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PREETMONINDER LIDDER

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GENETICS AND GENOMICS OF THE DST-MEDIATED DECAY PATHWAY IN *Arabidopsis thaliana*

By

Preetmoninder Lidder

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ABSTRACT

GENETICS AND GENOMICS OF THE DST-MEDIATED DECAY PATHWAY IN *Arabidopsis thaliana*

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Preetmoninder Lidder

The control of mRNA stability plays a fundamental role in the regulation of gene expression in plants and other eukaryotes. The basal mRNA decay machinery, sequence-specific decay components, and regulatory factors that respond to various stimuli can influence the control of mRNA stability. Despite the importance of mRNA stability in providing the cells with a means to rapidly tailor gene expression, little is known about this process in higher eukaryotes in general, and plants in particular. The overall goal of the research described in this thesis has been to elucidate the mechanisms and cellular factors which determine sequence-specific mRNA decay in higher plants. Towards this end, Arabidopsis mutants defective in the DST-mediated decay pathway were utilized.

The *dst* mutants were originally isolated as specifically elevating the steady-state level and increasing the half-life of DST-containing transcripts. To determine new molecular phentotypes of the *dst1* mutant and evaluate the biological significance of the DST-mediated decay pathway, cDNA microarray technology was employed. In addition to verifying the increase in the transgene mRNA levels, used to isolate these mutants, new genes with altered mRNA abundance in *dst1* were identified. RNA gel blot analysis confirmed the microarray data for all genes tested and was also used to catalog the first molecular differences in gene expression between the *dst1* and *dst2* mutants. Clustering analysis of genes altered in *dst1* exposed new co-expression patterns suggesting a link

between the *dst1* mutation and circadian rhythms. Earlier results from microarray expression data for unstable mRNAs in Arabidopsis showed that mRNA instability is associated with a group of genes controlled by the circadian clock. Experiments conducted during the course of this dissertation indicated that *Ccr*-like and *SEN1*, two transcripts which are regulated at the level of mRNA stability by the clock, are also direct targets of the DST-mediated decay pathway. Not only were *Ccr*-like and *SEN1* transcripts altered in their half-lives in *dst1*, but their stabilities were also altered in the mutant relative to the parental at different times during the day. This leads to aberrant circadian oscillation of these transcripts in the mutant demonstrating that the *DST1* locus is associated with circadian control. Previous experiments (e.g. with the *per* gene in Drosophila) have implicated differential mRNA stability in the control of CCGs but the observations in Arabidopsis are among the first to provide direct evidence for this.

Map-based cloning of the *dst1* mutant was initiated using *RAP2.4*, an endogenous target identified during the microarray experiments. The use of this marker circumvented some of the problems associated with the transgene to score homozygous mutants in the mapping population. A new mutant, *dst3*, was characterized in this thesis which should facilitate the investigation of the molecular components involved in DST-mediated degradation. Additionally, microarray experiments with *dst2* were carried out to identify molecular markers specific for *dst2* and further address the physiological role of the DST-mediated mRNA decay pathway in plants.

Taken together, the results described in this dissertation should constitute significant progress toward understanding the molecular machinery responsible for sequence-specific mRNA degradation in plants.

To my parents

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AFGC	Arabidopsis Functional Genomics Consortium
ARE	Adenylate/uridylate rich elements
CAPS	Cleaved amplified polymorphic sequence
CCG	Clock controlled gene
CT	Circadian time
dCAPS	Derived CAPS
DNA	Deoxyribonucleic acid
DST	Downstream element
EST	Expression sequence tag
GUS	β-glucuronidase
HPH	Hygromycin phosphotransferase
MIPS	Munich Information Center for Protein sequences
mRNA	Messenger RNA
ORF	Open reading frame
PCR	Polymerase chain reaction
per	Period
SAUR	Small auxin up RNA
SMD	Stanford Microarray Database
SNP	Single nucleotide polymorphism
SSLP	Simple sequence length polymorphism
TAIR	The Arabidopsis Information Resource
UTR	Untranslated region
ZT	Zeitgeber time

CHAPTER 1

CONTROL OF mRNA TURNOVER IN PLANTS

INTRODUCTION

Gene expression can be controlled at multiple levels in the cell and requires the integration of varied processes such as transcription, RNA processing and export, translation and post-translational events. In the past years, the significance of the plethora of mechanisms functioning at the post-transcriptional level has become exceedingly clear. The control of mRNA stability is a notable and well-known form of post-transcriptional gene regulation in eukaryotic cells. Compared to transcription, much less is known about the machinery for mRNA degradation. To provide a broad overview of the various components that contribute to the stability of mRNAs and the pathways by which mRNA decay occurs in plants, the focus of this chapter will be on the events affecting the turnover of mRNAs encoded by nuclear genes in Arabidopsis. Relevant examples from other higher plants will also be highlighted. For detailed knowledge on mRNA decay pathways in other systems, the reader is referred to excellent recent reviews on the topic (Caponigro and Parker, 1996; Guhaniyogi and Brewer, 2001; McCarthy, 1998; Mitchell and Tollervey, 2001; Ross, 1995).

The level of mRNA that is available in the cytoplasm for translation is a key control point in the regulation of gene expression. A useful way to consider the control of mRNA stability is in three interrelated levels (Gutiérrez et al., 1999). Recent studies indicate that the first and the most fundamental level is that of the basal mRNA decay machinery responsible for the decay of most cellular mRNAs. Superimposed on the general decay machinery is the second level of control that establishes the "inherent" degradation rates of various mRNAs, which can vary over a broad range. The average half-life of an mRNA in eukaryotic cells is on the order of several hours, but for some

mRNAs, sequence-specific recognition mechanisms can facilitate half-lives as short as minutes or as long as several days (Gutiérrez et al., 1999). The stability of some mRNAs is differentially regulated by exogenous or endogenous stimuli and such differential regulation of mRNA stability constitutes the third level of control.

There is much to be learned about the mechanisms and cellular factors that are responsible for mRNA turnover in eukaryotes in general and plants in particular. However one difficulty has been that most work on the general decay machinery has been carried out in yeast whereas work in mammals and plants has focused essentially on sequence-specific (constitutive and regulated) control of mRNA decay. Previous studies have suggested that there are distinct mechanistic differences between unicellular and multicellular eukaryotes with respect to mRNA decay. Therefore a thorough investigation of the mRNA decay machinery in multicellular systems is required to elucidate the fundamentals of how mRNA stability is controlled.

Arabidopsis thaliana, a member of the mustard family, has emerged as the primary plant model organism to study mRNA stability because of the various tools available for its analysis and the technical advantages of this system that surpass those of mammals for *in vivo* analysis. A genetic approach in Arabidopsis may offer the best opportunity to isolate and analyze the cellular factors involved in mRNA turnover. Also the biological functions of the potential factors that govern mRNA stability can be addressed using reverse genetic approaches that are hard to reproduce in mammalian systems. Insertion mutants of Arabidopsis are available from a variety of different collections. In addition, RNAi constructs, having a "panhandle" structure, can be used to inactivate the desired gene (Waterhouse et al., 1998). Further overexpression lines are

advantageous to assess the function of newly cloned proteins (Weigel et al., 2000) especially in conjunction with the inactivation lines. Finally, microarray technology (Schena et al., 1995; DeRisi et al., 1997) is a powerful approach to assay the expression of numerous genes involved in multiple aspects of mRNA stability and to monitor mRNA stability on a global scale. Arrays for Arabidopsis, containing approximately 14,000 clones, have been generated by the Arabidopsis Functional Genomics Consortium.

DETERMINATION OF mRNA DECAY RATES

The study of mRNA turnover in plants has been greatly aided by a number of methods for measuring mRNA decay rates. The derivation of the mRNA decay constant (k_d) is based on the assumption that the degradation of mRNA obeys first-order kinetics i.e. the turnover rate of a message is proportional to the amount of mRNA present at a given time and the rate constant for decay. Usually, the stability of a message *in vivo* is reported as a half-life, the time required for half of the existing mRNA molecules to be degraded. The half-life of an mRNA ($t_{1/2}$) is inversely proportional to its decay constant.

$$t_{1/2} = \ln 2/k_d$$

Determination of mRNA half-life begins by blocking mRNA synthesis, isolating RNA samples at various time intervals and monitoring the loss of a particular message by analyzing equal amounts of these samples with a message-specific probe. A semi-logarithmic plot of mRNA concentration as a function of time then yields a straight line with the decay constant as the slope, as defined by:

$$\ln (C/C_0) = -k_d t$$

where C_0 is the initial mRNA concentration and C is the mRNA concentration at time t. However, there are situations in which mRNA decay might not be stochastic. For example, in some eukaryotic cells, poly(A) shortening precedes decay of the mRNA body (Decker and Parker, 1993; Chen and Shyu, 1995) and only after the poly(A) tail has been shortened to a certain extent does first-order decay occur. Thus for mRNAs with biphasic kinetics, measuring half-life might not be straightforward and it is necessary to monitor the deadenylation step, or other steps which might precede decay of the body of the transcript, such as decapping. Over the past years, a number of methods to measure mRNA decay have been developed, a few of which are described in detail below. For a comprehensive review of techniques used for determining mRNA degradation rates, the reader is referred to some earlier reviews (Abler and Green, 1996; Ross, 1995).

The most common method to measure the decay rates of transcripts is to first shut off transcription and subsequently determine by RNA gel blot analysis the amount of transcript present as a function of time.

1. Chemical inhibitors: The inhibition of mRNA synthesis in plants is usually accomplished by treatment with drugs such as actinomycin D, cordycepin and α-amanitin. Actinomycin D interferes with transcription by intercalating into the DNA, while cordycepin acts as a chain terminating adenosine analog and has been shown to be a more efficacious inhibitor than actinomycin D in leaf tissue (Holtorf et al., 1999). α-amanitin is an inhibitor of eukaryotic RNA polymerases II and III and blocks transcription by binding to the polymerase. Since these drugs inhibit the transcription of many genes, half-lives of several mRNAs can be measured simultaneously, which is

especially important for genomic analysis. Such experiments are however most appropriate for measuring half-lives of short-lived mRNAs because of the potential deleterious effects of prolonged exposure to the drug. Also, it has been observed that the use of these drugs may increase the half-lives of some transcripts due to potential loss of labile factors involved in mRNA degradation (Dickey et al., 1994). In addition, the magnitude of the change in mRNA half-life in response to a particular stimulus may be dampened (Zhang et al., 1993).

2. Regulated promoters: A more specific method for measuring mRNA half-lives is to use a promoter that can be regulated to conditionally produce the transcript of interest. The rationale behind this approach is to first provide a stimulus that leads to a burst of mRNA synthesis followed by removal of the stimulus which causes rapid repression of synthesis. As a result, the mRNA of interest is synthesized for only a brief time, increases in abundance during that time and then degrades at a rate dependent on its half-life. Using this method, mRNA synthesis is repressed without resorting to toxic chemicals but with a high degree of synchrony (Ross, 1995). Also, it is unlikely that decay rates of mRNAs will be altered by depletion of labile turnover factors as they may be when global inhibitors of transcription are employed. An ideal inducible gene expression system should have low basal expression levels, high and specific inducibility, fast and efficient switch-off after the removal of the inducer and low toxicity. Inducible promoter systems that satisfy some of the aforementioned criteria have been developed in plants (Aoyama and Chua, 1997; Caddick et al., 1998; Padidam et al., 2003).

In contrast to the other positively regulated promoters, the negatively regulated Top10 promoter has been used most frequently (Gossen and Bujard, 1992). The Top10 promoter sequence, which has been used in plants, contains seven copies of the tetracycline operator DNA sequence fused to a truncated version of the CaMV 35S promoter (Weinmann et al., 1994). The Top10 promoter sequence is recognized by a transactivator that acts as a synthetic transcription factor. The transactivator is a chimeric fusion protein between the operator-binding portion of the bacterial tetracycline repressor fused to the activation domain of the herpes simplex virus transcription factor, VP16 (tTA) (Weinmann et al., 1994). In the absence of tetracycline, the tetracycline repressor region of tTA has a strong affinity for the operator DNA sequences within the Top10 promoter resulting in the expression of the desired gene. In contrast, in the presence of tetracycline, tTA binds tetracycline and is rapidly inactivated, leading to the repression of transcription. This strategy has been successfully used to measure Ferredoxin-1 mRNA stability in response to photosynthesis in transgenic tobacco plants (Petracek et al., 1998) and also to evaluate mRNA sequences that control the rate of *SAUR-AC1* transcript decay in transgenic tobacco cell lines (Gil and Green, 1996). Recently, it was shown that the Top10 promoter system is functional in Arabidopsis as well. Love et al. (2000) generated a homozygous Arabidopsis line in which tTA was expressed from a single transgenic locus. Individual plants from this line were then crossed with transgenic plants that expressed the ER-targeted green fluorescent protein (ER-GFP) from Top10 promoter. In the resulting progeny, GFP expression was seen to be stringently controlled by teracycline and the repression of the promoter was reversible upon tetracycline removal.

This system is thus an encouraging new approach for measuring mRNA half-lives in Arabidopsis.

3. RNA polymerase II: In yeast, an alternative method for inhibiting mRNA synthesis is to use a temperature-sensitive allele of the *RPB1* gene (*rpb1-1*), which encodes the large subunit of RNA polymerase II (Herrick et al., 1990). Use of the *rpb1-1* mutation to inhibit transcription again allows the half-lives of many mRNAs to be measured simultaneously and is also attractive because it promises to have fewer nonspecific effects compared to general transcriptional inhibitors. In theory it should be possible to extend the same technology to plants. Arabidopsis has a single gene for the large subunit of Pol II (Dietrich et al., 1990) and the residue corresponding to the ts Pol II mutation (*rpb1-1*) (Scafe et al., 1990) is conserved. Recent work by Maillet et al. (1999) also indicated that a null mutant of *RPB4* renders Pol II temperature sensitive in yeast and the kinetics for mRNA decay after a temperature shift are the same as in the *rpb1-1* mutant.

4. Microarray technology

With the application of genomics, monitoring mRNA stability can be achieved on a scale much larger than previously possible. Numerous groups have used DNA microarray analysis to determine mRNA half-lives on a global basis in both prokaryotic and eukaryotic systems (Bernstein et al., 2002; Holstege et al., 1998; Lam et al., 2001; Wang et al., 2002).

DNA microarrays have also been used to estimate mRNA half-lives for the genes of wild type Arabidopsis, by hybridizing spotted cDNA AFGC arrays to probes

corresponding to multiple time points after shutting off RNA synthesis with cordycepin (Gutiérrez et al., 2002). This study indicated that at least 1% of Arabidopsis transcripts represented on that array are rapidly degraded, with estimated half-lives of less than 60 minutes. Additional transcripts with half-lives of less than 120 minutes were also identified. The microarray results were very reliable and were confirmed by statistical analysis and by performing conventional half-life measurements with multiple time points quantitated on RNA gel blots (Gutiérrez et al., 2002). Ultimately, the ability to monitor mRNA decay on microarrays should help in the identification of transcripts that are differentially regulated at the level of mRNA stability in response to various stimuli.

STIMULI AFFECTING mRNA STABILITY

1. Plant hormones

Hormones regulate gene expression at many levels but an in depth understanding of their affect at the post-transcriptional level is lacking. The increase in the abundance of the mRNA encoding the light-harvesting chlorophyll *a/b* binding protein in response to cytokinin application in *Lemna gibba* occurs principally by a post-transcriptional mechanism (Flores and Tobin, 1988). mRNA stability has been shown to be responsible for the cytokinin-induced accumulation of *SrEnod2* mRNA in *Sesbania rostrata* (Silver et al., 1996). Downes and Crowell (1998) demonstrated that in cytokinin-starved soybean culture cells, *Cim1* mRNA is stabilized upon cytokinin treatment. The *Cim1* protein product is similar to the β-expansin proteins which are involved in cell wall expansion. *Cim1* abundance increases 20-60-fold within four hours of cytokinin addition to

cytokinin-starved soybean suspension cultures and analysis of *Cim1* stability revealed a greater than 4-fold increase in the half-life of the mRNA in response to cytokinin. In addition, *Cim1* accumulation is stimulated in the absence of cytokinin by the kinase inhibitor staurosporine and inhibited in the presence of cytokinin by the phosphate inhibitor okadaic acid, suggesting a role for protein dephosphorylation in cytokinin regulation of *Cim1* abundance (Downes and Crowell, 1998).

It has also been suggested that the increase in the abundance of the tomato *ERI* transcript in response to ethylene (Lincoln and Fischer, 1988) and the wheat *Em* transcript in response to ABA (Williamson et al., 1985) is controlled at the level of transcript stability but as yet there is no direct evidence.

2. Light

Light affects plant gene expression at many levels, including mRNA stability. The ferredoxin-encoding genes *Fed-1* from pea (Dickey et al., 1998) and *FedA* from Arabidopsis (Vorst et al., 1993) show increased mRNA accumulation in light-grown leaves. The *FedA* transcript is 20-fold higher in the light relative to the dark, while the transcriptional activity is only 2-fold higher. This discrepancy suggests that the transcript is either stabilized in the light or destabilized in the dark.

The pea *Fed-1* mRNA is 5-fold higher in the light than in the darkness. Using the Top10 promoter in transgenic tobacco plants, it was determined that *Fed-1* mRNA is post-transcriptionally regulated by light at the level of mRNA stability as its half-life is significantly reduced in the dark (Petracek et al., 1998). The region mediating the rapid degradation of the *Fed-1* mRNA includes the 5' UTR and a portion of the coding region

and is called the iLRE (internal light response element; Dickey et al., 1992). In order for the *Fed-1* mRNA to be light responsive, an open reading frame is required (Dickey et al., 1994), suggesting that the light effect on *Fed-1* mRNA is dependent on its synchronous translation. In addition, the insertion of nonsense codons within the *Fed-1* coding sequence disrupts the light regulation of *Fed-1* mRNA abundance (Petracek et al., 2000). When the *Fed-1* gene from pea is expressed in tobacco plants grown in light, *Fed-1* mRNAs are found in high molecular weight polysomes. Following a shift to the dark, the transcript rapidly dissociates from polysomes (Petracek et al., 1998), indicating that translation of the *Fed-1* mRNA is repressed by dark and this may trigger rapid degradation of the *Fed-1* mRNA. Treatment of plants with the electron transport inhibitor DCMU also results in destabilization of the *Fed-1* mRNA (Petracek et al., 1998). Therefore, it may be the cessation of photosynthesis when plants are shifted to dark that triggers *Fed-1* decay, rather than the mere absence of light.

In pea, a single pulse of high-fluence blue light results in an increased rate of *Lhcb* transcription, with no change in the steady-state level of *Lhcb* mRNA (Kaufman, 1993), suggesting that the excitation of the high-fluence blue light system results in destabilization of the *Lhcb* mRNA. Anderson et al. (1999) demonstrated that *Lhcb* RNA levels in etiolated Arabidopsis are also regulated in a similar manner by the high-fluence blue light system and the 65 bp 5' UTR of *AtLhcb* is necessary and sufficient for RNA destabilization. The blue light-induced destabilization response is not dependent on the phytochrome or the cryptochome receptors (Anderson et al., 1999; Folta and Kaufman, 1999) but the phototropin 1 photoreceptor seems to required for the blue-light mediated destabilization of the *Lhcb* transcript (Folta and Kaufman, 2003).

3. Sucrose

α-amylases are major amylolytic enzymes and play an important role in the degradation of starch. In rice germinating embryos and cultured suspension cells, expression of α-amylase genes is activated by sugar depletion and suppressed by sugar provision (Yu et al., 1996). The half-lives of *αAmy3*, *αAmy7* and *αAmy8* have been shown to be prolonged by sucrose starvation, with the mRNA half-life of *αAmy3* increasing from 1.5 h to 6 h in sucrose-starved cells (Sheu et al., 1996); however, the stability of these three mRNAs appears to be controlled by different mechanisms. The translation inhibitors cylcohexamide and anisomycin enhanced the accumulation of *αAmy3* mRNA regardless of whether or not the cells were provided with sucrose, while the accumulation of *αAmy7* and *αAmy8* mRNA was suppressed by the inhibitors, even in cells starved for sucrose. Moreover, cyclohexamide did not significantly alter the transcription rates of α-amylase genes, suggesting that labile proteins are involved in the stabilization of the *αAmy7* and *αAmy8* mRNAs but destabilization of the *αAmy3* mRNA. Examination of chimeric gene expression in stably transformed rice cells has shown that the regulatory sequences in the *αAmy3* 3' UTR act as potent determinants of mRNA stability in response to sugar availability (Chan and Yu, 1998).

A recent study conducted by Ho et al. (2001) showed that sugar activated or repressed gene expression in rice suspension cell cultures and this regulation operates at the levels of both transcription rate and mRNA stability. Amongst the sucrose-upregulated genes that were tested, the half-lives of actin, glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase and sucrose synthase P-2 mRNAs were approximately 1.6-2.6-fold longer in sucrose-provided cells than in sucrose-starved cells.

For all the tested sucrose-downregulated genes, the half-lives of mRNAs were 2.5-7.4-fold higher in sucrose-starved cells than in sucrose-provided cells (Ho et al., 2001).

4. Nitrogen

Nitrogen is an essential plant macronutrient and plants obtain nitrogen either from the soil or through symbiotic nitrogen fixation. Nitrate and nitrogen are reduced to ammonia, which is assimilated using two enzymes Gln synthase (GS) and Glu synthase (GOGAT). In higher plants, GS occurs as two isoforms, GS_1 in the cytoplasm and GS_2 in plastids. To determine if GS_1 genes in alfalfa were regulated at the post-transcriptional level, Ortega et al. (2001) analyzed transgenic alfalfa plants containing the soybean GS_1 gene under the control of the CaMV 35S promoter. A 3-4-fold drop in the level of the soybean GS_1 transcript in transgenic alfalfa plants, that were fed nitrate over their non-nitrate-fed counterpart, was observed. This difference in transcript levels could not be attributed to differential promoter activity and furthermore the transcript for the alfalfa endogenous GS_1 gene also showed a 3-fold drop in levels in the leaves of nitrate-fed plants. These results indicate that the decrease in GS_1 transcript levels in nitrate-fed plants is most likely due to increased turnover of the GS_1 transcript.

5. Methionine

Methionine biosynthesis is tightly regulated in plants and the first committed step in methionine biosynthesis is catalyzed by cystathione γ-synthase (CGS), suggested to be a major regulatory site of the pathway (Ravanel et al., 1998). Analysis of Arabidopsis *mto* mutants that overaccumulate soluble methionine demonstrated that the gene for CGS

is regulated at the level of mRNA stability (Chiba et al., 1999). Treatment of Arabidopsis calli with actinomycin D showed that turnover of *CGS* mRNA, in the absence of methionine, was faster in wild type than the *mto1-1* mutant. Methionine treatment accelerated the turnover in wild type but not in the mutant. It was also demonstrated that a 11-13 amino acid region encoded by the first exon of the *CGS* gene is sufficient and essential for down-regulating its own mRNA stability in response to methionine or one of its metabolites (Suzuki et al., 2001; Ominato et al., 2002) and ongoing translation is probably required (Lambein et al., 2003).

5. Biotic stress

In plants, an important process activated by infection or wounding is the remodeling of the cell wall (Mehdy and Brodl, 1998). A key constituent of the response to pathogens appears to be the degradation of a subset of preexisting mRNAs. An excellent example of the regulation of mRNA stability in response to biotic stress has been characterized in *Phaseolus vulgaris*. In bean cells treated with fungal elicitor, the transcripts of *PvPRP1*, a gene encoding a proline-rich protein believed to be a component of the cell wall, decrease to ~6% of the original level within 4 hr (Zhang et al., 1993). After actinomycin D treatment, the *PvPRP1* transcript has a half-life of 60 hr in the absence of fungal elicitors and 18 hr in the presence of fungal elicitors. Furthermore, transcriptional rates remain constant regardless of the presence or absence of the elicitor. A subsequent study by Zhang and Mehdy (1994) identified a 50 kDa protein, PRP-BP, which specifically binds to a U-rich sequence in the 3' UTR of *PvPRP1* mRNA (see later for details). The RNA-binding activity of PRP-BP is redox regulated *in vitro* and its

activity is induced in response to fungal elicitor treatment prior to the onset of *PvPRP1* mRNA degradation. On the basis of PRP-BP activation upon elicitor treatment and its specificity of RNA binding, it has been postulated that the interaction of PRP-BP with *PvPRP1* mRNA may be one component in the process of elicitor-induced *PvPRP1* mRNA degradation (Mehdy and Brodl, 1998).

In soybean cells, glucan elicitors induce a decrease of the *tubB1* (β-tubulin 1 isoform) transcript level, most likely by enhancing its degradation, while the transcript level of another tubulin isoform *tubB2* is not affected by this elicitor (Ebel et al., 2001). Direct evidence that the major control of this down-regulation is at the level of mRNA stability was provided by the observation that in the presence of cordycepin, the half-life of *tubB1* mRNA decreased upon addition of the elicitor. Pre-incubation with Ca^{2+} modulators blocked the decrease of *tubB1* mRNA levels, suggesting that calcium might be involved in the regulation of *tubB1* message levels. The down-regulation of *tubB1* mRNA levels induced by these elicitors could result from a general redirection of the available cellular resources to defense-related metabolism (Ebel et al., 2001). In mammalian cells, β-tubulin mRNAs are destabilized in the presence of free tubulin heterodimers (Theodorakis and Cleveland, 1992) and a similar mechanism could be occurring in plant cells where *tubB1*mRNA is degraded due to depolymerization of specific microtubules.

6. Abiotic stress

Abiotic stress may result from water deficit or excess, extreme heat or cold, toxic substances, salinity or drought and ultraviolet light. Like biotic stress, the regulation of

mRNA stability is an important step for the regulation of some genes in the abiotic stress responses. The steady-state level of transcripts encoding the pyrroline-5-carboxylate reductase of Arabidopsis (*At-P5R*) is upregulated under salt and heat stress and this induction is mainly due to an enhanced mRNA stability (Hua et al., 2001) mediated by its 5' UTR. In carrots, heat shock disrupts the function of the 5' cap and the poly(A) tail structures, resulting in the loss of translational competence but increases in mRNA stability (Gallie et al., 1995). In wheat, it has been reported that the activity of RNases decreases following heat shock and is in correlation with an increase in mRNA half-life (Chang and Gallie, 1997). In gibberellic acid-stimulated barley aleurone layers, heat shock reduces the half-life of α-amylase mRNA (Belanger et al., 1986) and other secreted hydrolases but does not affect the mRNA levels for non-secretory proteins (Brodl and Ho, 1991). Heat shock also decreases the levels of some wound-inducible mRNAs encoding extra-cellular proteins in carrot root disks and using cordycepin as a transcriptional inhibitor, it was shown that this decrease in mRNA levels is due to accelerated mRNA turnover during heat shock (Brodl and Ho, 1992).

Plants vary widely in response to cold temperature and there is evidence that altered gene expression occurs during cold acclimation (Thomashow, 1998). The increase in transcript levels for some *cor* (*co*ld-*r*egulated) genes from Arabidopsis, in response to cold stress, has been shown to be mainly at the posttranscriptional level, possibly due to increased transcript stability (Hajela et al., 1990). In alfala, transcripts of a cold acclimation-specific gene *cas18* are destabilized upon return to warm temperature (Wolfraim et al., 1993).

cis- ACTING DETERMINANTS OF mRNA STABILITY

The majority of mRNAs fall in the stable range of mRNA half-lives for a given organism (Taylor and Green, 1995; Ross, 1996). This observation led to the hypothesis that mRNAs might have sequence elements that can either act constitutively to establish the inherent instability of a particular transcript or modulate the stability of an mRNA in response to certain physiological, developmental, or environmental cues. Many *cis*-acting elements have been identified that target transcripts for rapid turnover in plants as well as other systems (reviewed in Abler and Green, 1996; Ross, 1995).

A number of studies conducted over several years have suggested a function for the 7-methyl-G cap at the 5' end and the poly(A) tail at the 3' end, two *cis*-acting determinants that are common to all plant mRNAs, as mRNA stabilizing structures. It is known that in the major mRNA degradation pathway of yeast these stabilizing structures are removed, or at least in the case of the poly(A) tail, greatly shortened, prior to degradation catalyzed by exoribonucleases (Decker and Parker, 1993).

1. DST element

In plants, an instability determinant called DST (Newman et al., 1993) has been studied in detail. The DST (**d**own**st**ream) element was first identified as a highly conserved sequence in the 3' UTRs of the soybean *SAUR* genes (McClure et al., 1989). The *SAUR* (small-auxin-up-RNAs) genes encode unstable transcripts whose half-lives have been estimated to be on the order of 10-50 minutes (McClure and Guilfoyle, 1987; Franco et al., 1990). Although the function of the SAUR proteins is unknown, the temporal and spatial expression of *SAUR* genes correlates with auxin-induced cell

elongation (McClure and Guilfoyle, 1989). Detailed studies of *SAUR* gene expression in Arabidopsis have been carried out on the *SAUR-AC1* gene, which contains the DST element in its 3' UTR. Examination of chimeric gene expression has shown that the promoter region is responsible for auxin induction, the 3' UTR is largely responsible for rapid mRNA turnover and the coding region contributes to low mRNA abundance but not by decreasing mRNA half-life (Gil and Green, 1996).

A synthetic dimer of the DST element has been demonstrated to be sufficient to destabilize normally stable reporter transcripts in stably transformed tobacco cell suspension cultures (Newman et al., 1993). 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 prototype DST, from *SAUR15A* of soybean, is about 45 base pairs in length and consists of three highly conserved subdomains separated by two variable regions (Newman et al., 1993). Site-directed mutagenesis studies have been performed in order to determine which features of the DST element are critical for its instability function (Sullivan and Green, 1996). Residues within the conserved second and third subdomains, the ATAGAT and the GTA regions respectively, have been found to be necessary for the DST element to function as an instability determinant.

As mentioned above, the DST element has 3 conserved subdomains, of which the ATAGAT and the GTA subdomains are critical for its instability function. Five- and six-base substitutions in the ATAGAT and the GTA regions resulted in inactivation of the instability function of the DST element in suspension cell cultures as well as in transgenic plants, while smaller two-base substitution mutations resulted in inactivation of DST in

transgenic tobacco leaves but had varying effects on DST function in tobacco cell culture (Sullivan and Green, 1996). These results suggest that the DST element might be recognized differently in different cell types.

