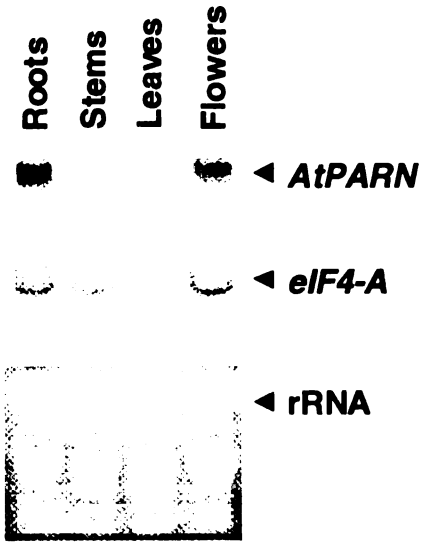

Figure 2-2-2. AtPARN mRNA is found throughout the plant. A Northern blot containing 20 μg of total RNA from roots, leaves, stems, and flowers of Arabidopsis plants was hybridized sequentially with *AtPARN* and *eIF4-A* probes. A photograph of the ethidium-bromide stained gel is included to highlight differences in abundance of chloroplast RNA in these samples.

The size of the *AtPARN* transcript was approximately 2 Kb, about that of the cDNA clone which was 2193 base-pairs excluding the poly(A) tail. Northern blot analysis indicated that AtPARN mRNA was more abundant in roots and flowers than in leaves and stems (Figure 2-2-2). These data must be interpreted with caution, however, because the differences in *AtPARN* mRNA abundance may reflect differences in loading due to the smaller amount of chloroplast rRNA in roots and flowers. The *eIF4-A* transcript abundance was to be used as loading control, however, the abundance of this message also varied in these samples.

Characterization of AtPARN activity in vitro

To analyze the enzymatic activity of the AtPARN polypeptide, the coding sequence was expressed in *E. coli* as an N-terminal six-histidine fusion. This construct facilitated the partial purification of the AtPARN polypeptide using a Ni^{2+}-NTA column. Denaturing polyacrylamide gel electrophoresis showed that two major proteins were eluted from the Ni^{2+}-NTA column, only one of which cross-reacted with anti-pentahistidine antibodies (data not shown). These proteins were approximately 70 kd, matching the predicted size of AtPARN, the smaller one may be the result of an N-terminal degradation that occurred after purification (Figure 2-2-3-A).

Poly(A) ribonuclease activity was analyzed using a trichloroacetic acid (TCA) precipitation assay that monitors the release of mononucleotides from a uniformly radiolabeled poly(A) substrate following incubation with AtPARN. Preliminary assays indicate that AtPARN has poly(A) ribonuclease activity (Figure 2-2-3-B). Thus far, only conditions optimal for HuPARN have been used in these assays.

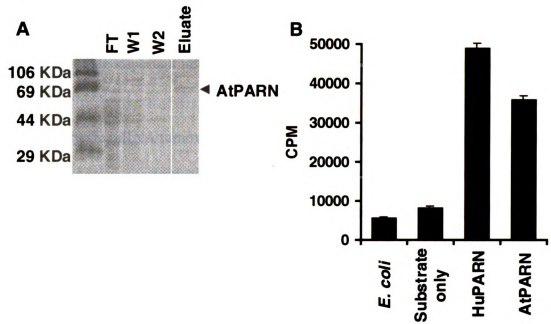

Figure 2-2-3. AtPARN has poly(A) ribonuclease activity. A. A denaturing polyacrylamide gel was loaded with approximately equal amounts of protein from the flow through (FT), the first wash (W1), the second wash (W2), and the eluate of a Ni^{2+}-NTA column. A photograph of the Coomassie stained gel is shown. B. The AtPARN eluate was tested using the TCA PARN assay (described in materials and methods). The average counts per minute (CPM) detected in two experiments is plotted ± the standard deviation. The eluate from BL21(DE3)pLysS cells (*E.coli*) that were not expressing AtPARN and the assay mixture with no protein added (Substrate only) served as negative controls. HuPARN was used as a positive control.

DISCUSSION

Deadenylation is the first step in the degradation of many yeast and mammalian mRNAs and the rate of deadenylation is important in determining mRNA stability in these systems (Shyu et al., 1990; Muhlrad et al., 1994, Ross, 1995; Caponigro and Parker, 1996). There is evidence that deadenylation may be involved in the degradation of at least one plant message, the oat phytochrome A transcript (Higgs and Colbert, 1994). A thorough study of poly(A) degrading activities in plants is necessary to determine the role

of this process in plant mRNA stability. In addition, studying a poly(A) ribonuclease in plants may provide important information about the mechanism of deadenylation in other eukaryotes.

Eukaryotic mRNA turnover pathways have been studied almost exclusively in yeast. In *Saccharomyces cerevisiae,* genes have been identified that encode every enzyme in the major mRNA decay pathway except the deadenylating nuclease. Thus far, the best candidate in any eukaryote for such an activity is HuPARN, a 3' to 5' exoribonuclease that degrades poly(A) preferentially (Korner and Wahle, 1997, Korner et al., 1998). The characteristics of this protein in vitro are consistent with a role in deadenylation (discussed above, Korner et al., 1997). Perhaps the best evidence that HuPARN may play a role in deadenylation of mRNA comes from studies in *Xenopus* oocytes. A deadenylating nuclease activity that catalyzes the default removal of poly(A) tails in mature oocytes was identified almost ten years ago (Varnum et al., 1990). Purification of a protein responsible for this activity and cloning of the gene that encodes it revealed strong similarity to *HuPARN* (Korner et al., 1998). Antibodies directed against HuPARN blocked the default deadenylation of transcripts in mature oocytes and ectopic expression of *HuPARN* promoted deadenylation in enucleated oocytes lacking *Xenopus* PARN. These experiments indicated that HuPARN is a deadenylase in vivo (Korner et al., 1998).

We have cloned a cDNA from Arabidopsis that bears significant similarity to *HuPARN.* Initial experiments indicate that this gene, called *AtPARN*, encodes a protein with poly(A) nuclease activity in vitro. Thus far only assay conditions that were optimal for HuPARN activity have been tested. The *AtPARN* mRNA is expressed throughout the

plant as might be expected for a gene involved in a ubiquitous process such as deadenylation of mRNA.

Future experiments will address the role of this gene in mRNA degradation in Arabidopsis. We have initiated the search for a transfer-DNA (T-DNA) insertion in AtPARN. A collection of ~68,000 plants that collectively have ~100,000 T-DNA inserts spread throughout the genome has been screened by PCR using primers corresponding to the borders of the T-DNA and the *AtPARN* genomic sequence (Hirsch et al., 1998). Initially, 30 large pools containing DNA from the entire collection were screened to identify those with inserts in *AtPARN*. These pools must be deconvoluted through a series of sub-pools in order to find single plants with the T-DNAs of interest. The PCR products corresponding to five T-DNA inserts in *AtPARN* have been sequenced. One T-DNA is inserted just after the first Exo domain within the third exon of the *AtPARN* genomic sequence. A pool of nine plants has been identified that contains plants with this T-DNA insertion. The T-DNA is ~8 Kb long, so insertions will likely result in a null mutation. Based on database searches and Southern blot analysis (data not shown), *AtPARN* appears to be the only *PARN*-like sequence in the Arabidopsis genome. Therefore, a mutant in this gene should be informative. Of course if AtPARN is important for the control of mRNA stability in plants, loss of function may be lethal. One of the advantages of isolating mutants by PCR screening is that heterozygotes can be identified if the mutation is recessive. Analysis of the progeny of these heterozygous plants allows one to determine if a mutation is lethal, an opportunity not available to classical geneticists. The chief goal of these studies will be to study the effect on poly(A) tail length in these mutants and analyze any phenotypic changes that become apparent.

A fusion of HuPARN to the green fluorescent protein (GFP) was localized to the nucleus and cytoplasm of Cos-1 cells (Korner et al., 1998). It is unclear why HuPARN would be found in the nucleus in Cos-1 cells, but one would expect a deadenylase involved in mRNA degradation to be found in the cytoplasm. In addition, *Xenopus* PARN isoforms are found in the nucleus and in the cytoplasm of mature oocytes (Korner et al., 1998). In *Xenopus*, PARN activity is thought to be regulated by sequestration in the nucleus. One interesting question raised by these studies is whether PARN is a specialized activity involved in the default deadenylation of mRNAs developing zygotes or whether it is involved in general mRNA degradation. The initial purification of mammalian PARN from calf thymus and the expression of *HuPARN* mRNA in many different types of human cells would suggest that this is not true of HuPARN, and whether this is true of Xenopus PARN remains to be tested (Korner et al., 1998). AtPARN is expressed throughout the plant, but we do not yet know where it is localized within the plant cell or whether it is expressed preferentially in certain cell types. We have initiated these localization studies by introducing an N-terminal GFP fusion into Arabidopsis plants.