Interestingly, the *SAUR-AC1* 3' UTR contains one canonical DST element located 80 bp downstream of the stop codon and 10 bp upstream of the poly(A) addition site (Gil et al., 1994). Earlier experiments have indicated that two copies of the synthetic DST element are required for instability function (Newman et al., 1993). 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 sites may be serving as multiple recognition sites for DST-mediated decay within the *SAUR-AC1* 3' UTR. Recently additional genes have been identified that have DST-like subdomains rather than a classical DST sequence like those in soybean *SAUR* genes (Pérez-Amador et al., 2001). Furthermore there is accumulating data in favor of multiple subdomains being sufficient for instability function (Feldbrügge et al., 2002).

Thus far, the DST element appears to be unique to plants. Visual inspection of known eukaryotic instability determinants has not led to the identification of any elements that contain all the characteristic features of a DST element. However, similarities have been noticed in sequence between the GTA region and animal mRNA elements which are bound by proteins containing a Pumilio-like RNA-binding domain (Feldbrügge et al., 2002; Zamore et al., 1999). To investigate whether the plant DST mRNA instability determinant is also recognized in mammalian cells, DST element variants were tested in mouse NIH3T3 fibroblasts, a well-defined model system to monitor mRNA decay in mammalian cells (Zubaiga et al., 1995). From the

aforementioned experiments, it seems that the plant DST element is not recognized in animal cells with the same sequence requirements as in plant cells. Therefore, its mode of recognition may be plant-specific (Feldbrügge et al., 2002). Interestingly, the GTA region interacts with the human Pumilio-like protein HsPUM in *in vitro* binding studies (Feldbrügge et al., 2002). This raises the intriguing possibility that this region is recognized by Arabidopsis proteins containing the same type of RNA-binding domain i.e. AtPUMs.

2. AUUUA sequences

The most widely studied instability determinants in mammalian cells are the AU-rich elements (AREs). AREs are found in the 3' UTRs of several of the most unstable mammalian transcripts, such as lymphokine, cytokine and proto-oncogene mRNAs (Chen and Shyu, 1995). Repeats of the pentamer, AUUUA, are often found in these AU-rich elements and have been shown to be important for their instability function (Shyu et al., 1991; Vakalopoulou et al., 1991). Many functional AREs mediate deadenylation as the first step in mRNA decay, although different classes of AREs exhibit different reaction kinetics (Chen and Shyu, 1995).

AUUUA repeats are likely to be of broad significance in higher eukaryotes, since they can target transcripts for rapid decay in plants (Ohme-Takagi et al., 1993), and recent evidence suggests that the ARE-mediated decay pathway is functional in yeast as well (Vasudevan and Peltz, 2001). Reporter transcripts containing 11 repeats of the AUUUA motif were degraded more rapidly in plants as compared to an AU-rich control lacking AUUUA repeats (Ohme-Takagi et al., 1993). AUUUA motifs present in the 3'

UTR of *PvPRP1* have been proposed to trigger mRNA degradation (Zhang and Mehdy, 1994) in common bean. Three AUUUA motifs are present in the 3' UTR of *tubB1* but none have been found in the 3' UTR of *tubB2*, which suggests differential regulation via degradation or stabilization of the message for the two different tubulin isoforms, as discussed earlier (Ebel et al., 2001). In rice, the entire *αAmy3* 3' UTR and two of its subdomains can independently mediate sugar-dependent repression of reporter mRNA accumulation (Chan and Yu, 1998). Examination of the nucleotide sequences has revealed that domains I and III each contain a stretch of a 9-bp AU-rich conserved sequence. Moreover, RNA structure prediction of the 3' UTR identified extensive regions of putative duplex formation, and regions encompassing domains I, II and III each contained a putative stem-loop structure. Also the 9-bp conserved AU-rich sequence is located in the loop regions of both domains I and III (Chan and Yu, 1998).

3. 5' UTR

Theoretically, the half-life of all mRNAs can be affected by how its 5' UTR influences its translational efficiency. Light-mediated changes in transcript stability are known to occur for the *Fed-1* mRNA in pea (Dickey et al., 1992). A major light response element, iLRE, in the pea *Fed-1* gene is located within the transcription unit and spans a portion of the 5' UTR and the first 20 codons of the coding region. A CATT repeat element, located near the 5' UTR, has been identified as being essential for light regulation (Dickey et al., 1998). This element is important for mRNA stability since two different mutations in the CATT repeat element altered dark-induced *Fed-1* mRNA disappearance. Recently it was demonstrated that mRNA containing the *Fed-1* iLRE

ceases translation and dissociates from polyribosomes soon after plants are transferred from light to darkness, providing support for the model that *Fed-1* mRNA is protected from degradation in light by association with ribosomes and/or the act of translation (Hansen et al., 2001).

The 5' UTR of the pea *Lhcb1*4* transcript contains a sequence involved in the regulation of transcript stability (Anderson et al., 1999). No known specific mechanisms by which the destabilization mediated through the 5' UTR occurs have been reported. However, it is plausible to postulate that the element somehow stalls translation, exposes the RNA which is then subject to digestion by RNase activity (Abler and Green, 1996). The first 92 bp region of the *At-P5R* 5' UTR is sufficient to mediate transcript stabilization during salt and heat stress (Hua et al., 2001). *In silico* analysis has predicted that extensive secondary structures in the 5' UTR and high GC content further stabilize the secondary structures. The *At-P5R* 5'UTR also contains a 26 bp sequence, repeated seven times, which has sequence identity with a part of the 3' non-coding region of a potential retrovirus (Hua et al., 2001). It is possible that the 26 bp region binds protein factors stabilizing the secondary structures during stress.

GENERAL mRNA DECAY MACHINERY

In contrast to the factors that are involved in the recognition of specific sequences within mRNAs, proteins which are responsible for general mRNA degradation can be considered to constitute the basal mRNA degradation machinery. The basal mRNA decay machinery catalyzes the degradation of mRNAs from many different genes, while

sequence specific mRNA binding proteins probably recruit the basal mRNA decay machinery to specific target molecules, or modulate the degradative activity of the basal machinery.

In yeast, mRNA turnover is mediated by mRNA decay pathways that use shared, as well as distinct components of the basal mRNA decay machinery (reviewed in Tourrière et al., 2002). It is likely that mRNA decay in Arabidopsis, as well as in other higher plants also occurs through multiple degradation pathways. There are several Arabidopsis homologs of the yeast basal mRNA decay machinery suggesting that mRNA turnover in Arabidopsis may resemble the mRNA decay pathways of yeast (Gutiérrez et al., 1999). However, plant-specific mRNA decay pathways and mechanisms are also likely to be functioning since apparent differences have been observed between the two systems (Kastenmayer et al., 2001).

The mechanisms responsible for mRNA degradation are most well understood in yeast. In yeast, the deadenylation-dependent-decapping pathway appears to be the main pathway for mRNA degradation. In this pathway, mRNAs are deadenylated by a complex of proteins including Caf1p and Ccr4p (Daugeron et al., 2001; Tucker and Parker, 2000). This deadenylation reaction shortens the poly(A) tail to a length of 10-12 adenylates (Decker and Parker 1993). Shortening of the poly(A) tail then triggers decapping catalyzed by Dcp1p (LaGrandeur and Parker, 1998) and associated factor Dcp2p (Dunckley and Parker, 1999), after which the decapped transcripts are degraded from the 5' end by the 5'-3' exoribonuclease Xrn1p (Muhlrad et al., 1994).
In addition to the major mRNA decay pathway, several minor mRNA decay pathways operate in yeast. The nonsense mediated decay pathway, responsible for the degradation

of mRNAs containing premature nonsense codons, is similar to the major decay pathway; however, 5'-3 degradation catalyzed by Xrn1p is not dependent on prior deadenylation (Muhlrad and Parker, 1994). Degradation of mRNAs is also catalyzed from the 3' end following deadenylation by a multi-protein complex, the exosome. The exosome and its associated co-factors in yeast consists of greater than 15 proteins, at least 10 of which are homologous to 3'-5' exoribonucleases from *E. coli* (van Hoof and Parker, 1999).

SEQUENCE-SPECIFIC DECAY

Sequence-specific mRNA degradation determines the degradation rate of particular mRNAs. The instability determinants can be thought to target certain transcripts to the mRNA decay machinery. In mammals, sequence-specific RNA binding proteins have been identified that interact *in vitro* with sequences that regulate mRNA stability. A number of proteins have been hypothesized to function in ARE-mediated decay based on *in vitro* activity, the best characterized of which are the human HuR/HuA protein and AUF1/hnRNP D. HuR belongs to a family of RNA-binding proteins related to the *Drosophila* embryonic lethal abnormal visual (ELAV) proteins. Overexpression of HuR *in vivo* (Fan and Steitz, 1998) and increased levels of HuR or other ELAV-like proteins *in vitro* (Ford et al., 1999) lead to stabilization of ARE-containing mRNAs. In contrast, *in vivo* depletion of AUF1/hnRNP D leads to a strong stabilization of diverse ARE-containing mRNAs whereas ectopic expression restores destabilization (Loflin et al., 1999). These data suggest that HuR and hnRNP D have antagonistic effects on the stability of ARE-containing mRNAs.

For another mammalian protein, tristetraprolin, a mouse insertional mutant was available, and demonstrating a role in ARE-mediated decay was more definitive. Half-life measurements showed that *TNF-α* mRNA, which contains AUUUA elements in its 3'UTR, was stabilized in cells from the tristetraprolin deficient mutant relative to wild-type (Carballo et al., 1998). Subsequently, the protein was shown to bind AUUUA sequences *in vitro* indicating that it functions to target *TNF-α* for ARE-mediated decay (Lai et al., 1999). More recently, it has been shown that tristetraprolin deficient mice are also defective in the deadenylation of ARE-containing *GM-CSF* mRNA (Carballo et al., 2000).

To date, no mRNA-binding proteins have been identified in plants that are known to play a role in the control of mRNA stability. One plant mRNA-binding protein which may function in controlling stability is a 50 kDa protein that binds to a sequence in the 3' UTR of the bean *PvPRP1* mRNA (Zhang and Mehdy, 1994). The *PvPRP1* transcript, which appears to encode a cell wall protein, is rapidly degraded following the addition of fungal elicitor. 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 in response to fungal elicitor treatment, consistent with this scenario.

Genetic approaches have rarely been applied to mRNA stability problems in multicellular organisms. The majority of the studies aimed at understanding the cellular factors in mRNA decay pathways have involved characterization of proteins that bind the instability sequences *in vivo*. Many RNA-binding proteins have been isolated that interact with AREs *in vitro* (discussed above). Isolation of sequence-specific RNA-

binding proteins, involved in mRNA stability, 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. This may be due to the presence of the plant vacuole, which is known to contain many non-specific ribonucleases and other hydrolytic enzymes that are released upon cell rupture during cell extract praparation.

One exception is the recent isolation of mutant mammalian cell lines that stabilize green fluorescent protein (GFP)-IL-3 reporter transcripts (Stoecklin et al., 2000). In this screen, two loci involved in rapid mRNA turnover mediated by the interleukin-3 (IL-3) 3'UTR were identified by screening mutagenized human HT1080 fibrosarcoma cells for elevated green flourescence. One of the mutants was rescued by a cDNA corresponding to butyrate-response factor-1 (BRF1), which is a Zn finger protein homologous to tristetraprolin (Stoecklin et al., 2002).

In order to understand the molecular basis of sequence-specific recognition and degradation of unstable mRNAs, an approach was devised to isolate *Arabidopsis* mutants defective in DST-mediated mRNA degradation (Johnson et al., 2000). This approach allows several important advantages, such as:

1. Genes identified in a mutant selection would be very likely to play a role in mRNA degradation *in vivo*. Of particular interest would be the cellular factors that might be involved in sequence-specific interactions.
2. Basic information about the mechanisms of mRNA degradation may be obtained by studying mutants since a genetic approach offers an alternative to biochemical approaches that have proved difficult.

dst1, *dst2* and *dst3* were isolated based on their ability to stabilize specific DST-containing transgene mRNAs (Johnson et al., 2000; Chapter 5). For this strategy, two transgenes, hygromycin phosphotransferase (HPH) and ß-glucuronidase (GUS), both containing a tetramer of the consensus DST element in the 3' UTR, were introduced into Arabidopsis as selectable and screenable markers, respectively. The presence of the DST elements decreased the mRNA levels for both transgenes, *HPH-DST* and *GUS-DST*, resulting in decreased resistance to hygromycin and low GUS activity. The *dst* mutants were isolated as hygromycin resistant plants with increased GUS activity due to stabilization of the corresponding mRNAs. In addition, *dst* mutants have elevated levels of *SAUR-AC1* mRNA, the only endogenous DST-containing unstable transcript characterized thus far (Gil and Green, 1996; Johnson et al., 2000). The *dst* mutants that we isolated are extremely rare (3/~800,000), are all codominant and exhibit no obvious morphological or developmental phenotype (Johnson et al., 2000). The simplest explanation to account for the mutant phenotype observed in the *dst* mutants would be that the gene corresponds to a sequence-specific RNA-binding protein or a ribonuclease. Alternatively, it could encode for a regulatory factor that controls one of these components.

To evaluate the physiological significance of the DST-mediated mRNA degradation pathway, we sought to identify additional molecular markers of *dst1* using DNA microarrays. In addition to the identification of novel targets of *dst1*, the microarray experiments provided the first indication of an association between circadian rhythms and DST-mediated decay (Pérez-Amador et al., 2001; Chapter 3).

PERSPECTIVE

Recent advances have begun to shed light on the mechanisms that underlie mRNA turnover in eukaryotic systems. Exciting progress has been made with *cis*- and *trans*-acting determinants of mRNA stability, but there is much to be learned about how other factors such as exogenous or endogenous stimuli interact with and affect mRNA decay. The identity of all the players participating in the mRNA degradation process as well as the rules governing specific RNA degradation remains to be elucidated. By combining the power of genomics with traditional genetic and biochemical approaches, it should be possible to better define the influence of transcript stability on gene expression.

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CHAPTER 2

NEW MOLECULAR PHENOTYPES OF THE *dst1* MUTANT REVEALED BY MICROARRAY ANALYSIS

Part of this chapter was published in "Pérez-Amador, M. A., Lidder, P., Johnson, M. A., Landgraf, J., Wisman, E., and Green, P. J. (2001). New molecular phenotypes in the *dst* mutants of Arabidopsis revealed by DNA microarray analysis. Plant Cell 13, 2703-2717. Experiments done by Pérez-Amador, M.A. or in collaboration with Pérez-Amador, M.A. are clearly marked.

INTRODUCTION

Cells must be able to adjust gene expression patterns quickly in order to respond to intra- and extracellular stimuli; therefore, it is necessary for certain transcripts to reach new steady-state levels rapidly. The steady state levels of eukaryotic mRNAs are determined by both their rate of synthesis and rate of degradation. The control of mRNA stability is a major determinant of steady-state messenger RNA levels in the cell and often has a great impact on the level at which a particular gene is expressed. Further, mRNA stability affects the rate at which new steady state RNA levels are achieved after changes in transcription; the more unstable the mRNA, the more rapidly it reaches a new steady state (Abler and Green, 1996).

Most recent studies on mRNA stability in eukaryotes have focused on transcripts that are relatively unstable, generally with half-lives of less than 60 minutes (Ross, 1995; Johnson et al., 1998). Unstable transcripts are particularly interesting because they often correspond to genes that must be rapidly or stringently controlled, such as those involved in regulating cell growth and differentiation. Transcripts that fall into this category include phytochrome mRNA (Higgs et al., 1995) and several auxin-induced transcripts (McClure and Guilfoyle, 1989) in plants, mating-type transcripts in yeast (Peltz and Jacobson, 1992) and several proto-oncogene transcripts in mammalian cells (Greenberg and Belasco, 1993).

The majority of mRNAs fall in the stable range of mRNA half-lives for a given organism (Taylor and Green, 1995; Ross, 1996). Thus, within the body of unstable mRNAs, specific sequence motifs must be present that can either act constitutively to

establish the inherent instability of a particular transcript or modulate the stability of an mRNA in response to certain physiological, developmental, or environmental cues.

The most widely studied instability determinants are the mammalian AU-rich elements (AREs). AREs are found in the 3' untranslated region (UTR) of several of the most unstable mammalian transcripts, such as lymphokine, cytokine and proto-oncogene mRNAs (Chen and Shyu, 1995). Repeats of the pentamer AUUUA are often found in these AU-rich elements and are important for their instability function (Shyu et al., 1991; Vakalopoulou et al., 1991). AUUUA repeats are likely to be of broad significance in higher eukaryotes, since they can target transcripts for rapid decay in plants (Ohme-Takagi et al., 1993), and recent evidence suggests that the ARE-mediated decay pathway is functional in yeast as well (Vasudevan and Peltz, 2001). It seems that all functional AREs mediate deadenylation as the first step in mRNA decay, although different classes of AREs exhibit different reaction kinetics (Chen and Shyu, 1995).

In plants, besides AREs, an instability determinant called DST (Newman et al., 1993) has been studied in detail. The DST (**d**own**st**ream) element was first identified as a highly conserved sequence in the 3' UTRs of the soybean *SAUR* genes (McClure et al., 1989). The *SAUR* (small-auxin-up-RNAs) genes encode unstable transcripts whose half-lives have been estimated to be on the order of 10-50 minutes (McClure and Guilfoyle, 1987; Franco et al., 1990). Although the function of the SAUR proteins is unknown, the temporal and spatial expression of *SAUR* genes correlates with auxin-induced cell elongation (McClure and Guilfoyle, 1989). The prototype DST, from *SAUR15A* of soybean, is about 45 base pairs in length and consists of three highly conserved subdomains separated by two variable regions (Newman et al., 1993). Mutagenesis

studies have demonstrated that residues within two of the conserved subdomains, the ATAGAT and GTA regions, are necessary for the instability function (Sullivan and Green, 1996).

In order to gain insights into the cellular components involved in the DST-mediated mRNA degradation pathway, we isolated Arabidopsis mutants defective in their ability to recognize DST elements. *dst1* and *dst2* were isolated based on their ability to stabilize specific DST-containing transgene mRNAs (Johnson et al., 2000). For this strategy, two transgenes, hygromycin phosphotransferase (HPH) and ß-glucuronidase (GUS), both containing a tetramer of the consensus DST element in the 3' UTR, were introduced into Arabidopsis as selectable and screenable markers, respectively. The presence of the DST elements decreased the mRNA levels for both transgenes, *HPH-DST* and *GUS-DST*, resulting in decreased resistance to hygromycin and low GUS activity. The *dst* mutants were isolated as hygromycin resistant plants with increased GUS activity due to stabilization of the corresponding mRNAs. In addition, *dst* mutants have elevated levels of *SAUR-AC1* mRNA, the only endogenous DST-containing unstable transcript characterized thus far (Gil and Green, 1996; Johnson et al., 2000). The only reported phenotype of the *dst* mutants is the elevation of these three mRNAs due to increased mRNA stability. Genetic analysis of two *dst* mutants isolated via this selection showed that they are incompletely dominant and represent two independent loci. The *dst* mutants exhibit no obvious morphological or developmental phenotype (Johnson et al., 2000). The main objective of the present work was to search for additional genes that were directly or indirectly regulated by this pathway.

Genes differentially regulated in mutant backgrounds have been identified in the past by a variety of techniques, such as differential display and subtractive hybridization (Kehoe et al., 1999). Now, with the application of plant genomics, monitoring gene expression can be achieved on a scale much larger than previously possible. DNA microarray technology (Schena et al., 1995) takes advantage of the vast amount of information generated by the genome sequencing projects, as well as the large number of expressed sequence tag (EST) clones isolated and sequenced from different organisms. In addition to dramatic changes in mRNA levels that can be identified by differential display or other techniques, DNA microarrays allow the detection of more subtle changes in gene expression (Kehoe et al., 1999). By comparing global patterns of gene expression in a mutant compared to wild type, we now have the unique opportunity to identify changes in biochemical processes due to the mutation. This approach may be especially fruitful when studying a mutation that affects the regulation of mRNA abundance and may be of particular interest when the mutation does not generate an obvious morphological phenotype.

Recently published studies have demonstrated how microarrays containing a large number of Arabidopsis genes can provide a powerful tool for plant gene discovery, functional analysis, and elucidation of genetic regulatory networks (Kreps et al, 2002; Mandaokar et al, 2003; Martzivanou and Hampp, 2003; Seki et al., 2001; Schaffer et al., 2001; Schenk et al., 2000). Plant microarray experiments have begun to focus on mutant analyses in addition to gene expression in wild-type plants. A pioneering microarray study carried out by Reymond et al. (2000) highlighted the promise of this approach, in which the coronatine-insensitive *coi1-1* Arabidopsis mutant was analyzed on DNA

microarrays, resulting in the classification of a large number of *COI1*-dependent and *COI1*-independent wound-inducible genes. Other studies have been carried out with Arabidopsis mutants since then providing further insight into various biological pathways (Goda et al, 2002; Scheible et al, 2003; Wang et al, 2002).

In this report, we describe the use of DNA microarrays containing more than 11,000 Arabidopsis expressed sequence tags (ESTs), representing approximately 7,800 unique genes, to examine gene expression in the DST-mediated mRNA degradation mutant *dst1*. Our results indicate that DNA microarrays are a powerful tool to identify new molecular markers affected by DST-mediated mRNA decay, some of which are likely direct targets of the pathway. Furthermore, our results suggest new experimental directions to pursue the biological significance of the pathway.

RESULTS

Generation of a 600-element DNA microarray

As a first step towards analyzing the *dst1* mutant for additional changes in gene expression, a DNA microarray representing approximately 600 Arabidopsis genes was assembled. The clones included were mainly ESTs from the MSU collection (Newman et al., 1994). Table 2.1 includes a description of these genes, grouped according to function or sequence similarity. Because the *dst1* mutant affects RNA metabolism, half of the clones included on the microarray were those predicted to be associated with some aspect of RNA metabolism. The criteria for the selection of many of these EST clones was their sequence similarity to potential RNA metabolism genes previously described in Arabidopsis or other organisms.

Table 2.1. Genes included on the 600-element DNA microarray (done by Pérez-Amador, M.A.)

Category	Number of genes
Pathogen-related	70
Lipid metabolism	40
Wounding/jasmonic response	25
MAPK	25
Protein transport vacuole/chloroplast	30
Senescence-associated	10
Auxin-induced	10
Polyamine metabolism	5
RNA metabolism	300
RNA binding proteins	70
Helicases	20
Transcription/translation	20
RNases	15
Splicing factors/snoRNP	15
Nonsense decay	8
Putative DST-containing genes	140
AU-rich genes	10
Other	20
Controls	76
Highly expressed/well studied	17
Ribosomal proteins	15
rRNA	2
Transgenes	5
Human clones	12
Other	25

Thus far, the *SAUR-AC1* transcript is the only known target for the DST-dependent mRNA degradation pathway in Arabidopsis (Johnson et al., 2000). To identify additional direct targets of this pathway, genes with possible DST elements were included. The strategy to identify genes containing potential DST elements was to combine the results of several pattern searches using different degenerate criteria, since our knowledge of what constitutes a functional DST element is still rather limited (Newman et al., 1993; Sullivan and Green, 1996). Because AREs also function in plants as mRNA instability elements (Ohme-Takagi et al., 1993), genes with AU-rich elements were selected in a similar manner, by searching for variations of the element AUUUA in an AU-rich context in the 3' UTR. As a positive control, the *HPH* and *GUS* coding sequences were also included so that the mutant phenotype of *dst1* could be confirmed.

Approximately 300 additional clones were included on the microarray to expand coverage beyond genes associated with RNA metabolism. A complete list of the clones on this array and the raw microarray expression data can be found at http://www.bch.msu.edu/pamgreen/Perez-Amador_etal/600_list.htm.

The expression levels for most genes are similar in *dst1* and the parental plants

The *dst1* mutant has a subtle phenotype; it exhibits a 3- to 4-fold elevation, relative to the parental plants, of *HPH-DST* and *GUS-DST* transgene mRNAs, which was the basis for its isolation. Each of these transgenes contains a tetramer of the prototype DST element (Newman et al., 1993; Sullivan and Green, 1996). The *dst1* mutant also shows a 3-fold elevation of the unstable *SAUR-AC1* mRNA, an endogenous Arabidopsis transcript known to contain a DST element. Prior to this work, these were the only known

dst1 phenotypes; *dst1* mutant plants appear normal with respect to morphology and development when compared to parental plants (Johnson et al., 2000).

To determine whether additional molecular differences existed, poly(A)$^+$ RNA from leaves of 40-day-old *dst1* and parental plants was extracted and used in a DNA microarray experiment. After hybridization and normalization of data, several genes could be identified with changes in mRNA levels greater than 1.5-fold. As expected, *HPH-DST* and *GUS-DST* were among genes with elevated mRNA levels in *dst1*. *SAUR-AC1* was also detected on the microarray to be elevated in the mutant. In addition, a DEAD box RNA helicase *RH15* (At5g11200) and EST 125O9T7 were identified as being elevated at the mRNA levels in the *dst1* mutant. When the sequence from EST 125O9T7 was compared to the TAIR database (http://www.arabidopsis.org/), the translation showed high similarity (63.7%) to the SAUR-AC1 protein and several other SAUR-like proteins as depicted in Figure 2.1A. As a result, this gene was named *SAUR-like1*. Most interestingly, although the 3' UTR of this gene lacks a classical DST element, it contains multiple DST-like subdomains, i.e., two ATAGAT-like and three GTA-like subdomains, as shown in Figure 2.1B.

RNA gel blot analysis was used to confirm the differential regulation of these genes identified by DNA microarray. In previous experiments, RNA gel blot analysis had proven an effective method to measure subtle differences in mRNA abundance between *dst1* and parental plants (Johnson et al., 2000). In addition, this approach has other advantages. RNA gel blot analysis has traditionally been relied upon for accurate analysis of gene expression at the level of mRNA abundance. Moreover, once differentially abundant mRNAs are identified using one combination of mutant and wild type, these

Figure 2.1 Analysis of EST 12509T7 identifies it as *SAUR-like1* (in collaboration with Pérez-Amador, M.A.).

A. Sequence alignment of SAUR-like1 with several SAUR-like proteins. Boxes indicate 5/5 or 4/5 amino acid identities.

B. Multiple DST subdomains in the *SAUR-like1* 3' UTR. Repeats of the ATAGAT- and GTA-like subdomains are indicated by arrows and bars, respectively. The determination of the 3' UTR was based upon the 5' and 3' sequences of EST 125O9T7 and 125O9XP, respectively.

mRNAs can be easily investigated in additional mutants. Finally, RNA gel blot analysis provides the means to test the biological reproducibility of data obtained in microarray experiments, because additional samples can be tested readily. Accordingly, the *dst2* mutant was included in this analysis in order to compare the molecular phenotypes between *dst1* and *dst2*. Like *dst1*, *dst2* has no known phenotype other than elevation of *HPH-DST*, *GUS-DST* and *SAUR-AC1* mRNA levels due to increased stability.

EST clones to be investigated were radiolabeled and used as probes on the RNA gel blots. The translation elongation factor *eIF4A*, which was used to normalize RNA gel blot data as described previously (Johnson et al., 2000), did not show altered mRNA levels in the *dst1* mutant in DNA microarray experiments, as expected. All blots were hybridized first to labeled *eIF4A* probe, then stripped and hybridized with the individual gene probes. Representative RNA gel blots are shown in Figure 2.2. *HPH* and *SAUR-AC1* mRNAs increased in abundance in both *dst1* and *dst2* mutants as expected (3.5-fold and 3-fold respectively). The mRNA levels for *SAUR-like1* were also elevated in both mutants, although to different extents (2.2-fold in *dst1* as opposed to 1.6-fold in *dst2*). *RH15* showed a 3-fold increase in mRNA level in *dst1* but did not show altered mRNA levels in *dst2*.

The 11,521-element microarray reveals additional genes with altered gene expression in the *dst1* mutant

To expand on the 600-element microarray analysis, high density arrays containing 11,521 clones (prepared by the AFGC DNA Microarray facility at MSU) were used. Poly(A)$^+$-selected RNA from leaves of five-week-old *dst1* and parental plants was extracted, labeled with Cy3 and Cy5-CTP, and used for the hybridization. Analysis was

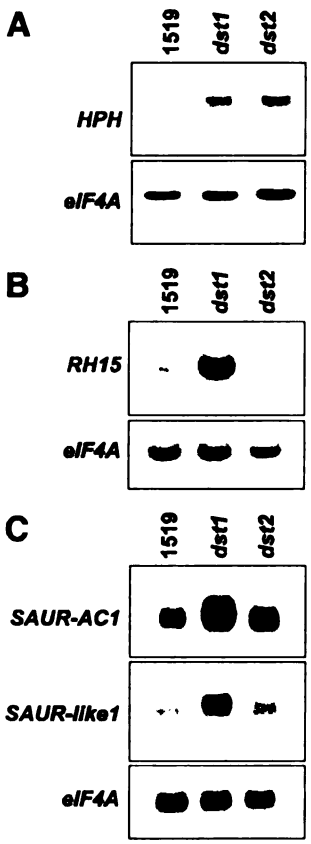


Figure 2.2. RNA gel blot analysis to confirm DNA microarray data (in collaboration with Pérez-Amador, M.A.)

Lanes contained 10 μg of total RNA extracted from WT (1519), *dst1*, and *dst2* plants. Each blot was hybridized sequentially with ^{32}P-labeled *eIF4A*, and with **A)** *HPH*, **B)** *RH15*, and **C)** *SAUR-AC1* and *SAUR-like1*.

carried out using four independently grown sample sets to control for biological variability. Each sample set consisted of the *dst1* mutant and parental plants. For each set, two microarrays were used, each with reverse labeling, for a total of eight microarray slides (raw data is available on http://genome-www4.stanford.edu/cgi-bin/SMD/cluster/QuerySetup.pl, under the experimenter name Green). The DNA microarray expression data was normalized as indicated in the "Methods" section to calculate the ratios of the fluorescence intensities of the two probes. The number of clones with a ratio greater than 1.5 fold was determined. Although a relatively low cut-off ratio of 1.5 was used, the data were reproducible and were confirmed by RNA gel blot analysis (see below). When the expression data, represented as the median of eight microarray slides, was plotted, 36 clones (31 ESTs and 5 clones for *HPH* and *GUS*) with ≥ 1.5-fold elevated or decreased mRNA levels could be identified (Figure 2.3). Most of these clones showed a difference of 1.5 fold or greater in at least seven of the eight slides analyzed. Each EST was mapped to the Arabidopsis genome by conducting a BLAST search against the Arabidopsis genome sequence database. Multiple ESTs representing the same gene were also identified in this manner and revealed that the 31 ESTs corresponded to 25 genes. The redundancy amongst the ESTs present on the array further allowed us to verify the microarray data, since ESTs for the same genes showed similar changes in expression. In some cases, ESTs corresponding to the same gene showed slightly different expression ratios (e.g., the four ESTs corresponding to *RAP2.4* ranged from 2- to 5-fold higher in *dst1*, with a mean of 3.9-fold); this variability could be due to either variable probe or target length, genetic redundancy, or a combination thereof. The

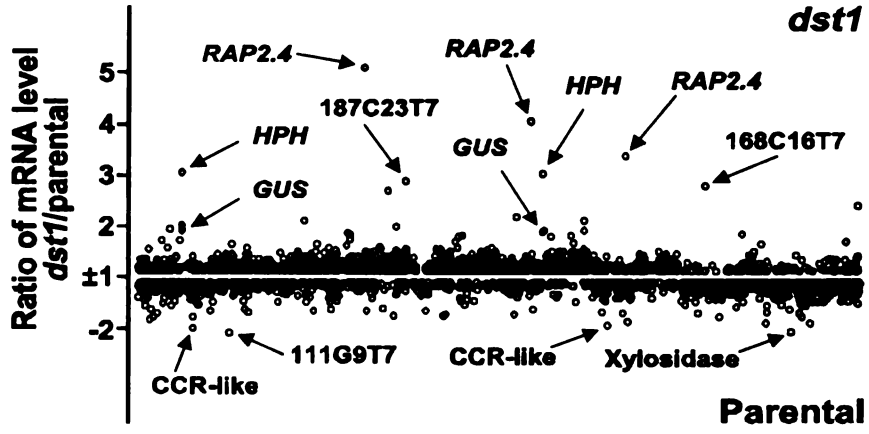


Figure 2.3. Comparison of mRNA levels in the *dst1* mutant and parental plants using the 11,521 element DNA microarray (in collaboration with Pérez-Amador, M.A.).