Genetic strategies are limited in mammalian cells and in *Xenopus*, therefore, the ability to test the function of AtPARN directly by isolating a T-DNA insertion mutant may provide us with the unique opportunity to address the role of this type of enzyme in cytoplasmic mRNA degradation in somatic cells.

MATERIALS AND METHODS

Cloning of AtPARN

Standard cloning procedures were used as described in Sambrook et al. (1989). Two products of RACE experiments corresponding to the 5' and 3' ends of the AtPARN cDNA were generated using primers complementary to the AtPARN genomic sequence and to the adapter sequence that was ligated to the ends of cDNAs from Arabidopsis seedling mRNA. The details of this protocol are described in the package insert of the Marathon cDNA kit (Clonetech). The 5' product consisted of the first 1.5 kb of the AtPARN cDNA and was amplified using primer pg-444 (5'-TGAAGTCAAAACCGAGATGATTGC -3'). This product was inserted into the EcoRV site of a derivative of pBlueskript SKII(-) called p948, the resulting clone was called p1843. The 3' product consisted of the latter 0.9 kb of the AtPARN cDNA and was amplified using primer pg-609 (5'-AGGTTGCATCTTTGCGCAGGC -3'). This product was inserted into the EcoRV site of p948, the resulting clone was called p1920. The overlap shared by these two PCR products contained a unique SphI site that allowed the two products to be joined, creating p1932.

The final cDNA clone was sequenced and checked against two available genomic sequences. No errors in the cDNA were found. The AtPARN Genomic region consists of ~3000 Kb on bacterial artificial chromosome f14j16 which has been mapped to chromosome 1 near position 80. Comparison of the cDNA with the genomic sequence revealed that *AtPARN* consists of 7 exons.

Analysis of *AtPARN* expression

Total RNA was isolated from the leaves, stems, and flowers of mature (6 week old) Arabidopsis (Col-0) plants. Root RNA was prepared from the roots of seedlings grown in liquid culture for 3 weeks (2.3% Gamborg's medium [GibcoBRL, Inc.], pH 5.0). RNA was prepared using the method described by Puissant and Houdebine (1990) with modifications described by Newman et al., (1993). Northern blot analysis was as described in Newman et al. (1993). The use of the translation initiation factor, *eIF4-A*, as a loading control for Northern blots was as described by Taylor et al., (1993). Hybridization probes corresponding to the *AtPARN* and *eIF4-A* coding regions were labeled using α-^{32}P dCTP by the random-primed method of Feinberg and Vogelstein (1983).

Expression of *AtPARN* in E. coli

The vector used to express HuPARN in *E. coli* (pGMMCS) was obtained from Wahle and coworkers (Korner et al., 1998). This is a derivative of the pET vectors (Novagen). To fuse six histidine residues to the N-terminus of the AtPARN coding sequence and to facilitate cloning into pGMMCS, the primers pg-718 (5'- T GCC ATG GCT *CAC CAT CAC CAT CAC CAT* GTC GAC ATG CGC CGG CAC AAG CGA TG – 3') and pg-727 (5' – CG TGC TCG AGT TAA TTA CTC GTA GCA GTT TCG – 3')

were used to amplify the AtPARN coding sequence by PCR. Stop and start codons are underlined, the codons encoding the six histidine residues are italicized. The resulting PCR product was inserted at the EcoRV site of p948 creating p1938. The AtPARN coding region was sequenced and no errors were found. An NcoI, XbaI fragment of this clone was then introduced into pGMMCS creating p1949, the AtPARN *E.coli* expression vector.