The graph was generated from the data obtained with ScanAnalyze software. Ratio values below 1 were transformed to -1/ratio in order to plot them as fold-difference (y-axis) against arbitrary clone number (x-axis). Five-point scale indicates genes with increased/decreased mRNA levels in the *dst1* mutant.

transgenes *HPH* and *GUS*, as well as the DEAD box RNA helicase gene *RH15*, were again detected with elevated mRNA levels in *dst1*.

Twenty genes, including the transgenes *HPH* and *GUS*, showed elevated mRNA levels while seven displayed decreased levels of mRNA in the *dst1* mutant compared with the parental plants, as listed in Tables 2.2 and 2.3, respectively. After correction for redundancy in the ESTs and excluding the transgenes *HPH* and *GUS*, a total of 25 endogenous genes whose expression levels were altered in *dst1* were identified. The probable biological functions of these genes, listed in Tables 2.2 and 2.3, were based on annotation by the MIPS *Arabidopsis thaliana* database.

RNA gel blot analysis confirms DNA microarray data

To test whether the transcript changes identified in the *dst1* mutant by microarray analysis were reliable, total RNA was obtained from the same plants that were used for each of the four microarray experiments and examined by RNA gel blot analysis. Several genes exhibiting altered mRNA levels in the *dst1* mutant as determined from the 11,521 microarray analysis were tested. All of the genes exhibited increased or decreased mRNA abundance as expected. Although mRNA changes were relatively small (1.5-3.9 fold), in all cases, differences in gene expression detected by the microarray translated into similar fold differences as determined by RNA gel blot analysis (Figure 2.4).

Again, RNA levels in *dst2* were also monitored in these experiments. Using this approach, molecular markers specific for each *dst* mutant were identified. Most of the genes that had increased mRNA abundance in the *dst1* mutant were unaffected in the *dst2* mutant. As demonstrated in Figure 2.5A, *RAP2.4*, *RH15*, and ESTs 120E6T7, F2H12T7, and 141P19T7 showed mRNA levels similar to the parental plants in the *dst2* mutant.

Table 2.2. Genes with increased mRNA levels in *dst1* vs. parental plants (in collaboration with Pérez-Amador, M.A.)

Gene	MIPS ID	Number of Clones	EST		Average ± SD
			MSU ID	Accession	
RAP2.4 (AP2 domain)	At1g78080	4	89I20T7	T20915	3.9 ± 1.1
			207H22T7	N37644	
			172J24T7	AA712560	
			272E2T7	AA651548	
Similar to cadmium induced (AP2 domain)	At1g22190 (a)	1	168C16T7	R64886	3.4 ± 1.9
HPH	--	2	-- --		3.2 ± 1.1
Chalcone synthase	At5g13930	1	187C23T7	R89978	3.0 ± 1.6
Similar to chalcone flavonone isomerase	At5g05270	1	113I3T7	T42455	2.8 ± 1.3
Putative membrane channel	At2g28900	1	141P19T7	T46371	2.4 ± 0.7
Unknown	At3g56360	1	180E15T7	H36869	2.2 ± 0.6
Putative transcription factor	At1g56650	1	99P9T7	T22223	2.1 ± 0.5
Calmodulin-like	At2g41410	1	F2H12T7	AA713007	2.2 ± 0.8
GUS		2	-- --		2.0 ± 0.4
Heat shock protein 90	At5g56010	1	204P1T7	H76891	2.2 ± 1.3
CCR4 associated factor1-like	At3g44260	1	222C9T7	N38276	2.1 ± 0.9
Unknown	At5g09850 (a)	1	120E6T7	T43948	1.9 ± 0.5
Unknown	At2g27830	1	E8D5T7	AA042743	1.9 ± 0.4
Unknown	Atg68490	1	209G16T7	N37313	1.9 ± 0.3
Unknown		1	222M14T7	N38314	1.8 ± 0.5
Unknown	At3g28270 (a)	1	139I3T7	T46268	1.8 ± 0.4
Similar to APR2	At1g62180	1	73F9T7	T45380	1.8 ± 0.6
Unknown	At3g26000	1	281D7T7	AA650744	1.6 ± 0.2
DEAD box RNA helicase RH15	At5g11200	1	91P21T7	T21481	1.6 ± 0.2

(a) EST sequence aligns upstream of the ATG codon of the deduced ORF of the indicated gene.

Table 2.3. Genes with decreased mRNA levels in *dst1* vs. parental plants (in collaboration with Pérez-Amador, M.A.)

Gene	MIPS ID	Number of Clones	EST		Average ± SD
			MSU ID	Accession	
Putative patatin protein	At2g26560	1	111G9T7	T42260	2.5 ± 1.0
Senescence-associated protein	At4g35770	1	170N19T7	R65416	2.0 ± 0.4
Similar to stem-specific protein	At4g27450	1	201O1T7	H76730	1.9 ± 0.4
Xylosidase	At5g49360	2	H5A10T7	W43767	1.9 ± 0.5
			G1G9T7	N96119	
STP1 glucose transporter	At1g11260	1	171P10T7	R65119	1.9 ± 0.6
Similar to CCR protein (*Ccr*-like)	At3g26740	3	E6E1T7 245H16T7 210A12T7	AA042331 N97142 N37331	1.8 ± 0.3
Unknown	At5g45510 (a)	1	G4A11T7	N96240	1.8 ± 0.5

(a) EST sequence aligns upstream of the ATG codon of the deduced ORF of the indicated gene.

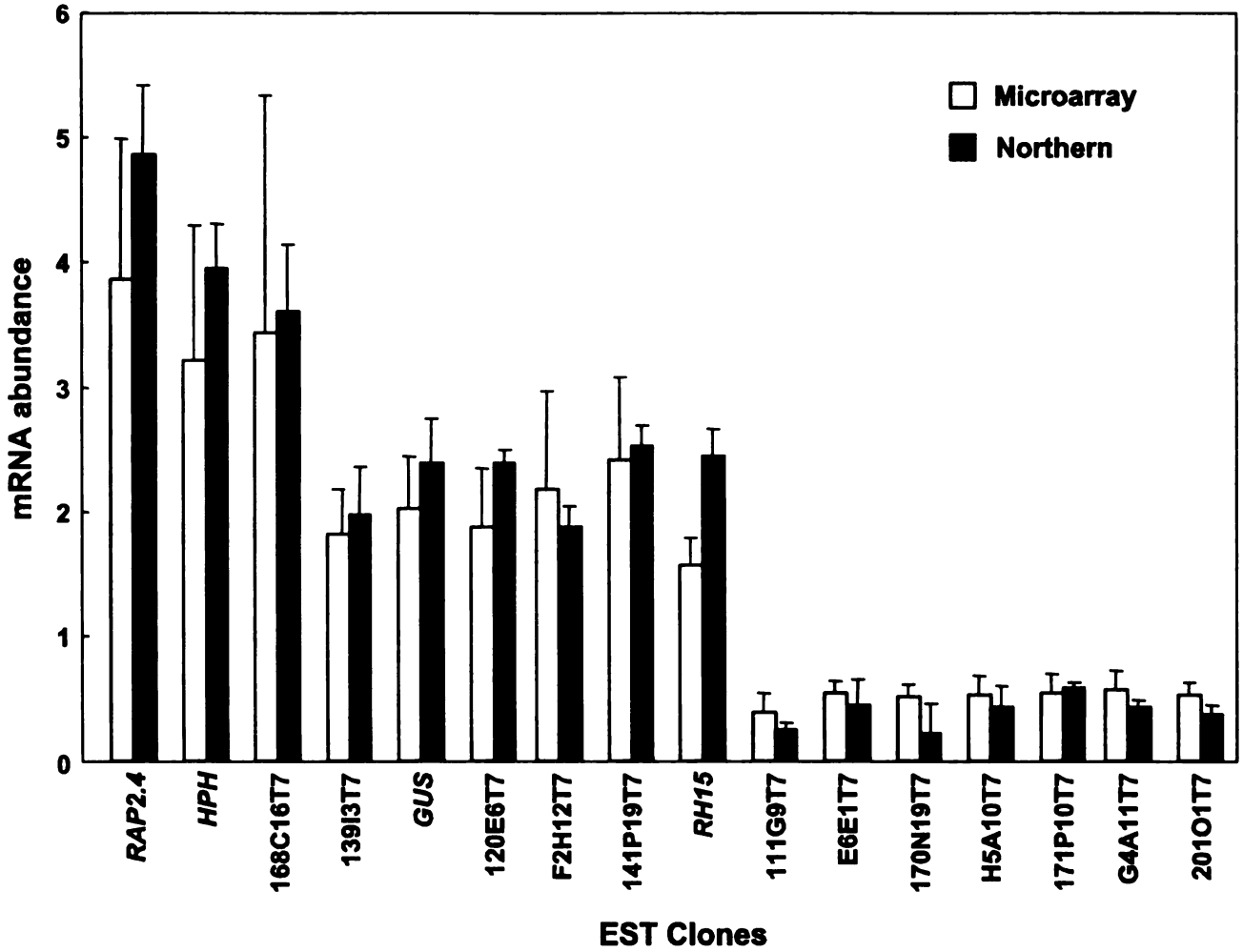


Figure 2.4. Histogram plot comparing the gene expression values obtained using microarray analysis and RNA gel blot analysis. The x-axis denotes the various EST clones tested while the y-axis represents the fold-difference for mRNA levels for each clone.

HPH and *GUS* mRNA levels were elevated in both mutants. The EST for one gene, 168C16T7, which shows high similarity to a cadmium-induced AP2 protein, had mRNA levels that were elevated in both mutants, although the elevation was higher in *dst1* than in *dst2* (approximately 3.5-fold versus 2-fold compared to the parental type). EST 139I3T7 which encodes an unknown protein may have marginally reduced mRNA levels in the *dst2* mutant.

Of particular interest was the analysis of the seven genes with lower mRNA abundance in *dst1* compared to parental plants. Most of these genes were either elevated (ESTs 111G9T7, E6E1T7, 170N19T7, and H5A10T7) or unaffected (ESTs 171P10T7 and 201O1T7) in the *dst2* mutant, as is evident from the histograms shown in Figure 2.5B. The only exception was EST G4A11T7; this EST codes for an unknown protein, and the mRNA levels for this gene were also diminished in *dst2*.

SAUR-AC1 and *SAUR-like1* were also analyzed by RNA gel blot analysis and were elevated in both the *dst1* and *dst2* mutants (as shown in Figure 2.2), although they were beneath our detection limit on the majority of the slides used for the microarray experiments. These signals were below detection because, in most experiments, the signals were too close to background to calculate a valid ratio. However, in the experiments in which we could detect a valid ratio, elevation in *dst1* was observed as expected (data not shown).

Identification of primary targets of the *dst1* mutation

The 3' UTRs of all the genes identified as being differentially regulated in *dst1* were analyzed for the presence of either a classical DST element or DST-like

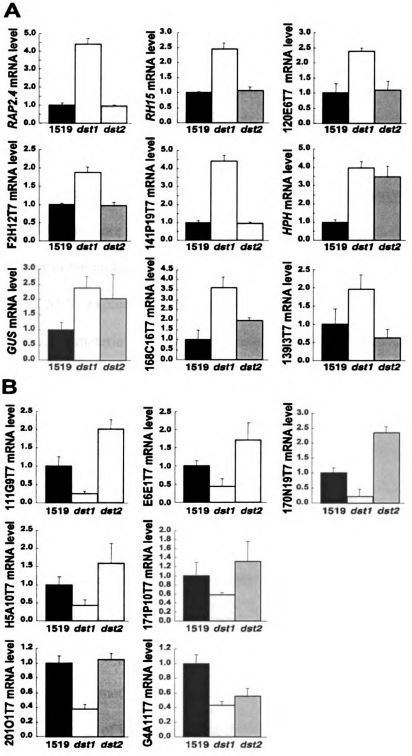


Figure 2.5. Histograms of the mutant (*dst1* or *dst2*)/parental ratios of mRNA expression for the indicated clones.

A) Increased levels of mRNA in *dst1*

B) Decreased levels of mRNA in *dst1*

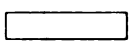
subdomains. Seven genes were identified that contain possible DST-like sequences in their 3' UTRs (listed in Table 2.4). Therefore, these genes could be hypothesized to be primary targets of the DST-mediated decay pathway which is deficient in the mutant. To determine whether the decay of some of the hypothesized primary targets required a functional DST1 pathway, mRNA turnover rates were measured in the *dst1* mutant and 1519 parental lines. Northern blot analysis of cordycepin time courses indicated that *RAP2.4* mRNA was more stable in *dst1* (Figure 2.6A and B) while *Ccr*-like mRNA decayed faster in the mutant as compared to 1519 parental Arabidopsis plants (Figure 2.6C and D). *SEN1* expression is not high enough to measure mRNA decay accurately in mature leaves, the material used for the microarrays and the decay curves in Figures 2.6A and C. However, half-life measurements of *SEN1* mRNA in seedlings (Figure 2.6E and F) showed that it too was less stable in *dst1* than in the parental 1519. This data indicates that *RAP2.4*, *Ccr*-like and *SEN1* mRNAs are indeed bona fide targets of the DST-mediated mRNA decay pathway in Arabidopsis.

Multiple replicates remove non-reproducible changes

An important aspect of our analysis of the *dst1* mutant was the ability to detect subtle changes in gene expression. As shown in Figure 2.4, using eight microarray slides, all of the changes that met our criteria were highly reproducible in independent RNA gel blots. However, it was of interest to examine the degree of reproducibility using a single pair of technical replicate slides and whether fewer than eight slides are sufficient because the number of slides to use for microarray experiments is not yet routine. This could be particularly significant for future experiments, since the 1.5-fold cut-off used

TABLE 2.4. Genes with possible DST-like sequences in their 3' UTR

GENE	3' UTR
RAP2.4 (AP2 domain)	GGGCAAAATAGGAAATTCAGCCGCTTGCAATGGAGTTTTTGTGAAATTGCATGACTGGCCCAAGAGTA ATTAATTAAATATGGATTAGTGTTAAATTTCGTATGTTAATATTTGTATTATGGTTTGTATTAGTCTC TCTGTGTCGGTCCAGCTTGCGGTTTTTTGTCAGGCTCGACCATGCCACAGTTTTCATTTTATGT
CAF1-like	GGGGTTTCTTTTTTTTTATAATTCAAATTCAATTAATTAACTCATTTTTTTTTTATTTTTTTTGGTA GTGTCAATTAATTAACTCAATTGATCCTTGTAGTTAAACGTTCTTTGGAAAGAAGAAATTTTAGAAAT TTGAAAATTCATTTGTTTAGTCCACATTTAAATCATATACAAATCGATATGAATTTAAAAACAG
Similar to APR2	GTTATGAGATAGAAAATAAAAAGAGGGTGATGAAGAATGAAAGAGAATTGAGATGAAGGAGTGATTTCATCTTGG TTATAATATTGTGGACGTCAGAGAATAAACAAGAGTTATTACTCTTCTACATGACTTTGTATATTTGTATTGTAG GGGTTTTTATAGACTCTTTTTGTTACCTCTATGTATCTTTTTTGGTCTAC
Putative patatin protein	GGATCTAAAGAGATCAATTATTGTATCTTGTATATTGTTGATGATTCGTTTGTTTCACTAATGCACGTTTG GATTTATATTAATAAAAAGGTTGCACCACATTGTGGTCACTTGTACGTTTGAATAAAGTCAATAAATATTTA ATGGATGGAAATGCTCTAAAATGTTTGGTCATTTTTCAACATACATTATACTAATAGAATAATGGCGAATA ATACATAGACACG
Senescence-associated protein (*SEN1*)	AAACAATCATAGCCATATCACCTCCTGTAATTATCAATAAATTCTGTAGATGTTCTCAACGCAAGTATCAA TCATCATTATTATTCTCTAAAACATAGTTTAACTGTCAAATCTATATTGGGTGAAAGCTTTACCGAACTTC GAACACCACAAAGTTTACGGACCAATTAAGGACGGGGATGCATGCCTACACACGTTTA
Xylosidase	CGGACGCATAAAAAAACCAACAAATAAGGAAAGCATTTTAACAAAGTGGAGTGTTTCCTCTTATTTATATA ATATAGAGAGATAGGTTTATTTTCTTATGAAATTATTACTCAAGAAAGTATGAATTTGTAAAGAACCACCA AAGGGTTGCTTCTTGGTTGTGTCTTTTTTCCTTTAATTTTTTTTTGGACCAAAGGTATTGTAACTTGTGGC TCCCAACATAAGAAATACAAGGAGCCCTTGTAAAATGCTGAAACTAAAGATAAATGATAAATTTGATATATACT
Similar to CCR protein (*Ccr*-like)	AAGAAACAAGATTTTATGTCATGTGAATATGTGTTTGTATTTCTACAAGTCTTTGTAACCACTCCTTAATTTA TGATATCTAGCTTCACTACTTAATCAAGATTGATGTTATGGATTAGACCCGAGTTCCCAAAGTTAATACTCTC TCTGTTCCAAGTTATTTGATGTTTTGGGTTTTACACAAGAATTAAGAAAAATTA



"classical" DST element
ATAGAT-like subdomain
GTA-like subdomain

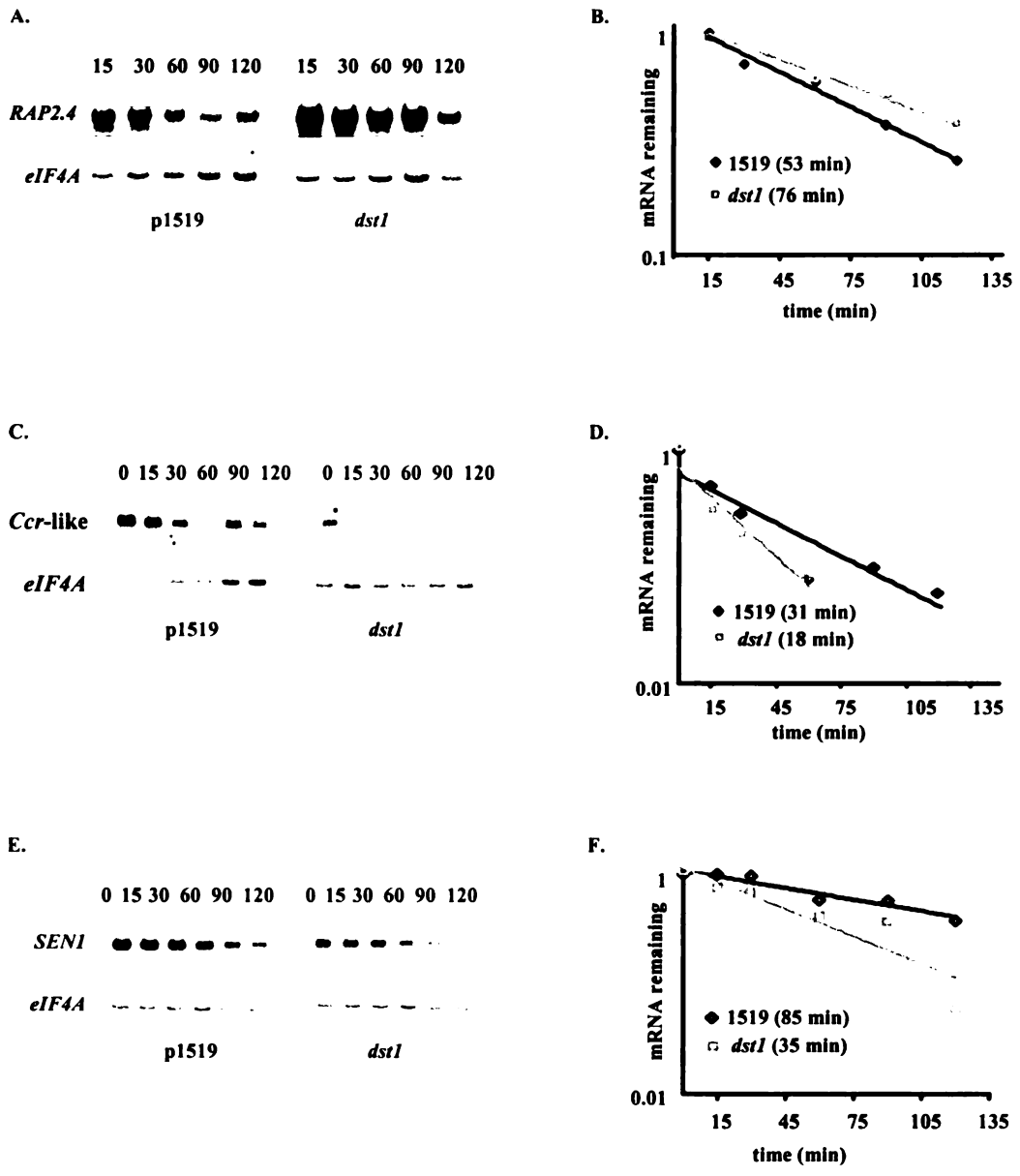


Figure 2.6. *RAP2.4*, *Ccr*-like and *SEN1* mRNAs are primary targets of the DST-mediated decay pathway.

Representative northern blot analysis of cordycepin time courses performed in the *dst1* mutant and 1519 parental Arabidopsis plants for (A) *RAP2.4*, (C) *Ccr*-like, and (E) *SEN1* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for (B) *RAP2.4*, (D) *Ccr*-like and (F) *SEN1* mRNAs. The stable *eIF4A* transcript was used as a reference for equal loading.

was lower than the commonly used threshold value of 2-fold (Wildsmith and Elcock, 2001; DeRisi et al., 1997), although a recent study reported that the minimum detectable fold-change for differential expression is 1.4 fold (Yue et al., 2001).

To monitor the reproducibility of the microarray hybridizations, for individual pairs of technical replica slides, the number of clones with a ratio greater than 1.5 was calculated. For each, a clone was considered reproducible if it was represented in the final set of 36 clones showing ≥ 1.5 fold difference and non-reproducible if it showed ≥ 1.5 fold difference in the slide pair but was not present in the final set. Using a cut-off value of 1.5 fold, a histogram was plotted to compare the percentage of reproducible and non-reproducible clones. From this histogram, it is evident that such a low cut-off yields non-reproducible changes (Figure 2.7A). This is expected because the cut-off value is close to the background. However, these non-reproducible changes can be identified and removed by conducting multiple experiments. When the reproducibility across repetitions was plotted as a function of the number of slides (worst to best pair and vice-versa, based on the percentage of visible gradient on the slide), it was seen that the percentage of non-reproducible clones decreased to less than 20% after four slides. In our experiment, it was possible to remove virtually all non-reproducible clones using seven slides (Figure 2.7B).

Cluster analysis of genes with altered expression levels in *dst1*

Clustering allows the grouping of genes with similar expression profiles and provides a holistic view since co-expressed genes involved in similar processes provide clues about the biological significance of the pathway being studied. We used cluster analysis to compare gene expression data for the 25 genes identified in this study using data from the 47 Arabidopsis microarray experiments (113 slides) available in the

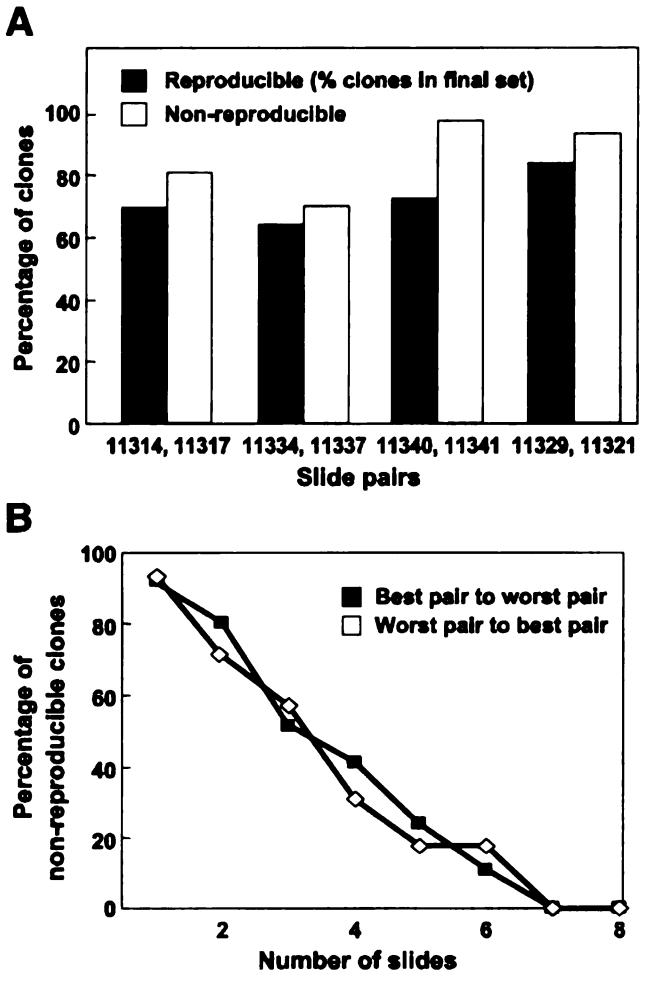


Figure 2.7. Assessment of the reproducibility of the microarray data using fewer replicates.

A. Histogram plot comparing the percentage of reproducible and non-reproducible clones. The percentage of clones (y-axis) is plotted against slide pairs (x-axis) used for the microarray experiments. Each slide pair denotes microarray slides that were technical replicas, i.e., reciprocal labeling was performed with each pair of targets. The slide numbers denote the ExptID in the Stanford Microarray Database.

B. Graph showing the percentage of non-reproducible clones (y-axis) plotted against the number of slides used for the microarray analysis (x-axis).

Stanford Microarray Database (SMD) (http://genome-www.stanford.edu/Microarray) at the time of the analysis. A portion of the resulting clusters is shown in Figure 2.8. As expected, when EST redundancy was not removed, ESTs representing the same gene clustered next to or in the immediate vicinity of each other, thus validating the observed pattern of gene expression (data not shown).

Although the cluster analysis was carried out using the limited number of genes that were identified in this study, certain common expression patterns could be found. The most prominent cluster is comprised of three genes that are regulated in a diurnal fashion and share several other expression characteristics. This cluster is characterized by genes whose transcripts are decreased in abundance in the *dst1* mutant and include genes encoding a protein similar to a stem-specific protein, senescence-associated protein, and xylosidase. These genes show tissue-preferential expression, with a high level of expression in leaves and a low level in flowers and roots, and are elevated by light. Two of the three genes in this cluster, senescence-associated protein and xylosidase, have unstable transcripts (see discussion), contain possible DST-like sequences in their 3' UTRs, and are also elevated in the *dst2* mutant.

Apart from the aforementioned cluster, a second cluster of coordinately expressed genes was identified. This cluster includes genes for chalcone synthase and a protein similar to chalcone flavonone isomerase, both of which show increased mRNA levels in the *dst1* mutant. These genes are regulated in a diurnal fashion as well but are decreased by light and have higher levels of expression in flowers compared to leaves or roots. In addition, these mRNAs are elevated in a mutant defective for protein import into chloroplasts.

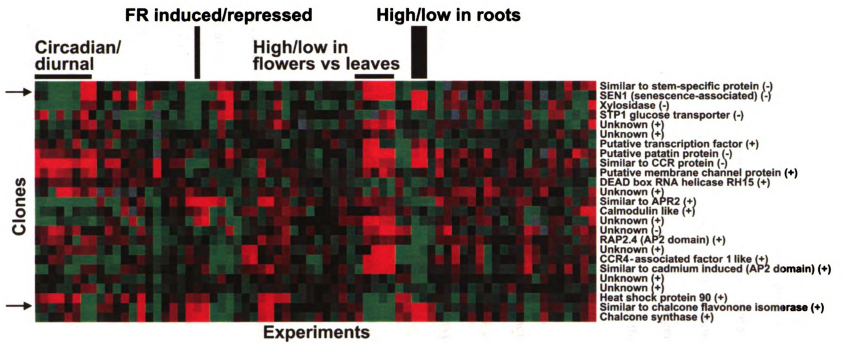


Figure 2.8. Cluster analysis of genes with altered mRNA levels in *dst1*.

Data from the 25 genes were clustered with data from 47 experiments from the SMD. Each gene is represented by a single row, and each experiment is represented by a single column. Arrows indicate the predominant clusters identified. (-) indicates genes with decreased mRNA levels in *dst1*; (+) indicates genes with increased mRNA levels in *dst1*. Image in this dissertation is presented in color.

No other common characterizing gene expression patterns could be seen. However, eight genes (senescence-associated protein, putative patatin protein, protein similar to CCR, putative membrane channel protein, protein similar to APR2, calmodulin-like protein, an unknown protein, and chalcone synthase) are regulated in a circadian manner, which points to a possible connection between the DST-mediated decay pathway and circadian rhythms. Four of these eight genes also contain DST-like subdomains in their 3' UTRs and could be direct targets of the *dst1* mutation.

DISCUSSION

Microarray technology has great potential for characterization of mutants, especially those with no visible aberrant phenotype. Here we report on the *dst1* mutant, which fits into this category. To evaluate molecular phenotypes of this mutant, DNA microarray technology was used, which allowed the identification of primary as well as secondary effects, and also revealed clues towards the identification of possible relationships among genes.