BL21(DE3)pLysS (Novagen) cells were transformed with p1949 by electroporation using the Gene-Pulser (BioRad) according to the manufacturer's guidelines. Induction of AtPARN expression was carried out in LB medium supplemented with chloramphenicol (33 μg/ml), ampicillin (100 μg/ml) and IPTG (1 mM) as described on pages 46 and 47 of the QIAexpressionist (1997, Qiagen Inc.). 10 ml cultures from three clones that expressed AtPARN to high levels were combined and a soluble lyste was prepared as described on page 63 of the QIAexpressionist (1997, Qiagen Inc.). AtPARN was purified using a Ni^{2+}-NTA (nitrlotriacetic acid) resin that was incubated with p1949 soluble lysates in a batch culture at 4°C as described on pages 66 and 67 of the QIAexpressionist (1997, Qiagen incorporated). The denaturing polyacrylamide gel shown in Figure 2-2-3-A consisted of 10% polyacrylamide and 5% SDS. Lysates from BL21(DE3)pLysS (Novagen) cultures were prepared in parallel and were resolved using Ni^{2+}-NTA resin so that eluates from these columns could be used as a negative control in PARN assays.

TCA PARN assay

This assay monitors the release of TCA-soluble products from homogeneously labeled poly(A). The substrate was prepared as described in Brown et al.(1996). The reaction mixture consisted of 500 units of yeast poly(A) polymerase (U.S. Biochemicals), 50 μCi α-^{32}P rATP, 0.5 μM 12 nucleotide oligo(A) (obtained from Dharmacon Research, Inc.), 167 mM rATP, 1X poly(A) polymerase buffer (U.S. Biochemical). The reaction was incubated at 30°C for 60 minutes. Polyacrylamide gel electrophoresis showed that labeled poly(A) was greater than 1 Kb in length. Unincorporated nucleotides were removed using a spin column (S-200, Pharmacia).

100,000 CPM of the poly(A) substrate was incubated with 10 μls of BL21 eluate, 10 μls of AtPARN eluate, or 1 μl of HuPARN or with the PARN assay mixture alone. The concentrations of AtPARN and HuPARN were not determined. The assay mixture and conditions were as described in Korner and Wahle (1997) and modified based on Brown et al. (1996). Briefly, eluates were added to a reaction mixture consisting of 2mM spermidine plus dilution buffer (5mM Hepes, pH 7.4; 2mM MgCl2; 14.4mM β-mercaptoethanol) in a total volume of 50 μls and were incubated at 37°C for 30 minutes. 200 μls of cold 20% TCA was then added and polynucleotides were precipitated at -20°C for 10 minutes. Pellets were spun down and 100 μls of the supernatant was counted by liquid scintillation. Two experiments that were conducted one after the other are reported in Figure 2-2-3-B.

ACKNOWLEDGEMENTS

I would like to thank Drs. Ambro van Hoof and Roy Parker of The University of Arizona who made us aware of the AtPARN genomic sequence before publication of Korner et al. (1998); and Dr. Eva Devlin in Dr. Elmar Wahle's group at the Universität Halle Institut für Biochemie, who provided pGMMCS and an aliquot of HuPARN. I thank Jonathan Vogel for his great interest in this project and for conducting the TCA PARN assays reported in Figure 2-2-3-B. I would also like to thank Preetmoninder Lidder who constructed a GFP-AtPARN fusion protein and is currently analyzing its localization in transgenic Arabidopsis. Thanks also to James Kastenmayer who generated the cDNA library that was used to clone *AtPARN* and Miguel A. Pérez-Amador who generated the Northern blot used for Figure 2-2-2.

REFERENCES

Beelman,C.A., Stevens,A., Caponigro,G., LaGrandeur,T.E., Hatfield,L., Fortner,D.M., and Parker,R. (1996). An essential component of the decapping enzyme required for normal rates of mRNA turnover. Nature *382*, 642-646.

Bernad,A., Blanco,L., Lazaro,J.M., Martin,G., and Salas,M. (1989). A conserved 3'----5' exonuclease active site in prokaryotic and eukaryotic DNA polymerases. Cell *59*, 219-228.

Brown,C.E., Tarun,S.Z., Jr., Boeck,R., and Sachs,A.B. (1996). PAN3 Encodes a Subunit of the Pab1p-Dependent Poly(A) Nuclease in Saccharomyces cerevisiae. Mol. Cell. Biol. *16*, 5744-5753.