Transcripts altered in *dst1* have predominantly increased levels, although some decrease in abundance

The *dst1* mutant is known to elevate mRNA levels of *HPH-DST* and *GUS-DST* transgenes by 3- to 4-fold (Johnson et al., 2000). The DNA microarray experiments carried out in this report were sensitive enough to detect reproducibly the elevations of these transcripts in multiple replicates of the experiment. Moreover, additional changes of similar magnitudes were detected for transcripts corresponding to 25 genes from among

those represented by the 11,521 clone array. Such a limited number of changes is not surprising, since the mutant does not exhibit any morphological abnormalities. It has been suggested that *dst1* and *dst2* correspond to weak alleles or affect genes that are part of gene families and therefore have only partial defects in mRNA stability (Johnson et al., 2000). The results of our microarray experiments are certainly consistent with this idea, since we detected only moderate changes in mRNA levels for a few genes in *dst1*. Increased transcript levels for some genes were expected, since *dst1* is known to increase levels of DST-containing transgene mRNAs and the endogenous DST-containing *SAUR-AC1*, presumably due to a defect in a component of the cellular machinery involved in the recognition and degradation of DST-containing transcripts.

Our analysis showed that expression of several genes decreases in *dst1*. Decreases in gene expression could be explained by the same defect if the DST recognition component acts as both a repressor and activator in a context-dependent manner similar to some transcriptional regulators. For example, *Drosophila* Drap1 and dCtBP are bifunctional transcription factors with distinct activation and repression functions (Willy et al., 2000; Phippen et al., 2000). Further support for this hypothesis comes from studies conducted on AUF1. It has been demonstrated that AUF1 acts as an RNA-destabilizing protein in the ARE-mediated mRNA decay pathway (Loflin et al., 1999; Buzby et al., 1999), but it has also been implicated as being part of the complex that mediates the stabilization of α-globin mRNA (Kiledjian et al., 1997). Similarly, the DST recognition component could be part of different multiprotein complexes that carry out separate functions.

The genes identified in this study encode for proteins involved in a variety of cellular processes. The DEAD box RNA helicase, *RH15*, is a part of a large gene family in Arabidopsis. RNA helicases are primarily RNA unwinding enzymes and have been implicated in a variety of molecular processes, including mRNA splicing, ribosome assembly, and translation initiation (Aubourg et al., 1999). Of the 18 endogenous genes that are elevated in the *dst1* mutant, two encode for proteins that have an amino acid motif known as the AP2 domain (see Table 2.2). The AP2 domain is essential for APETALA2 functions and contains an 18-amino acid core region that is predicted to form an amphipathic α-helix (Okamuro et al., 1997). The four ESTs that were most highly elevated in *dst1* correspond to the gene *RAP2.4*, which belongs to the *RAP2* (related to AP2) gene family. The AP2 polypeptide is distinct from known fungal and animal regulatory proteins, and it has been proposed that the RAP2 proteins may function as plant sequence-specific DNA binding proteins. Another interesting EST, 222C9T7, that was elevated in *dst1* corresponds to a protein that is similar to the CCR4 associated factor 1 protein (CAF1). CAF1 is a transcription-associated protein and is a member of the RNase D family of 3' to 5' exonucleases (Moser et al., 1997). Recently, it was shown that Caf1p is a critical component of the major cytoplasmic deadenylase in yeast (Tucker et al., 2001), suggesting a potential link between mRNA deadenylation and the DST-mediated mRNA degradation pathway.

Most transcripts that are affected in the *dst1* mutant relative to the parental type are expected to fall into one of two categories. One category consists of mRNAs that are the direct targets of the DST-mediated decay pathway, the primary transcripts, while the second category consists of transcripts, referred to as secondary transcripts, that are

elevated or diminished as a result of secondary effect of changes in the primary transcripts. A number of genes showing altered levels of gene expression in *dst1* may be due to secondary effects, since these genes do not appear to contain a consensus DST element or DST-like subdomains in their 3' UTR. Secondary effects would be expected if altered levels of DST-containing mRNAs influence the abundance of other RNAs. This situation is not unique to *dst1*; secondary effects would be expected in all microarray experiments comparing mutants with the wild type. However, this study allowed us to predict and subsequently confirm some of the primary and secondary effects of the *dst1* mutation based on the presence or absence of DST-like sequences, respectively. Although the precise sequences required for a functional DST element have not been fully elucidated, our data provide us with the advantage of identifying at least some of the most likely primary effects. For example, none of the 140 possible DST-containing sequences found by motif searching tools on the 600 element array, were affected in *dst1*. This suggests that the DST element is more complex in Arabidopsis than in soybean and that it is difficult to identify this element by simple sequence search tools. It appears that multiple subdomains of the DST element are sufficient for its function as an instability determinant, since 7 of the 25 genes identified contain DST-like subdomains. Earlier mutagenesis studies of individual subdomains are also consistent with this hypothesis (Feldbrugge et al., 2002). Further experiments are required to define the DST element, which would ultimately help refine our understanding of the requirements for DST recognition.

Molecular markers to expedite characterization of and differentiation between *dst1* and *dst2*

The most immediate utility of these results is the identification of new molecular markers for *dst1* that could be used to enhance mapping and prompt additional biological experiments. For example, the analysis of *dst1* showed that *RAP2.4* mRNA levels are more highly elevated than *HPH* or *GUS* mRNA levels. Thus *RAP2.4* could be a more useful marker for the detection of homozygous *dst1* mutants in future experiments.

Prior to this study, it was difficult to distinguish between *dst1* and *dst2*, because the known phenotypes of the two mutants were identical, and F_2 progeny of a cross between the mutants do not show additive increases in the abundance of DST-containing mRNAs (Johnson et al., 2000). By examining mRNAs in *dst2* that show altered levels in *dst1*, differences between the two mutants were identified. *dst1* and *dst2* represent mutations in independent genes (Johnson et al., 2000). The results from these experiments indicate that some targets of the decay pathway mediated by the two genes could be different. Further, there could be some degree of overlap leading to the coordinate regulation of common targets, as exemplified by *SAUR-AC1* and the transgenes. The cataloging of molecular markers specific for each mutant is also significant because specific markers should allow us to identify the double mutant without relying on map positions and subsequent crosses. This emphasizes another potential use of microarrays in identifying genes that can be used to examine and compare related mutants without necessitating individual microarray experiments for each. For example, a new *dst* mutant was recently obtained in our laboratory by activation tagging. The molecular markers identified in this study will facilitate

characterization of this mutant as well as subsequent mutants that affect this sequence-specific mRNA degradation pathway.

Circadian association of the *dst1* mutation

Utilizing the technique of cluster analysis, parallel expression profiling over many experiments was conducted. The clusters generated revealed co-expression characteristics of the genes affected in the *dst1* mutant that would not have been evident otherwise. Eight genes, which correspond to 32% of the 25 genes described in this analysis, were regulated in a circadian manner, while only 2% of 7,800 genes were found to cycle with a circadian rhythm in a study conducted by Schaffer et al. (2001). In another study by Harmer et al. (2000), 6% of 8000 genes showed circadian changes in mRNA levels. Based on comparison with their results, 4 of 25 (16%) genes in our experiments exhibited a circadian pattern of expression. These data point to a higher representation of circadian-regulated genes than would be expected by chance. Despite differences in results and experimental conditions between the two studies (e.g. only about 1500 genes were in common), four genes with altered expression in *dst1* were found to be circadian regulated in both studies adding more credence to our data. The significance of several genes in the cluster being regulated in a circadian manner is not yet clear, but it suggests an association between the *dst1* mutation and circadian rhythms. Further, Harmer et al. (2000) showed that *SAUR-AC1*, which is a target of the DST-mediated mRNA degradation pathway, is also circadian regulated. It is thought that a number of circadian-regulated transcripts are unstable and are expressed during a narrow window of time. It is possible that in the *dst1* mutant, the level of a potential regulatory factor or signal molecule that plays some part in the circadian clock function is altered, which leads to a

cascade effect and changes the expression of several circadian-regulated genes. If this is true, the circadian effects uncovered here would provide the first insight into the biological or physiological significance of the DST-mediated mRNA decay pathway. Elucidating the exact nature of this association and further testing of this hypothesis will require the cloning of *DST1*.

CONCLUSIONS AND FUTURE PROSPECTS

In addition to reproducing the known elevation of two transgene mRNAs in *dst1*, 25 additional transcripts were found to increase or decrease in abundance relative to the parental plant. Many of the corresponding genes were subsequently examined by RNA gel blot analysis to confirm the *dst1* microarray results and to evaluate their expression in the *dst2* mutant. In this way, several interesting and useful differences were uncovered between the two *dst* mutants. These new molecular markers should enhance subsequent analysis of the *dst* mutants, provide insight into their biological significance, and help identify other targets of the DST-mediated mRNA decay pathway.

The 25 genes identified in this study are most likely an underrepresentation of the molecular phenotypes of *dst1* due to the stringency of our parameters to avoid false positives. For example, ratios derived by the microarray results from weakly expressed genes, such as *SAUR-AC1* and *SAUR-like1*, which have low levels of expression in the absence of auxin (McClure and Guilfoyle, 1987), were significantly different from those determined by RNA gel blot analysis. The channel intensity values for these transcripts are close to the background fluorescence intensity levels and therefore are more susceptible to variation. Also, there may be additional targets of the DST-mediated decay

pathway not identified because the present generation of slides do not contain all of the genes of Arabidopsis. In the future, comprehensive microarrays with improved sensitivity should result in the discovery of a greater number of molecular phenotypes for *dst1*.

Beyond extending our knowledge of the DST-mediated decay pathway in plants, the current study provides additional general insights. Our study shows that subtle changes in gene expression can be measured reliably using multiple microarrays, which should enhance the global investigation of gene expression patterns under several conditions. This analysis demonstrates that new molecular phenotypes for mutants without a visible phenotype can be identified using DNA microarray technology. Also with full genome microarrays, it should be possible to catalog all the molecular phenotypes for any mutant. Further, new hypotheses and associations, such as the potential link between the *dst1* mutation and circadian rhythms, can be developed by employing the publicly available databases. Future mutant analyses via DNA microarray analysis should have even greater utility, particularly for the analysis of mutants identified by reverse genetic approaches (e.g., T-DNA insertions) that have no apparent mutant phenotype.

MATERIALS AND METHODS

Plant Material

All *Arabidopsis thaliana* plants described in this report are from the accession Columbia, grown in growth chambers under 16 hr light and 60% relative humidity at 20°C. Tissue from the parental line (p1519-31) and *dst1* and *dst2* homozygous mutants (Johnson et al., 2000) were harvested from 35- to 40-day-old plants. The *dst1* and *dst2*

lines used were from the second backcross to the parental line. All tissue was harvested from plants grown in parallel under the same conditions in different growth chambers.

Generation of a 600-element DNA microarray

EST clones were selected based on sequence similarity by BLAST analysis (Altschul et al., 1997) to known genes involved in RNA metabolism in Arabidopsis, in other plants, or in other systems, such as bacteria, yeast, and mammals.

Selected Arabidopsis EST clones were obtained from the PRL2 EST collection (Newman et al., 1994). These ESTs were cloned in lambda Zip-Lox (pZL1 clones, GibcoBRL, Rockville, MD) or pBluescript SK^- vector (pBSK clones, Stratagene, La Jolla, CA). To amplify the ESTs by PCR, we designed universal primers corresponding to the 3' and 5' ends flanking the cloning site of each vector backbone. pZL1 clones were amplified using the 5' primer 5' CGACTCACTATAGGGAAAGCTGG 3', and 3' primer 5' ATTGAATTTAGGTGACACTATAGAAGAGC 3'. pBSK clones were amplified using the 5' primer 5' CGACTCACTATAGGGCGAATTGG 3' and 3' primer 5' GGAAACAGCTATGACCATGATTACG 3'. cDNA clones isolated in our laboratory were cloned in pBluescript (primers as above) or pGEM-T (Promega, Madison, WI) vectors. To amplify clones in pGEM-T, we designed the 5' primer 5' CGACTCACTATAGGGCGAATTGG 3' and 3' primer 5' ATTTAGGTGACACTATAGAATACTCAAGC 3'.

Plasmid DNA from the MSU collection was diluted to a final concentration of 1-3 $ng \cdot \mu L^{-1}$ in TE (10 mM Tris·HCl pH 8.0, 1 mM EDTA). PCR reactions, in a final volume of 100 µL, contained 40 pmol of the corresponding primers, 0.2 mM of each dNTP, 2-5 ng of plasmid DNA and 2 units of Taq DNA polymerase in 1 × reaction buffer (10mM

Tris·HCl pH 8.0, 50 mM KCl, 2mM $MgCl_2$). After PCR, DNA was precipitated in ethanol and resuspended in 20 μL of 3 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M NaCitrate, pH 7.0). To check the quality and quantity of the amplified DNA, 1 μL of the final resuspension was analyzed by electrophoresis in 1.0% agarose gels. PCR products with low concentration (<200 $ng \cdot \mu L^{-1}$) or showing multiple bands were discarded and replaced with a new PCR amplification product derived from a different EST clone corresponding to the same gene. If no alternative EST was available, a new PCR amplification was performed using a gene-specific primer designed for the 5' end of the EST clone along with the corresponding 3' vector primer. In this way, we ensured that only high quality PCR products were included on the 600-element DNA microarray. To confirm the identity of the amplified DNAs, we sequenced 12 randomly selected clones. In each case, the sequence obtained matched the EST sequence deposited in the database.

For clones obtained from genomic DNA, DNA was extracted from total above ground tissue of mature Arabidopsis plants using the method of Dellaporta et al. (1983), and 100 ng was amplified as described for EST clones. In all cases, a single PCR product was obtained. Low abundance PCR products were re-amplified under the same conditions using 1 μL of the first PCR reaction as template.

DNA from the PCR reactions was transferred to master DNA plates and stored at 4°C until printing. The final concentration of DNA for printing was estimated to be 200-400 $ng \cdot \mu L^{-1}$. DNAs were arranged as four subgrids of 12 × 13 and printed twice on poly-L-lysine-coated slides. Printing, handling, and use of the 600-element DNA microarray was as for the 11,521 MSU DNA microarray as indicated below.

11,521 AFGC DNA Microarray

Microarrays were generated at the Arabidopsis Functional Genomics Consortium (AFGC) Microarray facility at Michigan State University. A total of 11,521 ESTs were spotted on super-aldehyde glass slides (Telechem International, Inc.; Sunnyvale, CA). Slides were washed and blocked according to the Telechem protocol.

Half-life measurements, total RNA Extraction, Poly(A)$^+$ RNA Purification, and RNA Blot Hybridization

Half-lives were determined as described by Seeley et al. (1992) with the following modifications. Two-week old Arabidopsis seedlings or rosette leaves from Arabidopsis plants were transferred to a flask with incubation buffer. After a 30 min incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and frozen in liquid nitrogen. Total RNA from leaf samples was extracted as previously described (Newman et al., 1993). Poly(A)$^+$ RNA was purified from 200 to 400 µg of total RNA using the Oligotex mRNA kit (Qiagen, Valencia, CA). RNA (10 µg of total RNA or 2 µg of poly(A)$^+$ RNA) was analyzed by electrophoresis on 2% formaldehyde/1.2% agarose gels and blotted onto nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). DNA probes were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) and purified from unincorporated nucleotides using probe purification columns (NucTrap, Stratagene, La Jolla, CA). The RNA blots were hybridized as described in Taylor and Green (1991) using the indicated ^{32}P-labeled probes. For a loading control, RNA blots were hybridized with a ^{32}P-labeled cDNA probe for the Arabidopsis translation initiation factor *eIF4A* (Taylor et al., 1993). Blots were stripped

between hybridizations in 0.1% SDS at 90 to 95°C for 1 hour. Quantification of hybridization signals was achieved using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Labeling of Poly(A)$^+$ RNA

For first strand cDNA synthesis, 1 μg of poly(A)$^+$ RNA and 1 μg oligo dT (GibcoBRL) in a total volume of 25 μl of DEPC-treated water was denatured at 70°C for 10 min and cooled down on ice. On ice, 8 μL of 5 × RT buffer, 4 μL of 0.1 M DTT, 2 μL of dNTPs (10 mM each), and 1 μL of Superscript II (GibcoBRL) were added, and the mixture was incubated at 42°C for 1 hr. To remove RNA, 0.5 μL of RNase H (4 units·μL^{-1}) was added, and the reaction was incubated for 30 min at 37°C. Single strand cDNA was purified in a Microcon YM-30 column (Millipore, Bedford, MA), concentrating to a volume of 10 μL TE (10 mM Tris·HCl, 1 mM EDTA, pH 8.0). Single-stranded cDNA was divided into two aliquots for Cy3- and Cy5-dCTP labeling, allowing for two hybridizations for each sample. The second-strand synthesis reaction contained 5 μL of single-stranded cDNA, 22 μL water, 4 μL Klenow buffer (500 mM Tris·HCl, pH 8.0, 500 mM NaCl, 100 mM $MgCl_2$), and 2 μL random hexamers (3 μg·μL^{-1}, GibcoBRL). The mixture was denatured at 95 °C for 3 min and annealed at room temperature for 5 minutes. Next, 4 μL of dNTP mix (250 μM dATP, dGTP, and dTTP, and 90 μM dCTP), 1 μL of dCTP-Cy3 or dCTP-Cy5 (Amersham, Arlington Heights, IL), and 2 μL Klenow (5 units·μL^{-1}) (GibcoBRL) were added, and the reaction was incubated for 2 hr at 37°C. All incubations were carried out in thin-wall 0.5 mL tubes in a RoboCycler 40 Temperature Cycler (Stratagene). Labeled dsDNA was purified using a QIAquick PCR purification kit (Qiagen) and eluted in 50 μL 2 mM Tris·HCl, pH 8.0. The probe was

dried down to 10 μL. To test the quality and quantity of the product, 1 μL was separated on a 1% agarose gel using a miniprotean gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The portion of the gel containing the DNA sample was placed on a glass microscope slide, dried on a heat block at 70°C, and scanned using a ScanArray 3000 or 5000 (GSI Lumonics). The rest of the probe was used to prepare the hybridization mixture.

DNA Microarray Hybridization and Analysis

For a single DNA microarray, 30 μL of hybridization solution was prepared by mixing 5.2 μL of 20 × SSC, 4.5 μL 2% SDS, 2.4 μL of tRNA (20 $\mu g \cdot \mu L^{-1}$), 9 μL Cy3-labeled probe, and 9 μL Cy5-labeled probe. The mixture was denatured at 100°C for 1 min and spun for 1 min to recover any condensate. The mixture was then hybridized to the array under a glass coverslip (24 mm × 40 mm, Corning) that had been washed in 95% ethanol, then 0.2% SDS, and rinsed in distilled water. The slide was then placed in a microarray hybridization chamber (ArrayIt Hybridization Cassette, TeleChem International, Inc.; Sunnyvale, CA) with 200 μL of 3 × SSC to ensure high humidity conditions.

Hybridization was carried out in a water bath at 65°C for 12 to 20 hours. After hybridization, the microarray was washed for 5 min in 1 × SSC/0.2% SDS, 5 min in 0.1 × SSC/0.2% SDS, and 30 sec in 0.1 × SSC without SDS, and finally dried by centrifugation at 600 rpm for 5 min.

The slide was scanned once in a ScanArray 3000 or 5000 (GSI Lumonics) for both channels 1 and 2 (corresponding to Cy3- and Cy5-labeled probes, respectively) at 10 μm resolution. The image files obtained were analyzed using ScanAnalyze software (v.

2.32, M. Eisen, Stanford University, http://genome-ww4.stanford.edu/MicroArray/SMD/restech.html). To ensure that only spots of high quality were used in the analysis, quality control measurements produced by the ScanAlyze software were employed. For example, the GTB2 value represents the fraction of pixels within each spot that are more than 1.5 × the background measurement. Spots with GTB2 values lower than 0.50 for either channel were removed and not considered for further analysis.

Data from each channel was transformed to the natural logarithm, and a Z-score was calculated to normalize the channel values in order to account for variation in RNA labeling. For the Z-score calculation [$Z = (\chi-\mu)/\sigma$, where χ = channel value, μ = mean of channel data, and σ = standard deviation of channel data], the trimmed mean and standard deviation using the middle 93% of the channel values were used in order to not bias the calculation due to extremely high or low values. The new data set has, by definition, a normal distribution with zero mean and unit variance. Values were retransformed from the natural logarithm by raising to the power e, and the channel ratio was calculated. From each of the four replica samples used with the 11,521 MSU DNA microarray, we generated two slides, with direct and reverse labeling. Ratios from reverse labeling were reversed in order to compare with the ratios from direct labeling. Therefore, for all slides, ratios above 1 and below 1 indicated elevated and decreased mRNA levels in *dst1* vs. parental plants, respectively. An average, standard deviation, and median of these eight ratios were determined and used as final ratio of mRNA levels.

Hierarchical clustering was performed using Cluster and Treeview software (Eisen et al., 1998; available at http://genome-www4.stanford.edu/MicroArray/SMD/restech.html).

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CHAPTER 3

CIRCADIAN CONTROL OF mRNA STABILITY: IMPACT OF THE *dst* MUTANTS

INTRODUCTION

Circadian clocks control rhythmic biological processes in many organisms and persist in the absence of environmental cues (Allada et al., 2001; Harmer et al., 2001; McClung, 2001). The Drosophila clock has been very well characterized and has been a model system to investigate circadian mechanisms. The transcription factors Clock (CLK) and Cycle (CYC) activate the transcription of the *period* (*per*) and *timeless* (*tim*) genes; PER and TIM proteins in turn negatively regulate the transcription of *per* and *tim* thus inhibiting the activity of CLK and CYC (Allada et al., 2001).

Genomic approaches to study circadian gene expression on a global basis in Arabidopsis, Drosophila and mammalian systems have revealed that 2-10% of mRNAs show circadian oscillations (Harmer et al., 2000; Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Miyazaki et al., 2003; Schaffer et al., 2001; Ueda et al., 2002). A recent study by Michael and McClung (2003) proposes that this could be an underestimate and using enhancer trapping, the authors showed that 36% of the Arabidopsis genome is under transcriptional control by the circadian clock.

For all the circadian clocks studied thus far, the core circadian oscillator is comprised of transcriptional feedback loops. However numerous lines of evidence in various systems suggest that posttranscriptional regulatory mechanisms are also required for clock function (Edery, 1999).

The oscillation in mRNA levels of all clock controlled genes cannot be accounted for by transcription alone. The best studied example to date corresponds to the *per* gene, one of the components of the central oscillator in Drosophila where comparison of *per* transcription rates and mRNA levels implicated a temporal regulation of mRNA half-life

(So and Rosbash, 1997). Further support for posttranscriptional regulation of *per* comes from transgenic flies that demonstrate daily cycling of *per* mRNA expressed from a constitutive promoter (Frisch et al., 1994). It has also been shown that sequences within *per*'s 5' UTR are required for regulating temporal RNA expression (Stanewsky et al., 2002) and this event depends on functional PER and TIM proteins (Suri et al., 1999). In addition, posttranscriptional regulation is thought to be involved in the adaptation of Drosophila to cold. An alternatively spliced form of the *per* transcript is generated at lower temperature causing an advance in the phases of both the mRNA and protein cycles (Majercak et al., 1999). Temperature appears to affect the translational control of a central clock component of Neurospora as well. The levels of FRQ (frequency) increase at higher temperatures although very little changes are seen for *frq* mRNA levels (Liu et al., 1998).

Several posttranscriptional mechanisms are responsible for the lag between the peak levels of mRNA and that of the resulting protein. In Drosophila, a kinase called DBT (doubletime) is believed to be responsible for phosphorylation of PER thus targeting PER for rapid degradation (Price et al., 1998). *dbt* mutants that have reduced kinase activity show defective PER degradation (Suri and Rosbash, 2000). Another kinase SGG (shaggy) is thought to be important for the phosphorylation of TIM (Martinek et al., 2001). Recent experiments indicate that SLMB (slimb), a component of the ubiquitin proteasome pathway, participates in controlling the levels of PER and TIM (Grima et al., 2002). Interestingly, TIM is photosensitive and is degraded by the proteasome in the presence of light (Naidoo et al., 1999).

The Drosophila CLK protein is also posttranscriptionally controlled, possibly due to changes in stability induced by phosphorylation at specific times during the day (Kim et al., 2002). Similar daily oscillations in the phosphorylated state of the Neurospora FRQ protein have been observed (Garceau et al., 1997), and *frq* mutants in which the phosphorylation site has been abolished have reduced rates of FRQ degradation (Liu et al., 2000). The Neurospora homolog of Drosophila Slimb, FWD1, regulates the degradation of FRQ through the ubiquitin-proteasome pathway (He et al., 2003). A recent report indicates that antisense *frq* transcripts are partly responsible for the entrainment of the Neurospora clock (Kramer et al., 2003). Antisense *frq* RNA is induced by light and in the dark it cycles in antiphase to sense *frq* RNA. The existence of antisense *frq* RNA could suggest the involvement of RNAi in the control of circadian gene expression.

Even though higher plants do not contain orthologs of clock proteins from other systems, oscillatory feedback loops seem to be conserved. The Arabidopsis circadian clock is suggested to be comprised of the MYB transcription factors *LHY* (Late Elongated Hypocotyl) and *CCA1* (Circadian Clock Associated 1) that are negative elements of a transcriptional feedback loop (Schaffer et al., 1998; Wang and Tobin, 1998) and have partially redundant functions (Mizoguchi et al., 2002). A pseudoresponse regulator *TOC1* (Timing Of *CAB* expression 1) might function as a positive element activating the transcription of *CCA1/LHY* (Alabadi et al., 2001).

Posttranscriptional modifications are also an integral part of the plant circadian clock. In Arabidopsis, a casein kinase II (CK2) phosphorylates CCA1 and influences CCA1 binding to DNA (Sugano et al; 1998). Moreover CK2 phosphorylates LHY *in vitro* and its overexpression leads to the shortened period of various circadian rhythms

(Sugano et al., 1999). The *tej* mutant results in a light-independent period lengthening of some clock genes and is caused by a mutation in a poly (ADP-Rib) glycohydrolase (Panda et al., 2002). Two Arabidopsis clock associated proteins, ZTL (Zeitlupe) and FKF1 (Flavin-binding, Kelch repeat, F box), contain an F-box motif that promotes ubiquitination of substrate proteins (Somers et al., 2000; Nelson et al; 2000). Kim et al. (2003) used Arabidopsis cell suspension cultures to demonstrate that the rhythmic changes in the levels of ZTL are caused by circadian phase-specific differences in protein degradation by the proteasome.

Transcript stability has been hypothesized to be responsible for the oscillations of the *CAB1* mRNA in Arabidopsis (Millar and Kay, 1991). Increased transcript stability has also been implicated in the accumulation of high steady state levels of *CAT3* mRNA in continuous dark (Zhong et al., 1997). Furthermore, based on nuclear run-on assays, the cycling of *NIA2* (Nitrate Reductase 2) is thought to occur through posttranscriptional regulation (Pilgrim et al., 1993). In rice, the circadian regulation of *CatA* (CatalaseA) expression has been postulated to be at the level of pre-mRNA stability (Iwamoto et al., 2000).

Microarray analysis has shown that a subset of unstable transcripts in Arabidopsis are controlled by the circadian clock (Gutiérrez et al., 2002). Additional microarray studies led to the finding that an unexpectedly high percentage of transcripts that were changed in abundance in a mutant deficient in DST-mediated decay, *dst1*, were circadian regulated, indicating that the biological significance of the DST-mediated mRNA decay pathway may be associated with the circadian clock (Pérez-Amador et al., 2001). In wild type plants, for two transcripts *SEN1* and *Ccr*-like, mRNA decay is regulated by the

circadian clock (Gutiérrez, 2003) and these transcripts are direct targets of the *dst1* mutant (see Chapter 2). These observations prompted us to test whether the *dst1* mutation affects the control of *Ccr*-like and *SEN1* mRNA stability differently at different times of the day. The results presented in this chapter demonstrate that the circadian clock regulated stability of specific plant mRNAs, *Ccr*-like and *SEN1*, is altered in the *dst1* mutant and the *DST1* locus is associated with circadian control. Furthermore our data indicates that control of mRNA stability adds another layer of regulation which impacts at the whole plant level for certain circadian processes and has a prominent role in clock controlled gene expression in plants.