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

Carpousis,A.J., Vanzo,N.F., and Raynal,L.C. (1999). mRNA degradation - a tale of poly(A) and multiprotein machines. Trends Genet. *15*, 24-28.

Deutscher,M.P. (1993). Promiscuous exoribonucleases of Escherichia coli. J. Bacteriol. *175*, 4577-4583.

Dunckley,T. and Parker,R. (1999). The DCP2 protein is required for mRNA decapping in saccharomyces cerevisiae and contains a functional MutT motif [In Process Citation]. EMBO J. *18*, 5411-5422.

Feinberg,A.P. and Vogelstein,B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6-13.

Fox,C.A. and Wickens,M. (1990). Poly(A) removal during oocyte maturation: A default reaction selectively prevented by specific sequences in the 3' UTR of certain maternal mRNAs. Genes Dev. *4*, 2287-2298.

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.

Higgs,D.C. and Colbert,J.T. (1994). Oat phytochrome A mRNA degradation appears to occur via two distinct pathways. Plant Cell *6*, 1007-1019.

Hirsch,R.E., Lewis,B.D., Spalding,E.P., and Sussman,M.R. (1998). A role for the AKT1 potassium channel in plant nutrition. Science *280*, 918-921. See also www.biotech.wisc.edu/Arabidopsis/

Jacobson,A. and Peltz,S.W. (1996). Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. Annu. Rev. Biochem. *65*, 693-739.

Joyce,C.M. and Steitz,T.A. (1995). Polymerase structures and function: variations on a theme? J. Bacteriol. *177*, 6321-6329.

Körner,C.G. and Wahle,E. (1997). Poly(A) tail shortening by a mammalian poly(A)-specific 3'-exoribonuclease. J. Biol. Chem. *272*, 10448-10456.

Körner,C.G., Wormington,M., Muckenthaler,M., Schneider,S., Dehlin,E., and Wahle,E. (1998). The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of *Xenopus* oocytes. EMBO J. *17*, 5427-5437.

LaGrandeur,T.E. and Parker,R. (1998). Isolation and characterization of Dcp1p, the yeast mRNA decapping enzyme. EMBO J. *17*, 1487-1496.

Mian,I.S. (1997). Comparative sequence analysis of ribonucleases HII, III, II PH and D. Nucleic Acids Res. *25*, 3187-3195.

Moser,M.J., Holley,W.R., Chatterjee,A., and Mian,I.S. (1997). The proofreading domain of *Escherichia coli* DNA polymerase I and other DNA and/or RNA exonuclease domains. Nucleic Acids Res. *25*, 5110-5118.

Muhlrad,D., Decker,C.J., and Parker,R. (1994). Deadenylation of the unstable mRNA encoded by the yeast *MFA2* gene leads to decapping followed by 5'-->3' digestion of the transcript. Genes Dev. *8*, 855-866.

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.

Puissant,C. and Houdebine,L.-M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. BioTechniques *8*, 148-149.

Qiagen Incorporated. The QIAexpressionist. March 1997. Qiagen Inc.
See also www.qiagen.com for protocols.

Ross,J. (1996). Control of messenger RNA stability in higher eukaryotes. Trends Genet. *12*, 171-175.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sheets,M.D., Wu,M., and Wickens,M. (1995). Polyadenylation of c-mos mRNA as a control point in Xenopus meiotic maturation. Nature *374*, 511-516.

Shyu,A.-B., Belasco,J.G., and Greenberg,M.E. (1991). Two distinct destabilizing elements in the c-*fos* message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. *5*, 221-231.

Taylor,C.B., Bariola,P.A., DelCardayré,S.B., Raines,R.T., and Green,P.J. (1993). RNS2: A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. Proc. Natl. Acad. Sci. USA *90*, 5118-5122.

van Hoof,A. and Parker,R. (1999). The exosome: a proteasome for RNA? Cell *99*, 347-350.

Varnum,S.M. and Wormington,W.M. (1990). Deadenylation of maternal mRNAs during Xenopus oocyte maturation does not require specific cis-sequences: a default mechanism for translational control. Genes Dev. *4*, 2278-2286.

Varnum,S.M., Hurney,C.A., and Wormington,W.M. (1992). Maturation-specific deadenylation in Xenopus oocytes requires nuclear and cytoplasmic factors. Dev. Biol. *153*, 283-290.