RESULTS

Diurnal control of *Ccr*-like and *SEN1* mRNA stability is affected in the *dst1* mutant

It was previously shown that DST1 function is important for the normal diurnal oscillatory expression of *Ccr*-like and *SEN1* transcripts (Gutiérrez, 2003). To determine if this alteration in diurnal oscillation was caused by defective mRNA degradation in the *dst1* mutant, mRNA decay rates were measured at two times during the day. Cordycepin time courses were carried out one hour after dawn, ZT1 (zeitgeber 1) and 8 hours after dawn (ZT8) in two week old 1519 and *dst1* plants grown in 16 hours light/8 hours dark conditions. In the morning (ZT1), *Ccr*-like mRNA was more unstable in *dst1*, relative to the parental (p-value = 0.0003), as expected from the microarray studies (Figure 3.1A and B). However this effect was reversed in the afternoon (ZT8) such that the transcript was now more stabilized in the mutant (Figure 3.1C and D; p-value < 0.0001). A similar effect was seen on *SEN1* mRNA decay kinetics with the transcript being rapidly degraded

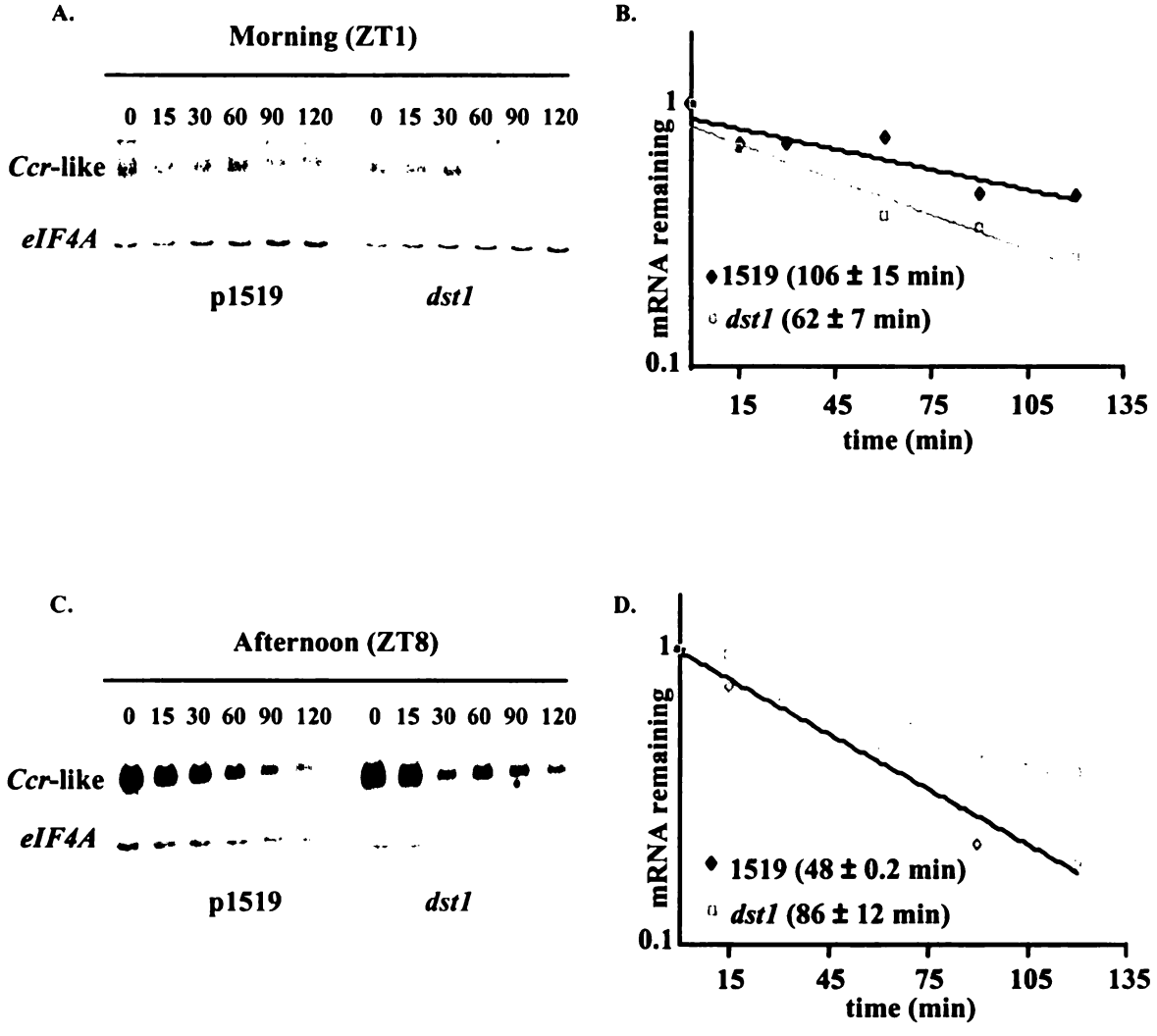


Figure 3.1. Regulation of *Ccr*-like mRNA stability is altered in the *dst1* mutant during the day.

Representative northern blot analysis of cordycepin time courses performed in 1519 and *dst1* plants (A) 1 hour after dawn (zeitgeber1 or ZT1), and (C) 8 hours after dawn (ZT8) for *Ccr*-like and *eif4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for *Ccr*-like (B) in the morning and (D) in the afternoon. The stable *eIF4A* transcript was used as a reference for equal loading. Half-life values are representative of 2 independent cordycepin time courses.

in the afternoon and stabilized in the morning in parental plants (Figure 3.2B and D). The opposite trend was seen for the *dst1* mutant with *SEN1* mRNA being less stable in the a.m. (p-value < 0.0001) and more stable in the p.m. (Figure 3.2B and D; p-value = 0.0061). Statistical analysis, as indicated above, showed that the differences in half-lives seen for *Ccr*-like and *SEN1* mRNAs in the mutant and parental plants are significant. These results suggest that normal *DST1* function is required for the proper timing of degradation of *Ccr*-like and *SEN1* transcripts under diurnal conditions.

DST1 function is required for normal circadian expression of *SEN1* and *Ccr*-like mRNAs

To evaluate the impact of the *dst1* mutation on the circadian oscillation of *Ccr*-like and *SEN1*, mRNA levels were examined under free running conditions. Arabidopsis seedlings were grown for 12 days in 16/8 LD cycles and on the morning of the 12th day transferred to continuous light. Seedling tissue for mRNA isolation was harvested every 3 hours starting on the morning of the 12th day (circadian time 0/CT0) up to the 14th day (CT45). As shown in Figure 3.3A and B, *Ccr*-like mRNA peaked approximately 3 hours later in the *dst1* mutant than in the parental plants. This effect of the mRNA peak lagging in the *dst1* was even more pronounced on the second day in constant light. A reduction in amplitude for *Ccr*-like mRNA was also seen in addition to the delay in phase. *dst1* influence on the circadian oscillation of *SEN1* was slightly more complicated since *SEN1* is known to be induced during the dark (Oh et al., 1996) possibly at the level of transcription (Chung et al., 1997). As is evident in Figure 3.4A, the dark induction of *SEN1* was not seen during the second day in continuous light. However, the *SEN1*

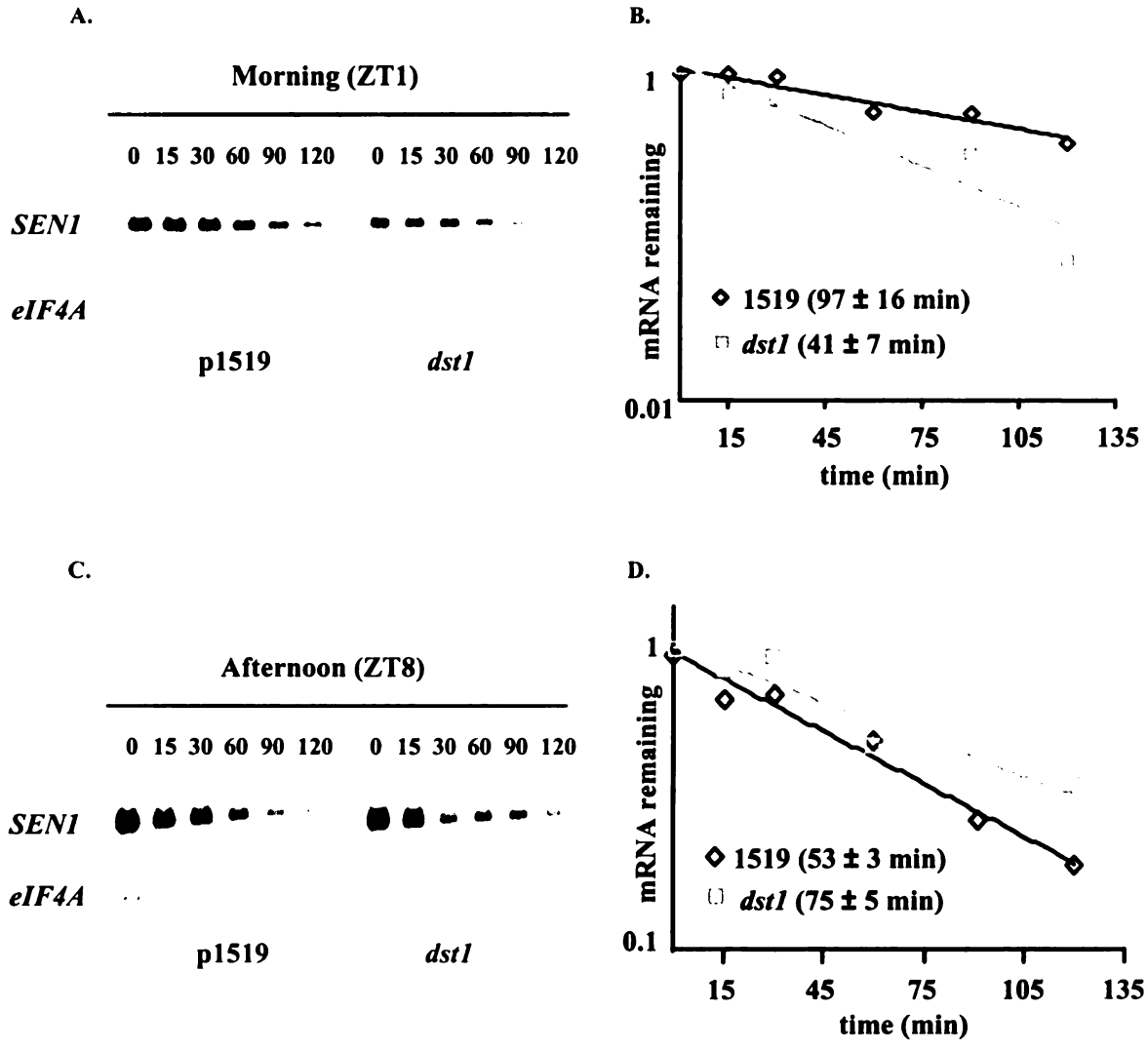


Figure 3.2. Regulation of *SEN1* mRNA stability is altered in the *dst1* mutant during the day.

Representative northern blot analysis of cordycepin time courses performed in 1519 and *dst1* plants (A) 1 hour after dawn (zeitgeber1 or ZT1), and (C) 8 hours after dawn (ZT8) for *SEN1* and *eif4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for *SEN1* (B) in the morning and (D) in the afternoon. The stable *eIF4A* transcript was used as a reference for equal loading. Half-life values are representative of 2 independent cordycepin time courses.

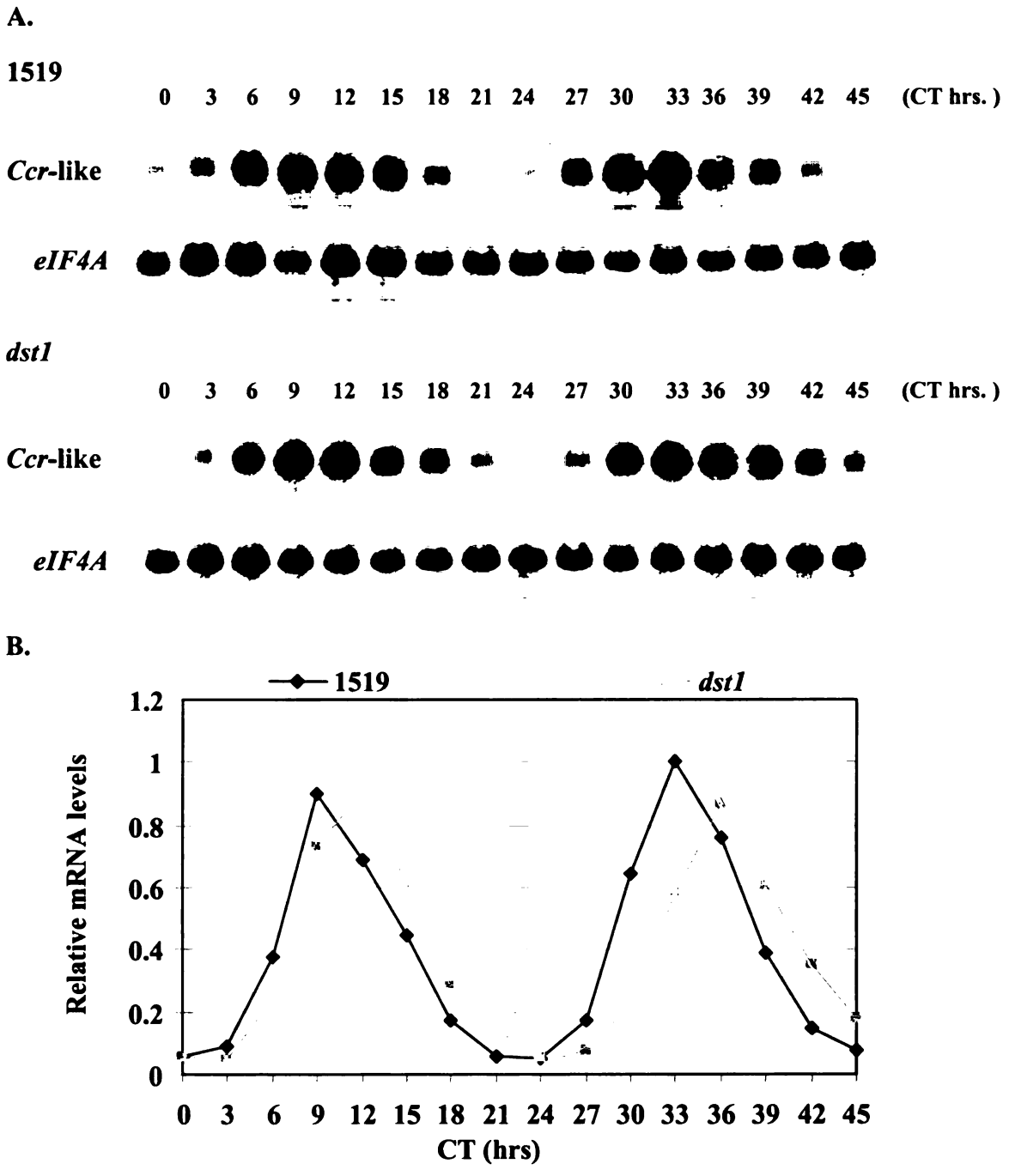


Figure 3.3. Circadian oscillation of *Ccr*-like mRNA is altered in the *dst1* mutant.

A) Representative northern blot analysis of time courses performed throughout two entire days in continuous light for *Ccr*-like mRNA in *dst1* mutant and parental 1519 plants. Samples consisted of 10 μg of total RNA isolated from the indicated times of the day after subjective dawn (CT=0). B) Quantitation of mRNA levels for *Ccr*-like. All values are the average of two independent experiments and are made relative to the highest mRNA accumulation in either of the two genetic backgrounds. The signal for *eIF4A* was used as a reference for equal loading.

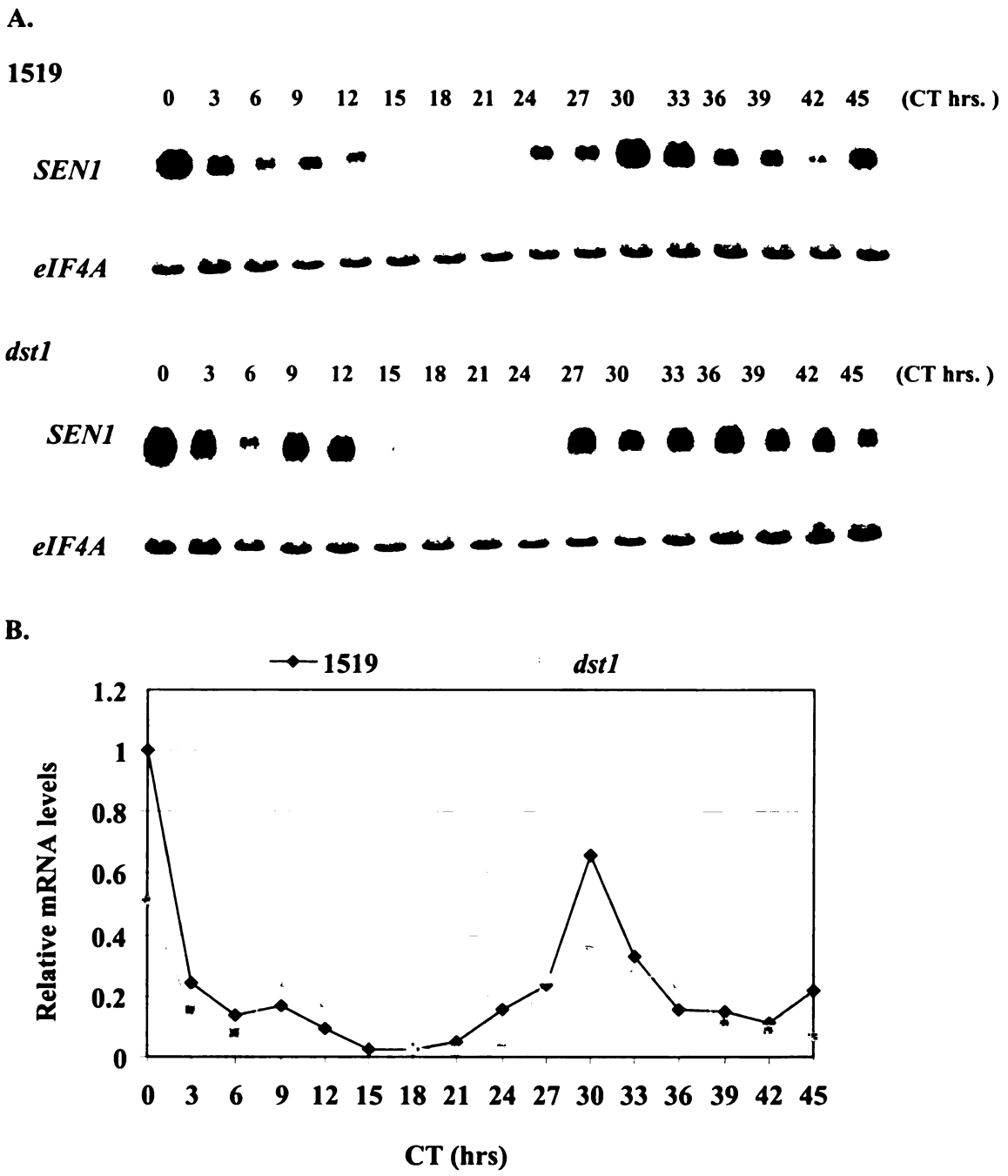


Figure 3.4. Circadian oscillation of *SEN1* mRNA is altered in the *dst1* mutant.

A) Representative northern blot analysis of time courses performed throughout two entire days in continuous light for *SEN1*mRNA in *dst1* mutant and parental 1519 plants. Samples consisted of 10 μg of total RNA isolated from the indicated times of the day after subjective dawn (CT=0). B) Quantitation of mRNA levels for *SEN1*. All values are the average of two independent experiments and are made relative to the highest mRNA accumulation in either of the two genetic backgrounds. The signal for *eIF4A* was used as a reference for equal loading.

transcript had reduced accumulation in the afternoon in the *dst1* mutant and this difference in p.m accumulation between the *dst1* and parental plants was more apparent on the second day (Figure 3.4A and B). Also the circadian expression of *Ccr*-like and *SEN1* transcripts under free running conditions was comparable to that seen previously under diurnal conditions (Gutiérrez, 2003). In order to study the effect of *dst1* on general clock controlled gene expression, circadian oscillation of *AtGRP7/CCR2*, which functions downstream of the master clock (Staiger, 2001), was tested. Oscillation of *CCR2* mRNA was unchanged in the *dst1* mutant compared to the parental (Figure 3.5A and B). Taken together, this indicates that the circadian oscillations of a subset of clock controlled genes, *Ccr*-like and *SEN*, are dependent on *DST1* function.

dst1 affects circadian control of mRNA stability

We hypothesized that the stabilization of *Ccr*-like and *SEN1* mRNAs caused by *dst1* in the afternoon under diurnal conditions would also occur in the subjective afternoon under free running conditions. To test this hypothesis, mRNA half-lives were measured in Arabidopsis seedlings that were transferred to continuous light for 2 days. Transcription was inhibited 1 hour (CT1) and 8 hours (CT8) after the subjective morning to determine mRNA decay rates. The impact of *dst1* was recapitulated for *Ccr*-like in continuous light with the transcript being more unstable in *dst1* in the subjective morning (Figure 3.6B; p-value < 0.0001) and more stable in *dst1* in the subjective afternoon (Figure 3.6D; p-value $= 0.0171$) relative to the parental. Even though some dampening in mRNA half-lives was seen under circadian conditions relative to the diurnal conditions, the differences in mRNA decay rates between the mutant and parental were statistically

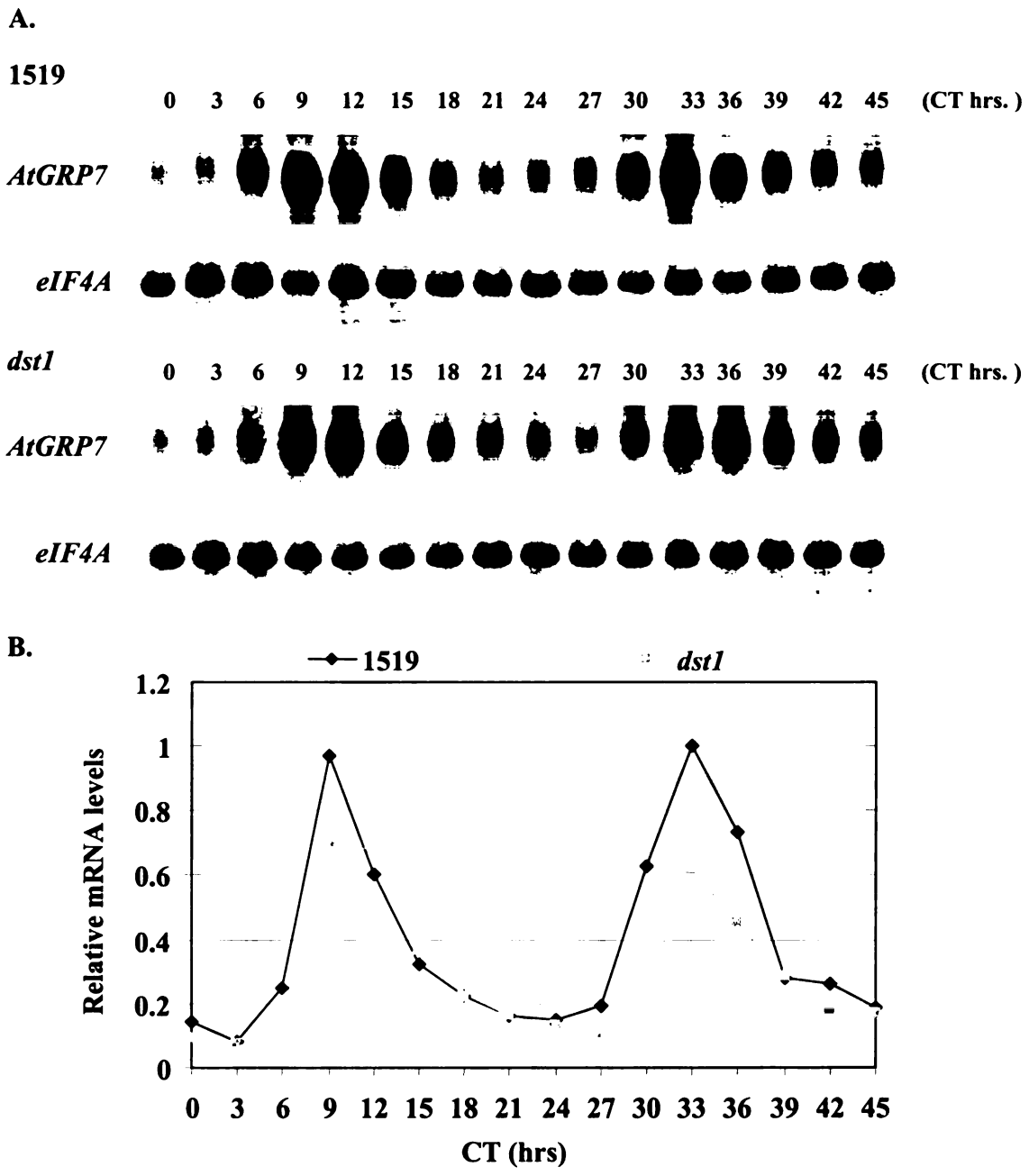


Figure 3.5. Circadian oscillation of *AtGRP7* mRNA is unaltered in the *dst1* mutant.

A) Representative northern blot analysis of time courses performed throughout two entire days in continuous light for *AtGRP7* mRNA in *dst1* mutant and parental 1519 plants. Samples consisted of 10 μg of total RNA isolated from the indicated times of the day after subjective dawn (CT=0). B) Quantitation of mRNA levels for *AtGRP7*. All values are representative of at least two independent experiments and are made relative to the highest mRNA accumulation in either of the two genetic backgrounds. The signal for *eIF4A* was used as a reference for equal loading.

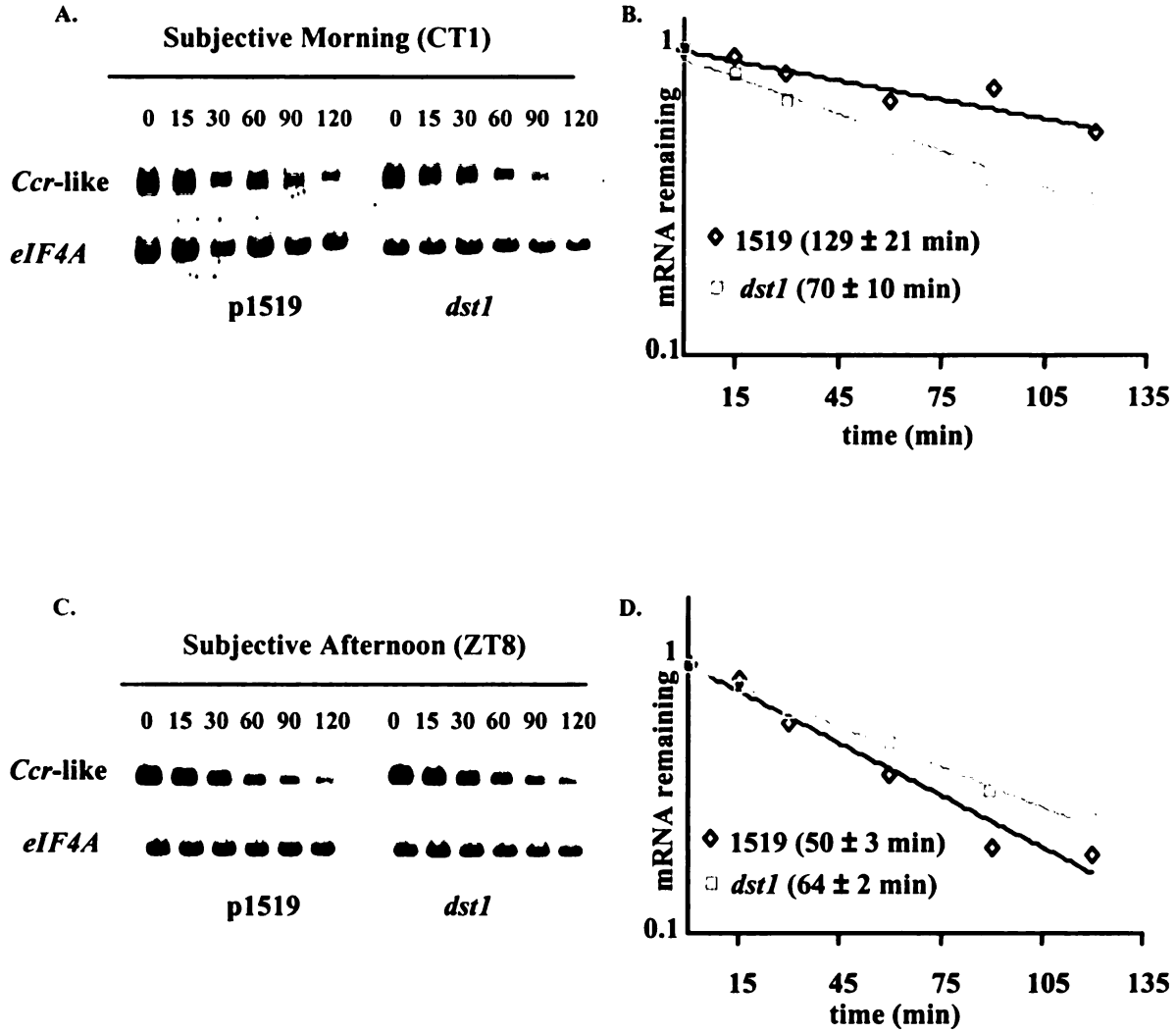


Figure 3.6. Circadian regulation of *Ccr*-like mRNA stability is altered in the *dst1* mutant.

Representative northern blot analysis of cordycepin time courses performed in 1519 and *dst1* plants (A) 1 hour after subjective dawn (circadian time 1 or CT1), and (C) 8 hours after subjective dawn (CT8) for *Ccr*-like and *eif4A* mRNAs. Samples consisted of 10 µg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for *Ccr*-like (B) in the subjective morning and (D) in the subjective afternoon. The stable *eIF4A* transcript was used as a reference for equal loading. Half-life values are representative of 2 independent cordycepin time courses.

significant. In the subjective morning, *SEN1* mRNA degrades faster in *dst1* than in parental (Figure 3.7A and B), whereas in the afternoon no significant differences in mRNA stability could be seen in *dst1* compared to the parental (Figure 3.7C and D). This could be attributed in part to the dampening observed under circadian conditions since the effect of *dst1* on *SEN1* mRNA stability under diurnal conditions was less dramatic than for *Ccr*-like (Figure 3.1 and 3.2).

Opposite effect of *dst2* on *SEN1* mRNA stability

For *Ccr-like* and *SEN1*, the *dst2* mutation is known to have an effect opposite to that of *dst1* in RNA gel blots of samples harvested in the a.m. (see Chapter 1). To confirm this at the level of mRNA stability, mRNA half-lives were measured at ZT1 and ZT8 in *dst2* and parental plants. As shown in Figure 3.8B, *SEN1* transcript was more stabilized in the *dst2* mutant in the morning. In the afternoon, *SEN1* mRNA decayed at a more or less similar rate in *dst2* and parental plants (Figure 3.8D), but on comparing the a.m. and p.m. half-lives for the transcript in *dst2*, it was observed that the transcript decayed at a much faster rate than in the parental (Figure 3.8). Similar experiments with *Ccr*-like are in progress.

Impact at the whole plant level: Classical circadian phenotypes are altered in the *dst* mutants

To address the question if the link between the DST-mediated decay pathway and the circadian clock impacted at the whole plant level, leaf movement, a classical circadian phenotype, was monitored in the *dst* mutants. The oscillation in the position of leaves can be monitored by video imaging and this technique has been used to study

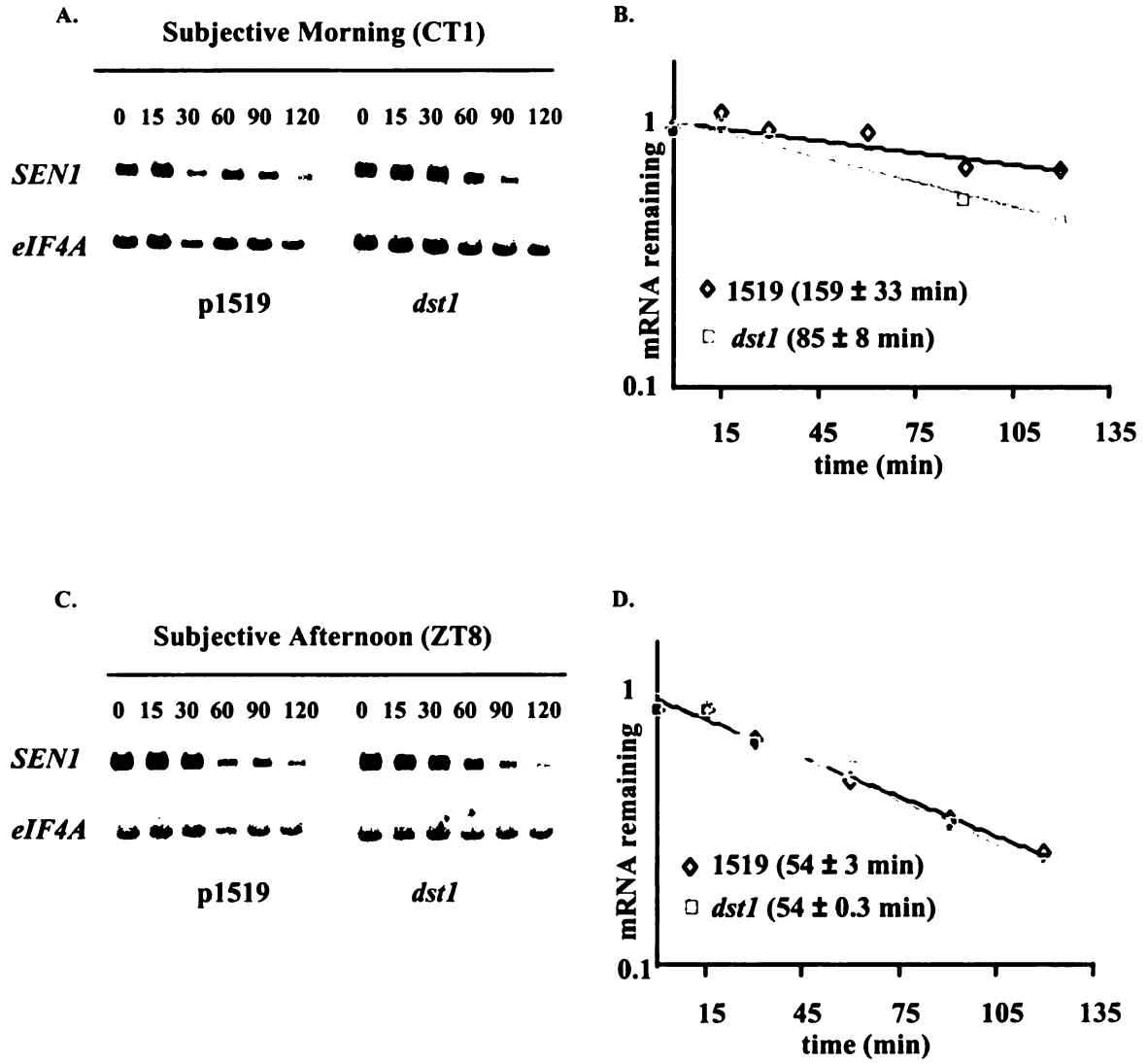


Figure 3.7. Circadian regulation of *SEN1* mRNA stability is altered in the *dst1* mutant.

Representative northern blot analysis of cordycepin time courses performed in 1519 and *dst1* plants (A) 1 hour after subjective dawn (circadian time 1 or CT1), and (C) 8 hours after subjective dawn (CT8) for *SEN1* and *eif4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for *SEN1* (B) in the subjective morning and (D) in the subjective afternoon. The stable *eIF4A* transcript was used as a reference for equal loading. Half-life values are representative of 2 independent cordycepin time courses.

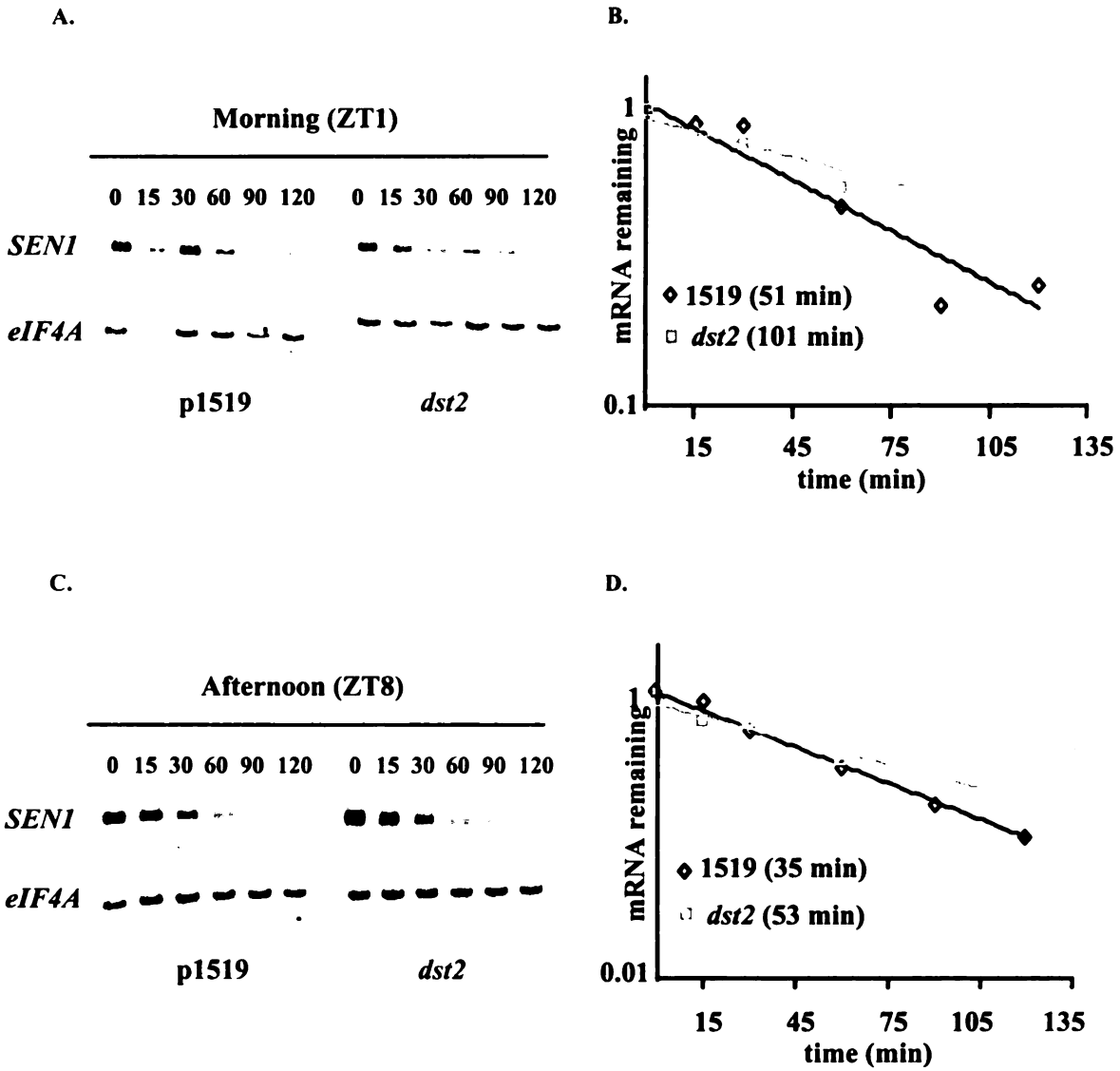


Figure 3.8. Regulation of *SEN1* mRNA stability is altered in the *dst2* mutant during the day.

Representative northern blot analysis of cordycepin time courses performed in 1519 and *dst2* plants (A) 1 hour after dawn (zeitgeber1 or ZT1), and (C) 8 hours after dawn (ZT8) for *SEN1* and *eif4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for *SEN1* (B) in the morning and (D) in the afternoon. The stable *eIF4A* transcript was used as a reference for equal loading.

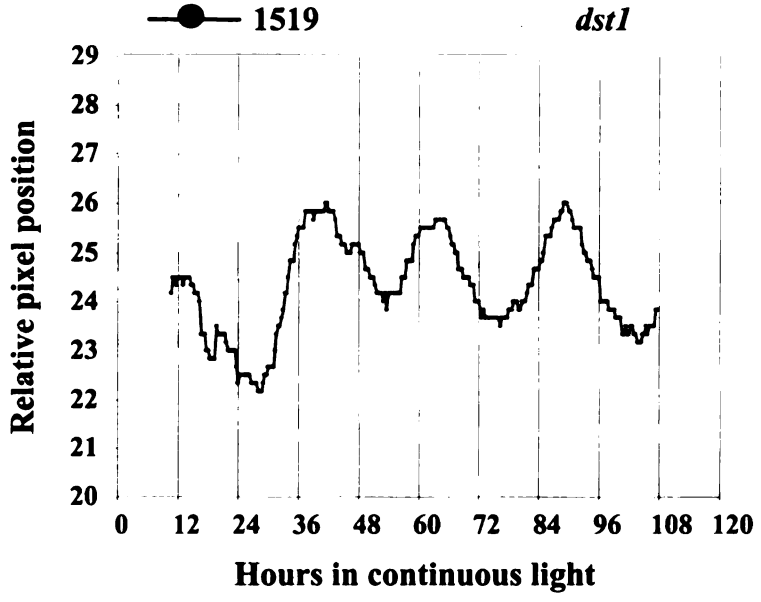
mutations in the circadian clock (Millar et el., 1995). Seedlings were grown for 5 days in 12 hr light / 12 hr dark, transferred to 24-well plates and released into continuous light. Cotyledon movement was then recorded for 7 days The potential significance of our mRNA steady state and half-life results was emphasized by the finding that *dst1* was phase lagging (Figure 3.9A) and *dst2* was phase leading (Figure 3.9B) in these experiments (in collaboration with Salome, P. and McClung, R.).

DISCUSSION

In this study, we have shown that the DST-mediated decay pathway is required for the normal oscillation of selected clock controlled genes in Arabidopsis and is critical for proper circadian regulation of the stability of these DST-containing transcripts.

The stability of the *SEN1* and *Ccr*-like mRNAs was altered in the mutant under diurnal conditions and the effect in the morning and afternoon was different (Figure 3.1 and 3.2). From these findings it can be concluded that *dst1* is required for the proper diurnal regulation of the stability of these DST-containing transcripts. A cycling mRNA with a longer half-life will show a phase delay and reduced amplitude when compared with an mRNA with a shorter half-life, assuming that the transcription rates are similar (Wuarin et al., 1992). Under circadian conditions, the *Ccr*-like transcript has an increased half-life in *dst1* compared to the parental (Figure 3.6) in the subjective afternoon and this posttranscriptional effect probably regulates the phase delay seen at the level of mRNA oscillation and leaf movement (Figure 3.3 and Figure 3.9A). The dampening in the mRNA decay rates seen under circadian conditions could be due to the fact that the clock is not reset in constant light leading to desynchronization such that the individual seedlings drift out of phase from each other.

A.



B.

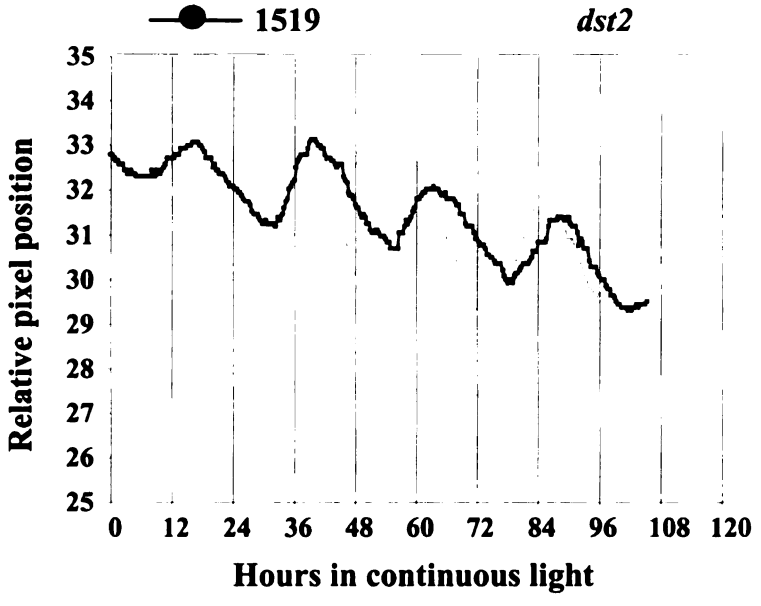


Figure 3.9. Circadian rhythm of leaf movement is altered in the *dst* mutants.

The graphs represent Arabidopsis leaf movement data taken by video cameras over a period of five days. Each peak represents the "up" position of the cotyledon as the underside of the petiole grows more than the upper side. Each trough is the "down" position as the upper side of the petiole grows more than the underside. The traces for A) *dst1* and parental are an average of 6 seedlings while the traces for B) *dst2* and parental are average of 12 seedlings.

An opposite effect was seen with *dst2* being phase leading in the leaf movement studies (Figure 3.9B). This observation is in agreement with the half-life changes seen in *dst2* and the parental plants. Even though the *SEN1* transcript is more stabilized in the mutant compared to the parental in the morning, the mRNA decays at a much faster rate in *dst2* (Figures 3.8, compare a.m. and p.m.). This results in a shift in phase with the peak being reached earlier in *dst2*.

It is evident that *cis*-acting elements as well as *trans*-acting factors are involved in the posttranscriptional regulation of clock controlled mRNAs. The 3' UTR of Drosophila *per* gene is AU-rich and it is known that AU-rich elements (AREs) are found in the 3'UTRs of several of the most unstable mammalian transcripts (Chen and Shyu, 1995). Both *Ccr*-like and *SEN1* contain DST-like elements in their 3'UTR and are primary targets of the *dst1* mutation. In addition, they belong to a unique subset of transcripts that are decreased in abundance in *dst1* but are increased in abundance in *dst2* (Pérez-Amador et al., 2001). The exact nature of the *DST1* mutation is not known as yet but it is possible that it corresponds to a sequence-specific RNA-binding protein. Clock controlled RNA-binding proteins have been identified in the algae *Gonyaulax polyedra* (Morse et al., 1989) and *Chlamydomonas reinhardtii* (Mittag et al., 1994) that bind to the 3' UTRs of several mRNAs that contain a UG-repeat region (Waltenberger et al., 2001). The circadian clock also controls the binding activity of these proteins which are hypothesized to function as translational suppressors (Mittag, 2003). Another example is the putative RNA-binding LARK protein of Drosophila that oscillates in abundance and regulates adult eclosion (McNeil et al., 1998).

Precedence for clock-controlled RNA-binding proteins in Arabidopsis has also been documented. *AtGRP7* mRNA as well as the protein undergoes circadian oscillations with slight delay of the protein peak relative to the RNA peak (Heintzen et al., 1997). Overexpression studies have shown that the transcript and protein are linked in a negative autoregulatory circuit (Staiger, 2001). This negative autoregulation was shown to be mediated through the binding of the protein to its own pre-mRNA resulting in the formation of an alternatively spliced transcript with a premature stop codon which is rapidly degraded (Staiger et al., 2003).

The role of mRNA stability in circadian control of gene expression has been further highlighted by the recent characterization of a Xenopus deadenylase, nocturnin that is expressed in a rhythmic manner (Baggs and Green, 2003). A nocturnin homolog is also present in Arabidopsis (Dupressoir et al., 2001). Since nocturnin is expressed in a circadian fashion, it is possible that this enzyme is responsible for the deadenylation of clock controlled mRNAs. A relevant example is the oscillation of vasopressin transcript levels in mammals where two species of mRNAs with differences in their poly(A) tail length are present at different times of the day (Robinson et al., 1988).

dst1 plants do not exhibit a severe effect on the clock at the molecular level since other clock controlled genes such as *AtGRP7* show normal oscillation patterns in *dst1* (Figure 3.5). Our data predicts that DST1 probably functions downstream of the master clock and affects a subset of clock controlled genes at the level of mRNA stability. This hypothesis is further supported by the fact that *DST1* maps to a region on chromosome 1 (see Chapter 4) that does not seem to contain any genes known to be involved in clock function. Cloning of the *DST1* gene should help us elucidate the precise relationship

between the DST-mediated decay machinery and the circadian clock. The link between sequence-specific decay and the circadian clock uncovered in our experiments could be extended to other systems as a powerful tool towards unraveling circadian clock mechanisms at the posttranscriptional level.

MATERIALS AND METHODS

Arabidopsis strains and growth conditions

All *Arabidopsis thaliana* plants described are from the accession Columbia. *dst1* mutant (from the second backcross to the parental line) and 1519 parental seeds were plated on agar plates containing 1x Murashige and Skoog salts, 1x Gamborg's vitamins, 1% sucrose and 50 μg/ml kanamycin for two weeks in an incubator set at 16 hours light (125 $\mu E/m^2$) /8 hours dark and 21°C. For the circadian (free running) experiments, Arabidopsis seedlings were grown for 12 days in 16 hours light/8 hours dark and on the morning of the 12^{th} day were transferred to continuous light for 48 hours. Lighting was provided by fluorescent light bulbs.

Half-life measurements, RNA preparation and analysis

Half-lives were determined as described by Seeley et al. (1992) with the following modifications. Two-week old Arabidopsis seedlings were transferred to a flask with incubation buffer. After a 30 min incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and frozen in liquid nitrogen. Total RNA was isolated using standard techniques. RNA was analyzed by electrophoresis on 2% formaldehyde/1.2%

agarose gels and blotted onto nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). DNA probes were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) and purified from unincorporated nucleotides using probe purification columns (NucTrap, Stratagene, La Jolla, CA). The RNA blots were hybridized as described in Taylor and Green (1991) using the indicated ^{32}P-labeled probes. For a loading control, RNA blots were hybridized with a ^{32}P-labeled cDNA probe for the Arabidopsis translation initiation factor *eIF4A* (Taylor et al., 1993).

SEN1 is a single gene in the genome of *Arabidopsis thaliana* (Oh et al., 1996). Ccr-like protein has no similarity to other protein sequences in the Arabidopsis genome as determined by BLASTCLUST (ftp://ftp.ncbi.nlm.nih.gov/blast/documents/README.bcl). In addition, BLASTN analysis (nucleotide *vs.* nucleotide comparison) using *SEN1* and *Ccr*-like transcribed sequences as query revealed no significant similarity to other transcribed sequences in the Arabidopsis genome. Hence, northern blot probes used in this study were made by the polymerase chain reaction using EST clones as template, 111D3T7 for *SEN1* and 245H16T7 for *Ccr*-like, and SP6 and T7 vector primers.

Statistical comparison of the half-lives measured at the different times of the day was performed using a repeated measure model by Xue Lan (Statistics Department, Michigan State University).

Leaf movement assay

Assessment of rhythmicity in leaf movement was carried out as described (Millar et al., 1995). For light entrainment, seedlings were grown under white light for 5 days in a 12 hr light /12 hr dark photoperiod. For temperature entrainment, seedlings were grown

under white light for 7 days in a 12 hr 22°C / 12 hr 12°C temperature regime. On the 5th or 7th day, seedlings were transferred to 24-well cloning plates and the plates were released into continuous white light and constant temperature of 22°C. Leaf movement was recorded every 20 min over 7 days by Panasonic CCTV cameras, model WV-BP120 (Matsushita Communications Industrial, Laguna, Philippines). Post-run analysis was performed using the Kujata software program [Millar et al., 1995), and traces were analyzed by FFT-NLLS [Plautz et al., 1997).

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CHAPTER 4

GENETIC MAPPING OF *dst1*

INTRODUCTION

Insertional mutagenesis techniques have been widely used to identify the function of unknown genes using T-DNAs (Alonso et al., 2003; Krysan et al., 1999) and transposable elements (Aarts et al., 1995; Weigel et al., 2000). However these methods cannot create partial loss-of-function or gain-of-function alleles. In contrast, chemical mutagenesis causes point mutations generating different types of alleles; this could be an advantage, especially if the genes in the regulatory pathway are essential. Furthermore, because EMS mutagenesis can result in a higher mutational frequency than T-DNA mutagenesis, fewer plants need to be screened in order to find a mutation in any given gene. The mutated genes then have to be isolated based on their map position.

Map-based cloning is an indirect approach; mapping narrows down the genetic interval that contains a mutation by sequentially excluding all other parts of the genome. Mapping with a high resolution requires a high density of genetic markers around the region of interest. This has been greatly facilitated by the sequencing of Columbia and Landsberg *erecta* (L*er*) *Arabidopsis* ecotypes which are sufficiently divergent to support the design of molecular markers at this high density. Also, the sequence of the *Arabidopsis* genome has been completed which should expedite pinpointing the genes after fine mapping.

Some molecular markers commonly used in mapping experiments include simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPS) and derived CAPS (dCAPS) (Lukowitz et al., 2000). These markers are co-dominant and are PCR-based. SSLP (Bell and Ecker, 1994) markers are based on the variability of short repetitive sequences. These markers are amplified using PCR and

exhibit ecotype-specific polymorphisms on agarose/polyarylamide gels. CAPS markers detect polymorphisms that occur in restriction sites (Konieczny and Ausubel, 1993) while dCAPS are capable of exploiting all single nucleotide changes whereby mismatches in PCR primers are used to create restriction sites (Neff et al., 1998). It is also possible to generate additional markers in the region of interest by utilizing single nucleotide polymorphism (SNP) changes as well as insertion-deletion (InDel) differences. On comparing Arabidopsis Columbia and L*er* genomic sequences, there is one SNP every 3.3 kb and one InDel every 6.6 kb (Jander et al., 2002). A total of 56,670 polymorphisms have been deposited on The Arabidopsis Information Resource web site (http://www.arabidopsis.org/Cereon) including 37,344 SNPs, 18,579 InDels, and 747 Large Indels.

To better understand the molecular mechanisms involved in sequence-specific mRNA decay, specially designed transgenic plants were used to select for mutants that are defective in DST-mediated mRNA degradation using an ethyl methane-sulfonate-mutagenized population (Johnson et al., 2000). The mutants that were isolated were extremely rare (3/~800,000), were all incompletely dominant and lacked any visible aberrant phenotype. This could be because the *DST* genes are essential and the *dst* mutants are weak alleles, or it is possible that the *DST* genes are part of gene families that have redundant members. Cloning of the *DST* genes should help in the elucidation of the components of the DST-recognition and decay machinery. *dst1* was chosen for cloning since it is the best characterized but these experiments should be applicable to the other *dst* mutants as well. The results described in this chapter demonstrate that *RAP2.4*, first identified in the microarray experiments (see Chapter 2), is as an excellent endogenous

molecular marker for scoring *dst1*. Using *RAP2.4* expression levels, homozygous *dst1* mutants were followed independent of the transgene and the mutation has been mapped to a 107 kb interval on the bottom of chromosome 1.

RESULTS

dst1 mutation does not appear to be linked to the transgene

The *dst1* phenotype is a 3-4 fold increase in *HPH* mRNA levels and heterozygous plants exhibit half this increase (Johnson et al., 2000). The requirement of the transgene to score the *dst1* mutants had several drawbacks since both the *dst1* mutation and the transgene were segregating in the mapping population. Consequently, in the F2, *HPH-DST* mRNA abundance reflected the dosage of the transgene and the *dst1* mutation. As a result larger number of plants had to be screened to identify homozygous *dst1* mutants. To overcome this dependence on the transgene, inheritance of *RAP2.4* mRNA abundance was examined for the mutant. Progeny of the second backcross to WT (1519-31) (F1) and the progeny of self-fertilizations of these plants (F2) were used in these studies. The F1 plants showed intermediate levels of *RAP2.4* mRNA abundance as expected (data not shown). Segregation of increased *RAP2.4* mRNA abundance in the F2 populations was also consistent with semi-dominance. Of 240 F2 plants segregating *dst1*, 57 were observed with high *RAP2.4* mRNA abundance (9.3 ± 3.3 fold higher than 1519-31), 111 had intermediate *RAP2.4* mRNA abundance (2.07 ± 0.8) and 72 showed *RAP2.4* mRNA abundance that was similar to parental (0.92 ± 0.2) (Figure 4.1). Segregation in the *dst1 F2* populations is most easily explained by a ratio of 1:2:1 as would be expected for the

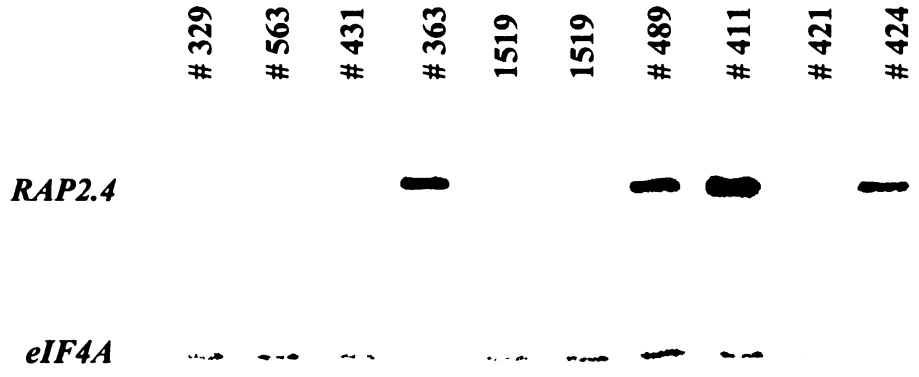

Figure 4.1. mRNA abundance of *RAP2.4* in F2 plants from the second back-cross of *dst1* relative to WT (1519-31).

8 representative plants are shown. Total RNA was prepared from rosette leaves and analyzed by Northern blot hybridization. Radiolabeled probes, prepared against the indicated transcripts, were used in sequential hybridizations of the same blot. The numbers indicate individual plants. #411 is an example of an F2 plant with high *RAP2.4* mRNA levels, #431 with intermediate and #329 with *RAP2.4* mRNA levels similar to WT (1519-31).

inheritance of a semi-dominant single gene, indicating that the mutation is not linked to the transgene (Table 4.1).

Use of *RAP2.4* as an endogenous marker to score homozygous mutants

It is known that the *dst1* mutation is associated with circadian rhythms (Pérez-Amador et al., 2001). In order to determine if *RAP2.4* could be used as a phenotypic marker for scoring the *dst1* mutation in the mapping population, it was critical to establish that *RAP2.4* was not circadian regulated. Towards this end, mRNA half-life experiments were carried out in *dst1* and 1519 plants in the morning and afternoon. A comparison of Figures 4.2B and 4.2D shows that *RAP2.4* mRNA is more stable in *dst1* than in the parental 1519 line when mRNA decay is monitored in the morning (zeitgeber time 1/ZT1) and in the afternoon (ZT 8) and all of the decay curves are nearly identical under the two conditions. These mRNA stability measurements indicate that the effect of *dst1* on *RAP2.4* is insensitive to the time of day. It is also of interest to note that *RAP2.4* was not regulated by circadian rhythms in the *dst1* microarray experiments.

Fine mapping of the *dst1* locus

The first step in a mapping experiment is to generate a mapping population. Since the mutation is in a Columbia (*gl1*) background, *dst1* was crossed to Landsberg *erecta* (*gl1-1*). F2 plants from this cross were scored by Northern blot analysis for 5-fold or higher *RAP2.4* mRNA abundance compared to parental 1519 plants. 78 *dst1/dst1* F2 plants were identified and genomic DNA was extracted from them for PCR. Mapping of the *DST1* gene to chromosome 1 was accomplished by linkage analysis to SSLP markers

Table 4.1. Segregation of increased *RAP2.4* mRNA abundance in the progeny of crosses between *dst1* and 1519-31 (*DST1*).

Cross		Class	*RAP2.4**	n	χ^2, P^f
DST1/dst1 x *DST1/dst1*	F_2	High	9.3 ± 3.3	57	
		Int	2.07 ± 0.8	111	1.9 P>0.3
		WT	0.92 ± 0.2	72	

Int, intermediate.

WT, wild-type.

* *RAP2.4* mRNA abundance: (*RAP2.4/eif4A*) segregating class/ (*RAP2.4/eif4A*) WT ± standard error.

f χ^2 calculated for 1:2:1 segregation of WT/Int/High *RAP2.4* mRNA abundance DST/DST, WT (1519-31).

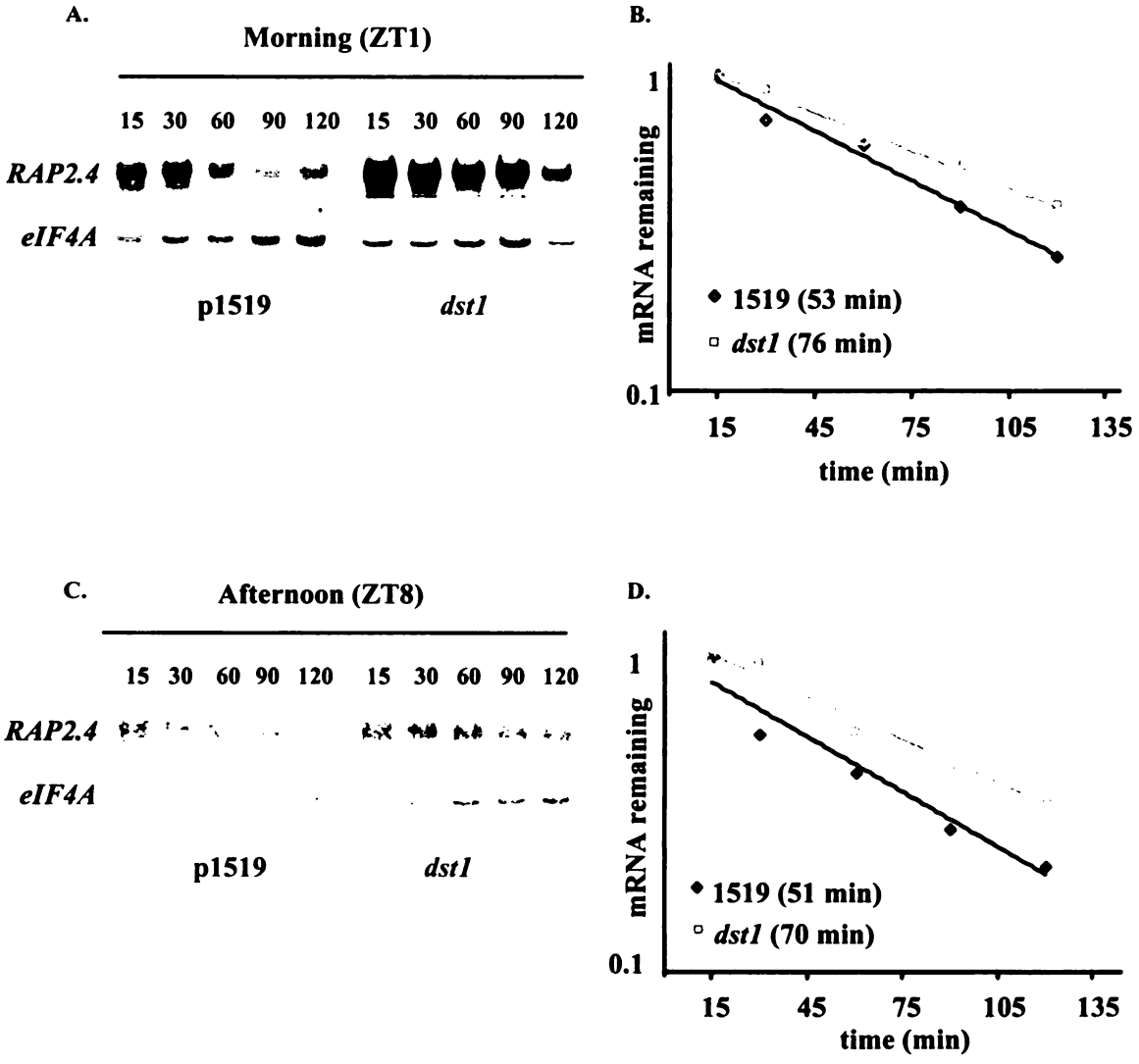

Figure 4.2. *RAP2.4* mRNA is stabilized in the *dst1* mutant and the decay kinetics are similar in the a.m and p.m.

Representative northern blot analysis of cordycepin time courses performed in 1519 and *dst1* plants (A) 1 hour after dawn (zeitgeber1 or ZT1), and (C) 8 hours after dawn (ZT8) for *RAP2.4* and *eif4A* mRNAs. Samples consisted of 10 μg of total RNA isolated from the indicated time points. Quantitation of the decrease in mRNA abundance and half-life estimation for *RAP2.4* (B) in the morning and (D) in the afternoon. The stable *eIF4A* transcript was used as a reference for equal loading.

(Figure 4.3). Recombination events between molecular markers and the *dst1* locus in the homozygous mutants were analyzed to confirm that *dst1* was located between markers f16p17 and f1n192. Additional polymorphic SSLP and CAPS markers were identified in this interval and two markers (f22c121 and f1n191) were found that were 5.8% recombination apart (Figure 4.4).

A larger F2 population was then generated for fine-resolution mapping. The closely linked SSLP markers (107 kb), f22c121 and f1n191, were used to screen a mapping population of 970 F_2 plants. 24 recombinants within the interval defined by the flanking molecular markers were identified. RNA gel blot analysis was used to determine whether the recombinants were homozygous mutant, homozygous wild type, or heterozygous at the *DST1* locus. This information together with additional markers is now being used to further narrow down the interval.

DISCUSSION

The strategy for scoring *dst1* was tailored in order to avoid dependence on the transgene and thereby expedite cloning. The reliance of the *dst1* phenotype on the transgene is an immense drawback for the generation of mapping populations due to the small magnitude of change in *HPH-DST* mRNA levels. This scenario is further complicated by the semi-dominance of *dst1*. A locus like *dst1* presents unique challenges as it does not exhibit any visible phenotype and therefore various approaches were devised to overcome this difficulty. Early on in the mapping process, scoring only the very highest *HPH-DST* mRNA levels as *dst1* homozygotes was tried but this proved to be very tedious because a number of homzygotes were lost due to low transgene dosage.

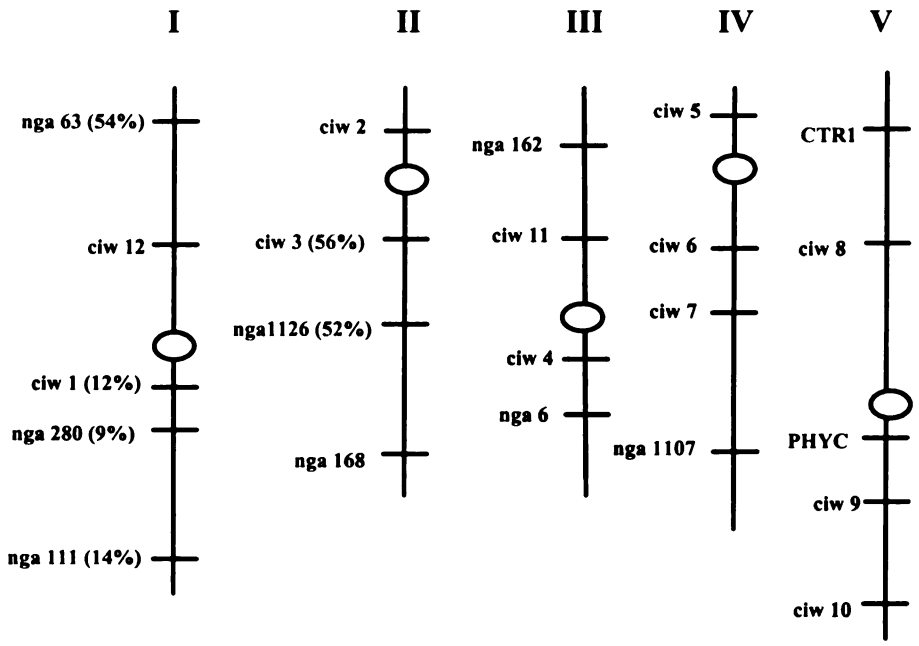

Figure 4.3. *DST1* maps to chromosome I.

Bulked segregant analysis was used to assign a rough map position to *DST1* on chromosome I. SSLP marker positions and the recombination frequencies in the mapping experiment are indicated.

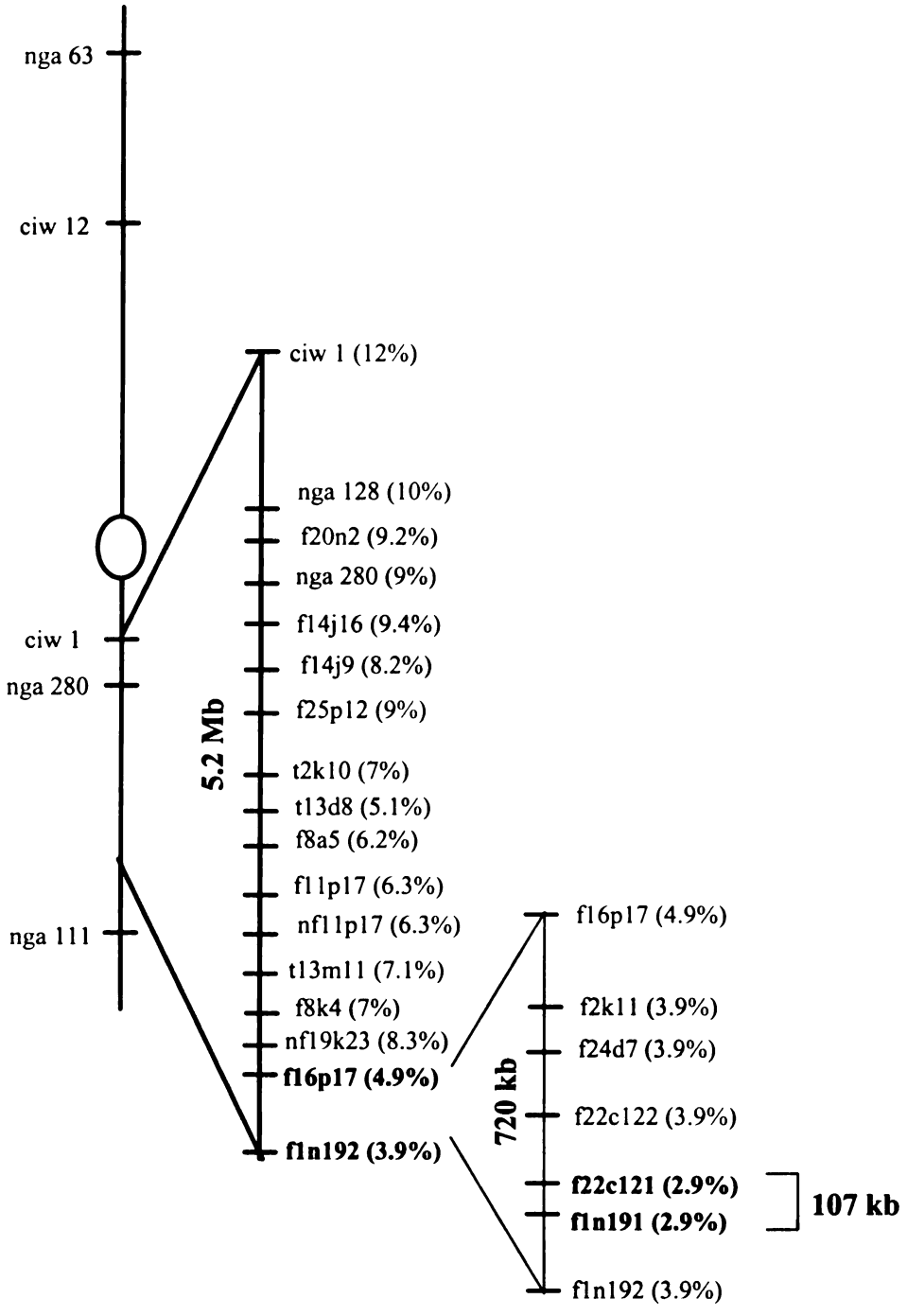

Figure 4.4. Map-based cloning of *DST1*.

SSLP and CAPS marker positions with the respective recombination frequencies are indicated.

Microarray technology was used as a novel tool towards the cloning of *dst1* since it identified a robust endogenous molecular marker. The experiments described in this chapter suggested that the *RAP2.4* transcript has several features which make it an ideal phenotypic marker for *dst1*. The 3' UTR of *RAP2.4* contains DST-like sequences and subsequent half-life analysis indicated that it is a direct target of DST-mediated mRNA decay. Also, *RAP2.4* mRNA levels exhibited greater increases in abundance than *HPH-DST* mRNA levels in *dst1* plants (5-fold compared to 3-4 fold). An added advantage was that *RAP2.4* could be used to screen mapping populations and follow the *dst1* phenotype even in plants lacking the transgene. A larger number of homozygous mutants could thus be scored in a more accurate manner because the presence of the transgene was not necessary. Finally, an appealing feature of *RAP2.4* was that even though it is a primary target of the DST-mediated decay pathway, it is not regulated in a circadian fashion. Harvesting hundreds of samples for RNA preparation from a mapping population is a lengthy and time-consuming process and using *RAP2.4* as a marker alleviated concerns about circadian variations occurring during that interval.

The 107 kb interval containing *DST1* spans 27 genes, including one t-RNA gene. Three genes in this region encode transcription factors, one of which belongs to the RAP2 (related to Apetala 2) family and *RAP2.4* mRNA levels are known to be elevated in *dst1*. Three genes encode proteins which could be functioning in protein degradation. There are several evolutionary links that seem to exist between RNA metabolism, protein degradation and ubiquitin signaling pathways suggesting that these processes interact with one another (Anantharaman et al., 2002). Numerous ubiquitin, ubiquitin E3 ligases and other proteins involved with the proteasome show fusions with various domains of

proteins functioning in RNA metabolism (Aravind and Ponting, 1998; Fang et al., 2000) implicating a role for RNA binding proteins in bringing the proteolytic machinery to the RNA-bound complexes.

Once a small interval containing *DST1* is defined, candidate genes in that region and/or the entire interval will be sequenced to identify the mutation. Construction of a cosmid library is in progress to aid in sequencing. Standard complementation tests to confirm the identity of *DST1* might not be straightforward due to the semi-dominant nature of the mutation. As such, the mutant gene might have to be introduced into wild type plants to phenocopy the mutation, which would also be greatly facilitated by the cosmid library being generated.

MATERIALS AND METHODS

Plant Material

dst1 mutant plants described in this report are from the accession Columbia (*gl1*) derived from EMS mutagenized Arabidopsis populations (Johnson et al., 2000). 1519 and *dst1* plants, from the second backcross to the parental line, were grown in growth chambers under 16 hr light and 60% relative humidity at 20°C. Rosette leaves were harvested from 35- to 40-day-old plants. All tissue was harvested from plants grown in parallel under the same conditions in different growth chambers.

Half-life measurements, total RNA Extraction and RNA Blot Hybridization

Half-lives were determined as described by Seeley et al. (1992). Rosette leaves from Arabidopsis plants were transferred to a flask with incubation buffer. After a 30 min

incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and frozen in liquid nitrogen. Total RNA from leaf samples was extracted as previously described (Newman et al., 1993). 10 μg of total RNA or was analyzed by electrophoresis on 2% formaldehyde/1.2% agarose gels and blotted onto nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). DNA probes were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) and purified from unincorporated nucleotides using probe purification columns (NucTrap, Stratagene, La Jolla, CA). The RNA blots were hybridized as described in Taylor and Green (1991) using the indicated ^{32}P-labeled probes. For a loading control, RNA blots were hybridized with a ^{32}P-labeled cDNA probe for the Arabidopsis translation initiation factor *eIF4A* (Taylor et al., 1993).

Genomic DNA extraction and markers for mapping

Genomic DNA was prepared from 1519 and *dst1* plants as previously described (Saghai-Maroofet al., 1984). Initial mapping of the *dst1* locus was carried out using SSLP markers (Bell and Ecker, 1994; http://genome.bio.upenn.edu/SSLP_info/SSLP.html). Primers were designed from available sequences and used to amplify genomic DNA by PCR. The primers used for genotyping the various markers are listed in Table 4.2. The CAPS PCR products were digested with the appropriate restriction enzymes according to the manufacturer's instructions. All PCR products were analyzed on high resolution agarose gels (4%) or 15% polyacrylamide gels.

Table 4.2. Oligonucleotides and restriction enzymes used to detect various SSLP and CAPS markers

Marker	Left oligonucleotide	Right oligonucleotide	Enzyme
464030	ggcccattcacaacagagat	ctcgcgaaagatcgagattc	AluI
464305	tcaggttccttctccgtcat	ccaagaacaaatggctgtcc	TaqI
464424	atcgacggaaaacaaaaagc	caaagcgcgtgaataacaac	Tsp509I
464476	tgggttatgtcgcgagagtt	gtcaattccttggctgcatt	AluI
464477	gcatccgttgaggattttgt	ccatgaacattgtgtatgtgagaa	AluI
464507	gcctaaacacagtgagggaga	tgcaaatgcaaacaacaaaga	Sau3AI
479633	actttaaagggcccaaatgc	cagctgtgttggtcatggag	HindIII
479657	tgccactattgactaggttttctg	cagcttctgcaaaacgaaga	Tsp509I
479671	acaacaggagcaggaaccac	gccctgatgcttacgacaat	HinfI
479760	ccactctcaacaattcccaag	acagatcgtgcttgatgtcc	Sau3AI
479762	atatccgggacatcaagcac	tggatccatctgtcttttaacg	Tsp509I
479788	gccaatgtctagccaccaaa	tctaccaccgtttagccactg	BalI
479790	ggctaatcagccacaaattca	gtcttcgaatcgggttgaaa	Tsp509I
479814	ttgccagtttggagatgaga	caacaaaaatcacataacgatttca	SpeI
479815	cctgcatgggaagaaaaaga	ggtcggtttgaccttcttca	HindIII
479833	tctgcttcggtttcgttctt	gctgttgaatcagagcacca	HinfI
479871	gctaagcacgcataggtgaa	ttcacgaggaaaattcaaacg	Hpy188I
23853199	tggtccaaggatacaaatcaca	aacgacattgtttcacctgct	Bstz17I
23950913	agtctgcgacgagtgaaggt	tcttccacctcttcacactca	Hpy188I
23952781	acgcccatctaattcccatt	ttttggcctcaacaaggttc	Tsp509I
23953329	gcttgaggaatccaaacaatg	ggttcgtcccagtcagagtc	BsrGI
23954354	ggtaaaaagaaaatgccattcg	cctcacggttatggatctgg	NheI
23954862	ccgtgagggatatagccaaa	tccaaggccattaagaccac	NspI
23955033	ggtagcgacagcgactgaat	gccattcagccgtaaactgt	NdeI
SNP17905	tttatttcggcccaagtctg	gacctcgcaacaattggact	Sau3AI
SNP17907	tgaagtggtgacggtaaaatga	catggaatcattttgtttagttcg	SphI
CER464688	atgaactgtggtacgcgaat	aaaatttctttcctttccgtttt	-
CER464689	cgccgttttcgttgataaat	ccgctctcctccattgatag	-
CER464692	tggttatggtttgacattattgc	attggcccatttgaagagtg	-
CER464693	gatggtcggctctcactctc	ttgagttggacggtggagat	-
F13O11_11m	aaaagaaaaggcttgggattg	tgggacacagaacttgttgc	-
F16G16_10m	aaaatcgacacatcactaagtcg	tgatttcgcaaaaacgaatg	-
F16M19_16b	cggtagatgatttccgatgtt	atgcgtttttcgtgaattgg	-
F16M19_8m	cgctttttcacgaatttaaacc	aactcgccattgacacaaca	-
F16P17_8b	acacgagagagcaatgatcc	ttcgcactgcaaattgctta	-
F1N19_10e	aacgaaacaggggactgaga	ttcttggctttcctcttcca	-
F1N19_9b	cgtctagtttcgcggtgttt	gcgtcatcaccatcatcatc	-
F22C12_11e	tgaaaacatgcaaagggaaa	ttcatattttcaatctcttgacttttt	-
F22C12_12e	ttgtatcttgtgtcaccgtcaa	tgatttgagtttaaaccatgttcg	-
F22C12_12ve	tcctcttcgtcttcttcgtca	aaagaccggccaaagaaaat	-
F22C12_14b	gatttcgccggtgatgttac	gtcggcccaattgattttt	-

F22C12_15b	ccagtccacaaggagtcca	tgcccacacaaacaatttca	-
F22C12_18b	cctgctcaaatgcgttacaa	tccacgagtcagaaggatga	-
F23N19_6m	agatggctgtgccttctagg	cagcattccccaactctttc	-
F23N19_7e	tgaagatctgaaatgaccctaaaa	tttctcaagcttttgaatgttttct	-
F24D7_17m	ttcagcatgcttattttattagcc	cgagcgccttactctgtgat	-
F24D7_18m	aaccagttttcaaatcaactgaag	tggtggtgttgaggtaccaa	-
F24D7_9e	tccctctcctcctttctcatt	gcaggcgatcagacattttt	-
F24O1_17m	tgtattgtcacaaaaatgcaaca	ctcggacccgttacaagaaa	-
F2K11_11m	cggttgtgactcccctaaaa	ttggctaagcatcttttccatt	-
F5I14_11b	tgcaacgaccaagaaattga	gaagaagtgcattgcttgaca	-
T12P18_6b	tgcgcagttatctccttttt	aaagagtataatgtcatgactcacgta	-
T12P18_8m	agataaatgcatcaacaaattgac	acccacctcacactctctcc	-
T13D8_15m	gaaaacggaatctgcaaaaca	aattggcttttaaaacaatgtca	-
T13D8_9b	agaaaaggcccatggaatct	agcagaaacagttggaaacga	-
T1F9_4e	gtaggaggagccatggattg	aacaaaagcaaatctatagttgttcac	-
T3P18_6b	ggtggaaaaggtggtggtaa	accactacgggaggaggtct	-

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CHAPTER 5

CHARACTERIZATION OF *dst3*, A NEW GENE IN THE DST- MEDIATED mRNA DEGRADATION PATHWAY

INTRODUCTION

Rates of mRNA degradation are highly variable in eukaryotic cells and these differences allow for precise control of gene expression (Gutierrez et al., 1999). *Cis*-regulatory elements have been identified that act as either instability or stability determinants in mammalian systems (Chen and Shyu, 1995; Holcik and Liebhaber, 1997). Several sequences that target transcripts for rapid turnover in plants have also been identified. These include the DST element (Newman et al., 1993), the AUUUA repeat (Ohme-Takagi et al., 1993), and premature stop-codons (van Hoof and Green, 1996). It is critical to understand the mechanisms by which the cell recognizes these sequence elements and targets the transcripts that contain them for rapid mRNA degradation.

Most studies aimed at understanding the cellular factors in mRNA decay pathways have involved characterization of proteins that bind the instability sequences *in vitro*. Isolation of sequence-specific RNA-binding proteins from plant cells has not been as successful probably due to the difficulty associated with preparing cytoplasmic protein extracts that are free of non-specific ribonucleases.

In order to gain insights into the molecular basis of sequence-specific recognition and degradation of unstable mRNAs, a genetic approach was devised to isolate *Arabidopsis* mutants defective in DST-mediated mRNA degradation (Johnson et al., 2000). Such a strategy has several distinct advantages. First, a gene that is identified by a mutant phenotype is, by definition, affecting the process *in vivo*. Second, basic information about the mechanisms of mRNA degradation may be obtained by studying mutants. Third, genetic analysis is not limited by pre-conceived mechanistic ideas. For

example, the cellular factor that recognizes an RNA degradation sequence may itself be an RNA molecule, not a protein. Finally, experimental complications that have limited the success of biochemical approaches, such as unstable or low abundance proteins or the presence of non-specific RNA degrading activities in protein extracts, are eliminated.

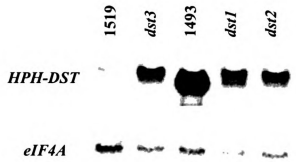
The mutagenesis strategy involved the generation of transgenic plants expressing *HPH* (hygromycin phosphotransferase) and *GUS* (β-glucuronidase) reporter genes (line 1519-31). The transcripts from both genes were destabilized by insertion of a tetramer of the DST instability determinant into their 3' UTRs (Johnson et al., 2000). Mutants in the mRNA decay pathway mediated by the DST element are expected to have increased *HPH* and *GUS* mRNA abundance and therefore, it should be possible to isolate them on the basis of these increased expression levels. *dst1*and *dst2* were isolated based on their ability to stabilize specific DST-containing transgene mRNAs, indicating that they harbor mutations that diminish the function of the DST-mediated decay pathway. Genetic analysis has demonstrated that *dst1* and *dst2* are semi-dominant mutations in independent single genes (Johnson et al., 2000). In this chapter, the isolation and characterization of a third gene, *dst3*, in the DST-mediated mRNA decay pathway is described.

RESULTS

dst3 exhibits increased *HPH-DST* and *GUS-DST* mRNA levels

A third putative mutant was isolated during the selection of 794,000 mutagenized M_2 seeds (Johnson et al., 2000). The abundance of the *HPH-DST* mRNA for *dst3* is shown in Figure 5.1. The level of this transcript is approximately 3.5 fold higher relative to p1519-31 and this increase in mRNA abundance is similar to that observed in *dst1* and

A.



B.

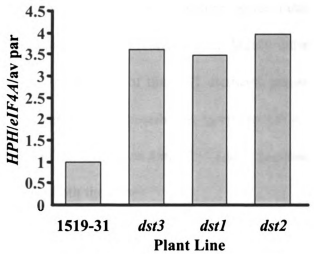


Figure 5.1 *HPH-DST* mRNA levels are elevated in *dst3*.

A) *HPH-DST* mRNA abundance was measured in the rosette leaves of *dst3*, p1519-31, the non-destabilized control 1493 and in previously isolated mutants *dst1* and *dst2*. Each lane contained 10 μg of total RNA. The abundance of *HPH-DST* mRNA was normalized to that of the loading control, *eIF4A*.
B) Quantitation of the increase in *HPH-DST* mRNA levels in the *dst* mutants.

dst2 (Figure 5.1B; Johnson et al., 2001). Increased *HPH-DST* mRNA abundance has been observed consistently in the progeny of two backcrosses of *dst3* to 1519-31. An analogous increase in *GUS-DST* mRNA levels was also observed, although the elevation of this transcript is higher in *dst3* than in *dst1* and *dst2* (Figure 5.2). Two transcripts have always been detected for *GUS* in the parental line and the *dst* mutants. The higher molecular weight transcript is probably due to the recognition of a cryptic downstream polyadenylation signal. Both transcripts are regulated in a similar manner in the *dst* mutants, although for quantitation purposes, the abundance of the lower molecular weight transcript (the expected size) was measured. Since both the *HPH-DST* and *GUS-DST* transcripts were elevated in the mutant, 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 reporter transcripts. To examine this possibility, genomic DNA was isolated from the mutant plants and the DST tetramer was amplified and sequenced. The DST tetramer was found to be unaltered for both the genes.

Genetic analysis of *dst3*

For the genetic analysis of *dst3*, the inheritance of *HPH-DST* mRNA abundance was examined. Progeny of the first backcross to WT (1519-31) (F1) and the progeny of self-fertilizations of these plants (F2) were used in these studies. Ten F1 plants were examined. *HPH-DST* mRNA abundance was on average 1.9 ± 0.53 fold higher in *dst3* F1 compared to WT (1519-31). These levels of *HPH-DST* mRNA abundance are intermediate between WT (1519-31) and mutant levels as would be expected if heterozygotes showed a semi-dominant phenotype. Segregation of increased *HPH-DST*

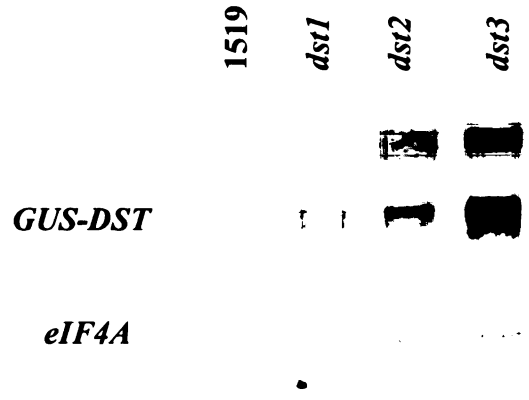


Figure 5.2 *GUS-DST* mRNA levels are elevated in *dst3*.

GUS-DST mRNA abundance was measured in the rosette leaves of *dst3*, p1519-31, and in previously isolated mutants *dst1* and *dst2*. Each lane contained 10 μg of total RNA. The abundance of *GUS-DST* mRNA was normalized to that of the loading control, *eIF4A*.

mRNA abundance in the F2 populations was also consistent with semi-dominance. Of 80 F2 plants segregating *dst3*, 20 were observed with high *HPH-DST* mRNA abundance (3.2 ± 0.59 fold higher than WT[1519-31]), 38 had intermediate *HPH-DST* mRNA abundance (1.7 ± 0.22) and 22 showed *HPH-DST* mRNA abundance that was similar to WT (1519-31) (0.97 ± 0.24) (Figure 5.3). This pattern of segregation in the *dst3 F2* populations is most easily explained by a ratio of 1:2:1 as would be expected for the inheritance of a semi-dominant single gene (Table 5.1).

dst3 is not allelic to *dst1* or *dst2*

To determine whether *dst3* was allelic with *dst1* or *dst2*, a complementation test was performed. The data from these experiments are summarized in Table 5.2. Like *dst1* and *dst2*, *dst3* is partially dominant, a characteristic of these mutations that complicates standard complementation tests. The F1 progeny of a cross between *dst3* and *dst1* or *dst2* showed intermediate *HPH-DST* mRNA abundance levels implying the lack of an additive interaction (Table 5.2). The results of *DST3-/-* crosses to *DST1-/-* or *DST2-/-* were similar to those of the cross to p1519-31 (*DST+/+*), indicating that *dst1*, *dst2*, and *dst3* are likely to affect three distinct genes. If *dst1* or *dst2* had been allelic with *dst3*, these crosses would have resulted in F1 plants with high levels of *HPH–DST* mRNA abundance. In addition some F2 plants with *HPH-DST* mRNA levels similar to 1519-31 were recovered (Table 5.2) which would not be possible if the *dst* mutations were in the same gene or tightly linked.

Increased stability of *HPH-DST* mRNA in *dst3* compared with 1519-31

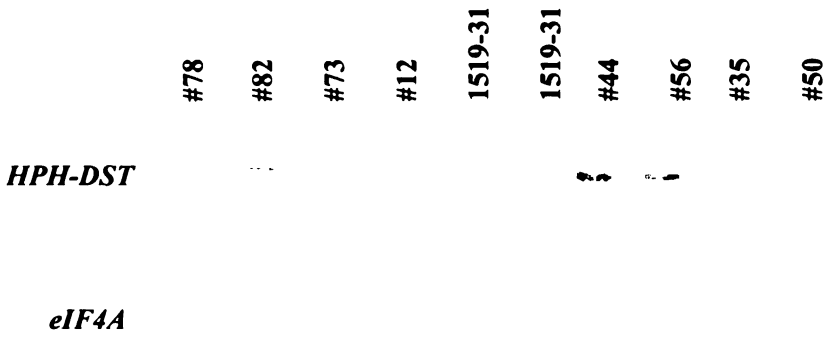


Figure 5.3. mRNA abundance of *HPH-DST* in F2 plants from the first back-cross of *dst3* relative to WT (1519-31).

8 representative plants are shown. Total RNA was prepared from rosette leaves and 10μg was analyzed by Northern blot hybridization. Radiolabeled probes, prepared against the indicated transcripts, were used in sequential hybridizations of the same blot. The numbers indicate individual plants. #56 is an example of an F2 plant with high *HPH-DST* mRNA levels, #82 with intermediate and #12 with *HPH-DST* mRNA levels similar to WT (1519-31).

Table 5.1. Segregation of increased *HPH* mRNA abundance in the progeny of crosses between *dst3* and 1519-31 (*DST3*).

Cross		Class	*HPH-DST**	n	χ^2, *P*[*f*]
DST3/DST3 x *dst3/dst3*	F_1	Int	1.9 ± 0.53	10	
DST3/dst3 x *DST3/dst3*	F_2	WT	0.97 ± 0.24	22	
		Int	1.70 ± 0.22	38	0. 3 P>0.8
		High	3.20 ± 0.59	20	

Int, intermediate.

WT, wild-type.

* *HPH-DST* mRNA abundance: (*HPH-DST/eif4A*) segregating class/ (*HPH-DST/eif4A*) WT ± standard error.

f χ^2 calculated for 1:2:1 segregation of WT/Int/High HPH-DST mRNA abundance DST/DST, WT (1519-31).

Table 5.2. Segregation of increased *HPH* mRNA abundance in the progeny of crosses between *dst3* and *dst1* and *dst2*.

Cross		Class	*HPH-DST**	n
dst3/dst3 x *dst1/dst1*				
	F_1	Int	1.6 ± 0.1	15
	F_2	WT	0.9 ± 0.2	16
		Int	1.6 ± 0.3	30
		High	3.7 ± 0.9	21
dst3/dst3 x *dst2/dst2*				
	F_1	Int	2.5 ± 0.2	16
	F_2	WT	0.9 ± 0.2	9
		Int	1.8 ± 0.4	31
		High	3.1 ± 0.6	21

Int, intermediate.

WT, wild type.

* *HPH-DST* mRNA abundance: (*HPH-DST/eif4A*) segregating class/ (*HPH-DST/eif4A*) WT ± standard error.

The elevation in *HPH-DST* transcript levels in *dst3* is probably due to the DST-mediated decay pathway being deficient in the mutant. To test this hypothesis, mRNA decay rates for *HPH-DST* mRNA were measured in *dst3* and WT plants. A change in mRNA stability would indicate that the mRNA is a primary target of the decay pathway. Half-life analysis was carried out in rosette leaves using cordycepin as the transcriptional inhibitor. The *HPH* mRNA abundance was standardized relative to the abundance of *eif4A* transcript. As is evident in Figure 5.4A and B, the decay of *HPH-DST* mRNA was slower in *dst3* compared to the parental and the differences in steady state mRNA levels became even more prominent at later time points. The half-life of *HPH* mRNA was calculated to be 1.5-fold greater in *dst3* than in 1519-31 suggesting that the *dst3* mutation results in an increase in mRNA stability that is responsible for the increased abundance of the *HPH-DST* transcript. It has been noted before that differences in half-life measurements between the parental and the *dst* plants are reflective of about 17% of the differences based on steady state mRNA levels (Johnson et al., 2000), and a coordinate dampening in the mRNA half-life value for *dst3* relative to the parental line was observed.

DISCUSSION

dst3 was identified using a targeted genetics strategy and exhibited elevated levels of DST-containing mRNAs. mRNA decay kinetics showed that the increased *HPH-DST* mRNA abundance is caused by a corresponding increase in message stability. The three mutants isolated by this strategy were extremely rare suggesting that the genes involved in the DST-mediated decay pathway are essential. Further, all three *dst* mutants are weak

A.

B.

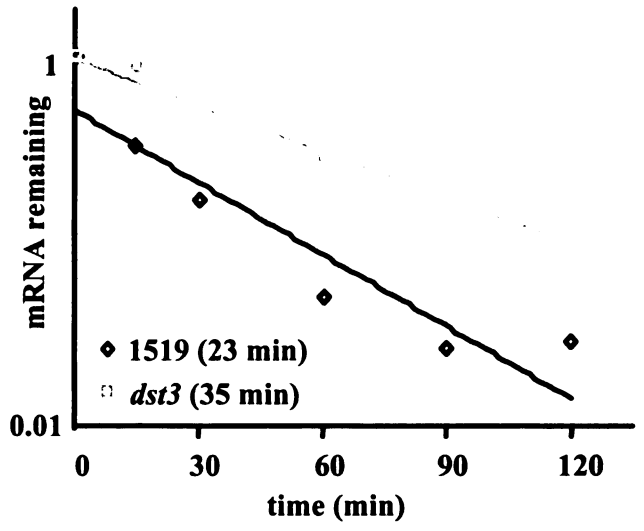


Figure 5.4 Analysis of *HPH-DST* mRNA stability in leaves from 1519 and *dst3* plants.

A) Representative northern blot analysis of cordycepin time courses over a period of 120 minutes. Samples consisted of 10 µg of total RNA isolated from the indicated time points.
B) Quantitation of the decrease in mRNA abundance and half-life estimation for *HPH-DST*. The stable *eIF4A* transcript was used as a reference for equal loading.

alleles allowing partial functioning of the DST-mediated decay pathway since they did not restore *HPH-DST* mRNA levels to those found in plants with non-destabilized mRNAs (Figure 5.1). These findings may indicate that stronger alleles were lethal or that there is redundancy in the DST pathway. If so, the genes may be members of the same gene family.

dst1 and *dst2* are partially dominant mutations (Johnson et al., 2000), and interestingly, so is *dst3*, the new mutation described in this study. A semi-dominant mutant phenotype could result if the defective DST-recognition factors no longer recognized the DST sequence, but still bound the interacting proteins required for mRNA decay. On the other hand, a semi-dominant phenotype could arise if a defective DST-binding protein retained the ability to bind a DST subdomain but had reduced ability to interact with or recruit proteins required for mRNA degradation. Another alternative would be that a *dst* gene product encodes a DST-specific ribonuclease that binds the DST sequence but has decreased RNase activity, thereby providing partial protection to mRNAs bound by a mutant version.

Studies conducted with the previously identified *dst* mutants, *dst1* and *dst2*, have indicated that both genes have distinct and overlapping roles in the DST-mediated mRNA decay pathway (see Chapter 2). It will be of interest to examine how *dst3* cross talks with *dst1* and *dst2*. In addition, the continuing analysis of all possible mutant combinations should conclusively define the roles of each gene as they work alone or in combination.

Prior to this work, only two mutants involved in DST-mediated mRNA degradation were known. Considering the rarity of the mutants isolated, the identification of an additional mutant, *dst3*, is important. In theory it should be possible to generate

more mutants in this pathway since the screen was not saturating, in spite of being very tedious. Finally, the cloning of *dst3* should lead to a better understanding of the DST-mediated decay pathway in plants.

MATERIALS AND METHODS

Plant Material

dst3 mutant plants described in this report are from the accession Columbia (*gl1*) derived from EMS mutagenized Arabidopsis populations (Johnson et al., 2000). 1519, *dst1*, *dst2* and *dst3* plants were grown in growth chambers under 16 hr light and 60% relative humidity at 20°C. Rosette leaves were harvested from 35- to 40-day-old plants. All tissue was harvested from plants grown in parallel under the same conditions in different growth chambers.

Half-life measurements, total RNA Extraction and RNA Blot Hybridization

Half-lives were determined as described by Seeley et al. (1992). Rosette leaves from Arabidopsis plants were transferred to a flask with incubation buffer. After a 30 min incubation, 3'-deoxyadenosine (cordycepin) was added to a final concentration of 0.6 mM (time 0). Tissue samples were harvested at regular intervals thereafter and frozen in liquid nitrogen. Total RNA from leaf samples was extracted as previously described (Newman et al., 1993). 10 μg of total RNA or was analyzed by electrophoresis on 2% formaldehyde/1.2% agarose gels and blotted onto nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). DNA probes were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) and purified from

unincorporated nucleotides using probe purification columns (NucTrap, Stratagene, La Jolla, CA). The RNA blots were hybridized as described in Taylor and Green (1991) using the indicated ^{32}P-labeled probes. For a loading control, RNA blots were hybridized with a ^{32}P-labeled cDNA probe for the Arabidopsis translation initiation factor *eIF4A* (Taylor et al., 1993).

Analysis of the *HPH-DST*x4 sequence element in *dst3*

Genomic DNA was prepared from *dst3* F_2 plants from the first backcross to p1519-31 as described in Saghai-Maroof et al.(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 proof-reading polymerase (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.

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CHAPTER 6

MICROARRAY ANALYSIS OF *dst2*

INTRODUCTION

The expression level of thousands of genes can be monitored simultaneously using DNA microarrays. Therefore, studies using DNA microarray technology can be used to address mechanistic questions on a global basis and identify targets of various metabolic pathways. DNA microarray technology has been exploited to characterize the molecular phenotypes of mutants (Pérez-Amador et al., 2001; Goda et al, 2002; Scheible et al, 2003) and to investigate the intra- and interspecies variations in genome expression patterns (Enard et al., 2002). Genomic approaches have also been particularly useful to discriminate the functions of individual members of a gene family which may have partial redundant functions and would be difficult to dissect using classical genetic techniques (Wang et al., 2002).

As pointed out in Chapter 2, microarray analysis of the *dst1* mutant was critical in providing the first clue about the physiological significance of the DST-mediated decay pathway in Arabidopsis. In addition, some of the genes identified as being differentially regulated in *dst1* were subsequently examined by RNA gel blot analysis and found to be either coordinately or oppositely regulated in *dst2*. In this chapter, preliminary microarray studies were carried out with *dst2* to expand our understanding of this mutant and discover new molecular markers specific for *dst2*.

RESULTS

15k element microarray reveals genes with altered gene expression in *dst2*

15 k slides, prepared by the AFGC microarray facility at MSU, were utilized to examine global gene expression changes in the *dst2* mutant. Poly(A)$^+$-selected RNA from

the rosette leaves of five-week old *dst2* and parental plants was labeled with the fluorescent dyes and used for hybridization. Two biological replicate experiments were performed, each with a reverse labeling technical replicate. After normalization of the data, 33 ESTs and multiple clones for *HPH* and *GUS* showed a difference of 1.5-fold or greater in all four slides. Of the 33 ESTs, 29 clones corresponded to 22 annotated Arabidopsis genes while 4 ESTs did not correspond to any annotated open reading frames. Multiple ESTs corresponding to the same gene that behaved in a similar fashion further corroborated the microarray data.

Twenty four genes, including the transgenes *HPH* and *GUS*, showed elevated mRNA levels while four displayed decreased levels of mRNA in the *dst2* mutant compared with the parental plants, as listed in Tables 6.1 and 6.2, respectively. After correction for redundancy in the ESTs and excluding the transgenes *HPH* and *GUS*, a total of 26 endogenous genes whose expression levels were altered in *dst2* were identified. The probable biological functions of these genes, listed in Tables 6.1 and 6.2, were based on the latest annotation by the TIGR *Arabidopsis thaliana* database.

Identification of the putative primary targets of the *dst2* mutation

As a first step towards determining the primary targets of *dst2*, the 3'UTRs of the genes that were differentially regulated were analyzed for the presence of DST-like subdomains. 11 genes were identified that contained possible DST-like sequences in their 3' UTR (listed in Table 6.3). Direct targets of the decay pathway mediated by *DST2* are expected to be stabilized in the *dst2* mutant. It will therefore be of interest to measure mRNA decay rates for these transcripts and determine if the changes in expression levels

Table 6.1 Genes with increased mRNA levels in *dst2* versus parental plants

Gene	MIPS Number	Number of clones	EST MSU ID	Average ± SD
GUS	NA	5	NA	4.5 ± 1.2
HPH	NA	6	NA	3.0 ± 1.5
Similar to DNA topoisomerase IV subunit A	At3g15950	4	117I22T7 146L7T7 183N7T7 113J18T7	2.4 ± 0.5
Chalcone synthase	At5g13930	1	187C23T7	2.3 ± 0.2
Identical to Cytochrome P450	At4g31500	1	148G2T7	2.2 ± 0.8
Formate dehydrogenase	At5g14780	2	172I13T7 192D16T7	2.1 ± 0.3
Zn finger (C3HC4-type RING finger) protein family	At3g05200	1	123F8T7	2.1 ± 0.2
Anthranilate synthase, beta subunit	At1g25220	1	241P6T7	2.1 ± 0.3
Expressed protein	At3g49590	1	G10A5T7	2.0 ± 0.2
Polyubiquitin (UBQ3)	At5g03240	1	146J8T7	2.0 ± 0.6
Expressed protein	At1g15350	1	F10G9T7	2.0 ± 0.3
Zn finger (C3HC4-type RING finger) protein family	At3g16720	1	ATHATL2A	2.0 ± 0.1
Expressed protein	At5g21940	2	152A16T7 40B7T7	1.9 ± 0.3
No annotated ORF	NA	1	M44D8STM	1.9 ± 0.1
No annotated ORF	NA	1	M13C10STM	1.9 ± 0.2
SKP1 interacting partner 5 (SKIP5)	At3g54480	1	214F2T7	1.9 ± 0.5
C2H2-type zinc finger protein family	At2g24500	1	62A9XP	1.9 ± 0.1
NRAMP metal ion transporter 6, (NRAMP6)	At1g15960	1	F4H9T7	1.8 ± 0.2
Elongation factor 1-alpha (EF-1-alpha)	At1g07930	1	162G19T7	1.8 ± 0.2
Scarecrow-like transcription factor 13 (SCL13)	At4g17230	1	162M13T7	1.8 ± 0.1
O-methyltransferase family 2	At4g35160	1	F10C12T7	1.8 ± 0.2
Cadmium induced protein (AS8)	At4g19070	1	G8D8T7	1.8 ± 0.3
Expressed protein	At1g77220	1	G4B1T7	1.7 ± 0.0
RNA and export factor binding protein, putative	At5g02530	2	154H21T7 G9F7T7	1.6 ± 0.1

Table 6.2 Genes with decreased mRNA levels in *dst2* versus parental plants

Gene	MIPS Number	Number of clones	EST MSU ID	Average ± SD
Similar to ubiquitin-specific protease 12	At1g58270	2	220F21T7 193G23T7	1.9 ± 0.2
Polysaccharide lyase family 1 (pectate lyase)	At3g07010	1	226D20T7	1.9 ± 0.2
No annotated ORF	NA	1	M15C10STM	1.6 ± 0.0
No annotated ORF	NA	1	M32E9STM	1.6 ± 0.1

Table 6.3. Genes with possible DST-like sequences in their 3' UTRs	
Gene	**3' UTR**
NRAMP metal ion transporter 6, (NRAMP6)	attgaagaattagaacatttcagatagaagaaatagtgtttacttattgtaatctattgcatagaataa aaatgaagttgtttacttaagtgtaatctattacatagatcaaacatgaagacttgactacaaaatcc atcaaacggtttggccaaagaactgagtgtacagattcataaccagacgagacatattgaaaca gaggtttcacataaccagagacaacaaacaaaagcaacttcaatggttaaggatacttaagggg gatacttgagggaattaataagcttttcaagaccc
Expressed protein	gaaaaacaaaaggtggtgcaagtgcaaacgaagctgaaacaattctgaagatgccttttttgtg gaagtctcatcttcgtcacatctaacaaagaagttgtagatttatgggatgatgcttactctgctgg atttaaacacaggtaacaacagatgatgctactaactagttttcctctacctttacacaaacttattg agaagttagatttgtatctcgtaatgacagtttcttacacaaaaataaaataaaatacaaatttgtcat gactaatatcttatgaaacatcttctcatatattgtgtttgttct
Zn finger (C3HC4-type RING finger) protein family	tagttgacttttcttttctcatctttttcatttgtttttgtttgggattgttgataaatacgcaaatacattttc aatttttttaattgaattatccttgttttgtttaaaattctctgtaacgtacatatgaagtcagatcgaaaa cggattcacttttgaaaactaatgtataaaaaagcttataacaaaacaacggacaatgatgtgag gtgcgctgtggtgcaggtgggtttctcggtttttcaatattaaaaaagtcgtattcgtggacaaaga atatagctatccgatcccctgataaaatatatgctaatagaatata
Similar to DNA topoisomerase IV subunit A	tcacttaattgaactaatgagattcttgattgtggttaaagcacatgttcaattagttgtggttcttgtg ttttattttcttgtgttgttttgtttgagacttttgtttgttgcttatatcaggggagagaggttgaaaaac ctttgagagagatagataaatgagagatttgtgaaacacaaataatgttttagtcgtcttataataaa acctttttttttcttatatatgctcattgataatactgcaaaaaggtaaaatcatgttttttttttttttaatat gaatcatgatattcagg
Zn finger (C3HC4-type RING finger) protein family	tgacttgtcacgtgttggtgtctgattggtttaatgttaaccgggagtaaaaaaaggaattactaca agtcaacaggcttttgtctaggtgttgatttcggcgcccaaggacacgtggcgtaaactgagctt ccaggaatcaatattcaccgtctattatgattagataggttagatagatttgtgtaacgatgtacaaa gtcatctacaatattgaatctatttccatttatttaccatattcttttttttttttataattttcgaagttctac aaactcttttatgtaaaacacaatccaatggtcataattgtgataaagactttgcataatt
SKP1 interacting partner 5 (SKIP5)	tgatttttgctggttttatttatttttcttcttctgtctgtaacattagtaatctgtgtgatgatgggaaatg atccatcttcatttgtactttgtgtttccccttaagaccagacaggtcctttgttgtatgcttattatgttc agaaataatgtccttttaggacgaattgccgaattcatttcaattggatttttataatttctgcttagtg tatcttttgtgataaatggtttctttcttcactgttatcaaaatatt
Scarecrow-like transcription factor 13 (SCL13)	tgatgatggctgggttcacgggttggccggtcagcacatctgcagcgtttgcagcgagtgagat gctgaaagcttatgacaaaaactacaaactgggaggccatgaaggagcgctctacctcttctgg aagagacgacccatggctacatgttccgtgtggaagccaaacccaaactagattgggtaagtta tagtgatgatggttacttgagtggataaagaagagcacaacaaaaacacatctgtcgctgtaaat tttttaggatgtgcaatgatgttttaagttgtaacacaacctaagttatatatgtatacaaaccaaacc tggtggttgtttttctcttgtaaattgtcatgtggttgtgggtgggttgctagtaatgaaatataacca aaacattgattaggtca
Cadmium induced protein (AS8)	taatgctacccaccacgaggatcttaagcatctgagacgcatttcgagatagttactttgatggaa tttgtgaatgtgactgaaatattcagcacaaatcagaacatggttcctgtcttgtttggagaagcac tatgaaccaaacctgaagcccttgtgatttagagttacagagatacattaaaagggttcgggattt cacagtagcattcatagacagttcaggttagttaaacgtgcttcacaaaaagaaaaaaaacaga agtgtagttgaggtcacaccacaggtttatgctttgctcttaatcaaatatttatctttctctttttgtta tttgtccttttaattccatttgctttattccttacttgtggggattaaatgtttgcagagaaagataattc gactgaataaatacacttttacctgt

Identical to Cytochrome P450	tgatgctatatatatcattaggacgtttctgctggtagatatggcgtgaccaatggttatttttcattg caatatccctttttgttttaatgagtactatgttctcattttaacgaataaaaatgtatcagtgctcttgtt tttggactagaaaagaaagtagtccgatgtttaatattcgggtcccttaatattccctctggtttaca atattttaagctatcttagtaaacatctat
O-methyl-transferase family 2	taagaggaggacatgaaagatatatctttcctttgaggaaaactcaataaattagtgattgttgattt ggtgtttttatacagaacgacgtgtgggtgaataattgtgttgtgtgataataagtaaggcttgtttc agtatcagcagagttgtttttttatttgtatttttcacatttttttatcattaatttccttgtgagtggcacct ctaatttacttttttaattgttataggctatataagatagataaccttatacaatgtcaaatcttacaatt acattatgaacatgaa
RNA and export factor binding protein, putative	tagaaaggggagattaaacctattgcatgtctacatgtattccttcctttagtgccgatatgcttttgt attagtatgatctttagattgaatgtcaatgggtctgacattttcgagctttagcttttgttttttcttctct tggtgagcttcttagctttttgcagttgagttcagagaaacaagcattgtgtgtaccgtggaactca aaactcttctaaaatatcaattcaaaacacaattctatctactctcta

ATAGAT-like subdomain

GTA-like subdomain

detected on the microarray slides are indeed due to a corresponding change in mRNA stability.

RNA gel blot analysis of previously identified transcripts

Prior microarray studies with *dst1* followed by Northern blot confirmation demonstrated that some transcripts were affected in both the *dst1* and *dst2* mutants (see Chapter 2). Intriguingly, only one of these genes showed a greater than 1.5-fold difference on all four slides (chalcone synthase) although some of them could be detected on two out of the four slides. Also some of the ESTs for the relevant genes on the 15k arrays were different from the previously used 11k arrays (see Discussion).

In order to validate the quality of the RNA used for labeling, a few of the formerly characterized transcripts were checked by RNA gel blot analysis. As illustrated In Figure 6.1, *HPH* mRNA levels were elevated in both *dst1* and *dst2*, *RAP2.4* mRNA levels were increased in *dst1* but unchanged in *dst2* and mRNA levels for a putative patatin gene were diminished in *dst1* but elevated in *dst2*. All the transcripts tested increased or decreased in abundance as expected (see Chapter 2). Experiments to confirm additional transcripts are in progress.

DISCUSSION

Microarray analysis identified 26 genes as being differentially regulated in the *dst2* mutant. The genes identified code for proteins involved in variety of metabolic processes. Four ESTs corresponding to a protein similar to DNA topoisomerase IV subunit A showed the highest mRNA elevation in *dst2*. Topoisomerase IV is a type II

Figure 6.1. RNA gel blot analysis of previously identified transcripts.

Lanes contained 10 μg of total RNA extracted from WT (1519), *dst1*, and *dst2* plants. Each blot was hybridized sequentially with ^{32}P-labeled *eIF4A*, and with **A)** *HPH*, **B)** *RAP2.4*, and **C)** 111G9T7, EST corresponding to putative patatin protein.

isomerase involved in the topological changes of DNA during replication (Zechiedrich and Cozzarelli, 1995). Four transcription factors were identified, three of which seem to contain DST-like sequences in their 3'UTR. If these are the primary targets of *DST2*, it could be hypothesized that changes in the mRNA levels for these transcripts would lead to downstream secondary effects. Interestingly, one of the transcription factors was a scarecrow-like protein (SCL13) and it was recently shown that a certain species of micro-RNAs (miR171) is responsible for the cleavage of several members of scarecrow-like mRNAs in Arabidopsis (Llave et al., 2002). Micro-RNAs interact with perfect complementarity guiding cleavage of the target mRNA (Hammond et al., 2001) and *SCL13* seems to be a direct target of *dst2*, suggesting the possibility of miRNAs being involved in DST-mediated decay.

The small number of genes reported in this chapter is most likely a conservative estimate of the actual targets of *dst2*. Although the high stringency used in the data analysis possibly reduced the number of false positives, some bonafide targets of the *dst2* mutant could have been missed. This is especially relevant because two different printings of the 15k microarray slides were used for this study. Even though most of the cDNAs present on the two separately printed slides corresponded to the same genes, different ESTs were used. ESTs corresponding to the same gene can exhibit variable expression ratios, depending on the probe or target length, genetic redundancy, or a combination thereof.

It is perhaps pertinent to note that on comparing the technical replicates for each set of printings, many more differentially regulated mRNAs could be detected, some with much greater fold changes and multiple ESTs showing similar changes in expression

(data not shown). RNA gel blot analysis to confirm some of these targets, in addition to the ones shown in Tables 6.1 and 6.2 should be useful in ascertaining molecular markers specific for *dst2*. Once a robust marker is found for *dst2*, that is unaffected in *dst1* and *dst3*, it could be used to facilitate the mapping of *dst2*. *dst2*- specific markers should also aid in testing double mutants as well as provide unique clues about the possible genetic interactions (epistasis relationships) among the multiple loci involved in the DST-mediated degradation pathway. Finally, additional microarray experiments should be informative about the impact of *dst2* at the whole plant level relative to the other *dst* mutants.

MATERIALS AND METHODS

Plant Material

All *Arabidopsis thaliana* plants described in this report are from the accession Columbia, grown in growth chambers under 16 hr light and 60% relative humidity at 20°C. Tissue from the parental line (p1519-31) and *dst1* and *dst2* homozygous mutants (Johnson et al., 2000) were harvested from 35- to 40-day-old plants. The *dst1* and *dst2* lines used were from the second backcross to the parental line. All tissue was harvested from plants grown in parallel under the same conditions in different growth chambers.

15k AFGC DNA Microarray

15,532 and 15,488 element microarrays were generated at the Arabidopsis Functional Genomics Consortium (AFGC) Microarray facility at Michigan State University. The ESTs were spotted on super-amine glass slides (Telechem International,

Inc.; Sunnyvale, CA). Slides were washed and blocked according to the Telechem protocol.

Total RNA Extraction, Poly(A)$^+$ RNA Purification, and RNA Blot Hybridization

Total RNA from leaf samples was extracted as previously described (Newman et al., 1993). Poly(A)$^+$ RNA was purified from 100 μg of total RNA using the Oligotex mRNA kit (Qiagen, Valencia, CA). RNA (10 μg of total RNA or 2 μg of poly(A)$^+$ RNA) was analyzed by electrophoresis on 2% formaldehyde/1.2% agarose gels and blotted onto nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). DNA probes were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) and purified from unincorporated nucleotides using probe purification columns (NucTrap, Stratagene, La Jolla, CA). The RNA blots were hybridized as described in Taylor and Green (1991) using the indicated ^{32}P-labeled probes. For a loading control, RNA blots were hybridized with a ^{32}P-labeled cDNA probe for the Arabidopsis translation initiation factor *eIF4A* (Taylor et al., 1993). Blots were stripped between hybridizations in 0.1% SDS at 90 to 95°C for 1 hour. Quantification of hybridization signals was achieved using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Labeling of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was labeled using the aminoallyl labeling procedure. 0.5 μg of poly(A)$^+$ RNA and 6 μg random hexamers in a total volume of 18.5 μl of DEPC-treated water was denatured at 70°C for 10 min, set at room temperature for 3 minutes and cooled down on ice. On ice, 6 μL of 5 × RT buffer, 3 μL of 0.1 M DTT, 0.6 μL 50X aminoallyl-dNTP mix, and 2 μL of Superscript II (200U/μL) were added, and the mixture

was incubated at 42°C for 3 hrs. The RNA was hydrolyzed by adding 10 μL 1N NaOH and 10 μL 0.5M EDTA and incubating at 65°C for 15 minutes. 10 μL 1N HCL was then added to neutralize the pH. Unincorporated aa-dUTP and free amines were removed with the Qiagen PCR purification kit using phosphate wash and elution buffers. Aminoallyl-labeled cDNA was resuspended in 9 μL 0.1M Na_2CO_3, mixed with the appropriate NHS-ester Cy dye and incubated in the dark for 1 hr. The labeled cDNA was purified using the Qiagen PCR purification kit. To test the quality and quantity of the product, 2 μL of the labeling reaction with 2 μL glycerol was separated on a 1% agarose gel using a miniprotean gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The portion of the gel containing the DNA sample was placed on a glass microscope slide, dried on a heat block at 70°C, and scanned using Affymetrix 428™ scanner. The rest of the probe was used to prepare the hybridization mixture.

DNA Microarray Hybridization and Analysis

For a single DNA microarray, 50 μL of hybridization solution was prepared by mixing 50 μL slide hybridization buffer (Ambion) and 4 μL Cy5- and Cy3-labeled probe. The mixture was denatured at 95°C for 10 minutes. The mixture was then hybridized to the array under a glass coverslip that had been washed in 95% ethanol, then 0.2% SDS, and rinsed in distilled water. The slide was then placed in a microarray hybridization chamber (ArrayIt Hybridization Cassette, TeleChem International, Inc.; Sunnyvale, CA) with 200 μL of 3 × SSC to ensure high humidity conditions. Hybridization was carried out in a water bath at 65°C for 12 to 20 hours. After hybridization, the microarray was washed for 5 min in 1 × SSC/0.2% SDS, 5 min in 0.1 × SSC, and 15 sec in 0.05 × SSC without SDS, and finally dried by centrifugation at 600 rpm for 5 min. The slide was

scanned once in an Affymetrix 428™ scanner for both channels 1 and 2 (corresponding to Cy3- and Cy5-labeled probes, respectively).

The image files obtained were analyzed using GenePix Pro 3.0 software. Data from each channel was transformed to the natural logarithm, and a Z-score was calculated to normalize the channel values in order to account for variation in RNA labeling. Values were retransformed from the natural logarithm by raising to the power e, and the channel ratio was calculated.

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CHAPTER 7

FINAL REMARKS AND FUTURE PROSPECTS

An important challenge for plant biologists is to understand all of the mechanisms used by plant cells to regulate gene expression levels. Transcriptional regulation has justifiably enjoyed a great deal of experimental attention over the years. However, there are several important modes of regulation that occur once messenger RNA (mRNA) molecules are generated. The mechanisms responsible for the post-transcriptional regulation of gene expression are only beginning to be understood in plants. Several messenger RNA sequences that control abundance, localization, and translation initiation have been identified, yet the factors that recognize these sequences are largely unknown.

The genetic studies coupled with the functional genomic approaches described in this thesis should lead to an enhanced understanding of sequence-specific decay in Arabidopsis and other higher plants. Cloning of the *DST1* gene should be accomplished in the near future and the characterization of its product should provide insight into the decay machinery that recognizes and degrades DST-containing transcripts. The simplest hypothesis is that *DST1* encodes an RNA-binding protein or a ribonuclease. It is also likely that DST1 is a regulatory factor that controls one of these components or has some unanticipated function. An intriguing possibility is that the DST element is recognized by specific complementary noncoding RNAs.

If DST1 is a sequence-specific RNA-binding protein, it could be hypothesized to influence deadenylation by modulating the rate at which a single ribonuclease functions or by recruiting kinetically distinct ribonucleases. In addition, if DST1 is indeed a DST-specific RNA binding protein, it would be of interest to examine its interaction with specific subdomains of the DST element. Once the identity of *DST1* is known, knock-out

and overexpression lines can be generated to further elucidate the function of the DST1 protein.

Microarray studies and subsequent half-life analyses have indicated that *DST1* is most likely involved in the degradation of mRNAs in discrete pathways. Identification of the protein complexes that DST1 interacts with will be the next step in determining the biological role of the DST-mediated degradation pathway in Arabidopsis. Protein-protein interactions of DST1 could be examined *in vitro* with other Arabidopsis proteins predicted to be involved in mRNA decay, such as AtXRN4 (5'-3 exoribonuclease), AtPARN (poly(A) ribonuclease) and components of the exosome. Alternatively, the yeast two-hybrid system could be used to detect novel proteins that associate with DST1.

Recent experiments have suggested that DST-mediated mRNA decay might be developmentally regulated. Early in the analysis of *dst1* and *dst2*, it was noted that the mRNA turnover defect of these mutants, at least for *HPH-DST* mRNA, was more pronounced in older plants compared with seedlings (Johnson and Green, unpublished data). This result indicates that it may be important to assay the impact of mutations that affect posttranscriptional regulatory pathways throughout plant development even when it has been assumed that the element is not developmentally regulated. Future studies will be required to determine whether components of the DST-mediated mRNA decay pathway are under temporal regulation or are modulated in response to other stimuli such as circadian rhythms.

Distinct DST-containing mRNAs could also be differentially regulated within the same cell and this regulation might be due to the different mRNA decay pathways mediated by the various *DST* genes. Likewise, it could be postulated that some of these

DST-containing mRNAs require a sequence in addition to the DST element and proper cooperation between multiple RNA elements is necessary for mRNA stabilization or destabilization. Furthermore, specific types or numbers of subdomains present in certain DST-containing mRNAs might be contributing to the modulation of DST-mediated decay.

The progress outlined in this thesis opens up fresh avenues for evaluating the association of DST-mediated decay with the circadian clock. The circadian experiments carried out with the *dst1* mutant should be applicable to *dst2* and *dst3* as well. Analysis of the effect of the different *dst* mutants on circadian gene expression should further aid in unraveling the mechanisms that underlie posttranscriptional control of CCG expression at the level of mRNA stability.

The *dst* mutants have and will continue to serve as excellent tools to address how specific mRNAs are targeted for increased turnover. Although the functioning of the machinery is only partially known, it is clear that the DST-mediated decay pathway can regulate particular mRNAs in a rapid, coordinated fashion. Apart from basic research, detailed knowledge of this pathway should have a potential impact on applied research as well. The inhibition of rapid mRNA turnover through interaction with the DST complex might be a means of increasing the amount of a specific protein in plant cells, a highly desirable trait for commercial applications.