CHARACTERIZATION OF THE *FERREDOXIN-1*

mRNA INSTABILITY ELEMENT(S)

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LIST OF ABBREVIATIONS

Δ	Deletion
μg	Microgram
μL	Microliter
μM	Micromolar
3-AT	3-Amino-1,2,4-triazole
AD	Activation domain
ATA	Aurintricarboxylic acid
$CaCl_2$	Calcium chloride
CDC	Cell division cycle
DBD	DNA binding domain
DTT	Dithiothreitol
dsRBDS	Double-stranded RNA-binding domains
EDTA	Ethylenediaminetetraacetic acid
Fed-1	*Ferrodoxin-1*
g	Gravitational field
GST	Glutathione S transferase
HCl	Hydrochloric acid
His	Histidine

h	Hour
KCl	Potassium chloride
kb	Kilobase
kDa	Kilodalton
Leu	Leucine
LiCl	Lithium chloride
Mg^{++}	Megnessium ion
$MgCl_2$	Megnessium chloride
min	Minutes
mM	Millimolar
$MnSO_4$	Manganese sulphate
NaCl	Sodium chloride
nt	Nucleotide
OD	Optical density
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonylfluoride
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RPM	Revolutions per minute
RRM	RNA-recognition motif
SDC	Synthetic defined complete
SDS-PAGE	Sodum dodecyl sulfate-Polyacrylamide gel electrophoresis
Trp	Tryptophan

UTR	Untranslated region
Ura	Uracil
UV	Ultraviolet
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
YC	Yeast complete

CHAPTER 1

INTRODUCTION

Post-transcriptional regulation of gene expression in plants

Control of plant gene expression is exerted at transcriptional and post-transcriptional levels. Although much has been learned in terms of transcriptional regulation, the study of post-transcriptional gene regulation is still limited to relatively few laboratories. However, a complex picture is emerging, showing post-transcriptional control of gene expression at multiple levels including mRNA stability and translation. Regulation at the level of mRNA stability and translation has been observed for a number of plant genes [reviewed in (Abler and Green, 1996; Bailey-Serres, 1999). Considerable progress has been made towards the identification of *cis*-elements and *trans*-acting factors involved in mRNA stability and translational regulation [reviewed in (Abler and Green, 1996; Gallie, 1996; Gutiérrez et al., 1999), which are discussed in the later sections of this chapter.

Both external and internal stimuli can regulate post-transcriptional processes. Various external stimuli have been documented to regulate gene expression post-transcriptionally. Heat shock affects the stability and translational status of many plant mRNAs. For example, heat shock destabilizes the α-amylase mRNA and mRNAs coding for secretory proteins in barley aleurone layers

(Belanger et al., 1986; Chu et al., 1997). It is hypothesized that following a heat shock, the signal recognition particles (SRPs) bound to nascent secretory proteins are not released from the endoplasmic reticulum leading to a premature termination of translation and thus resulting in degradation of respective mRNAs (Chu et al., 1997). In wheat, heat shock also brings about translational changes by phosphorylating initiation factors eIF-4A and eIF-4B (Gallie et al., 1997). Another external stimulus, sugar, also regulates gene expression of rubisco small subunit (*RbcS*) mRNA in suspension culture cells of tomato (Sinha et al., 2002). α-Amylase mRNA in cultured rice cells is more stabilized in the absence of sucrose (Sheu et al., 1994). Hypoxia, anoxia and fungal elicitors alter stability and translation of specific plant mRNAs [reviewed in (Bailey-Serres, 1999) and (Gutiérrez et al., 1999)]. *Arabidopsis* pyrroline-5-carboxylate reductase (*At-P5R*) mRNA translation is inhibited by both salt and heat stress (Hua et al., 2001).

Internal stimuli, such as hormones, regulate gene expression at both transcriptional and post-transcriptional levels. In ripening tomato fruit, most genes are regulated by ethylene at the transcriptional level. However ethylene-responsive proteinase inhibitor (*eri*, previously known as E17) mRNA in tomato is controlled at the level of mRNA stability through stabilization of the mRNA in the presence of ethylene (Lincoln and Fischer, 1988). Cytokinins have also been shown to stabilize chlorophyll A/B binding protein of light-harvesting complex II (LHCP) mRNA (Flores and Tobin, 1988) and soybean β-expansin, *Cim 1* mRNA (Downes and Crowell, 1998). In the germinating rice seed aleurone layer, gibberellin regulates rice α-amylase mRNA (*Ramy3D*) expression at the post-transcriptional level (Nanjo et al., 2004).

Light influences multiple levels of nuclear gene expression

Of the external stimuli that influence plant gene regulation, light is one of the most extensively studied and has been demonstrated to influence expression of many nuclear [reviewed in (Petracek and Thompson, 2000)] and chloroplast-encoded genes [reviewed in (Bruick and Mayfield, 1999)] through a combined input of both transcriptional and post-transcriptional processes. A clear example of regulation at several levels is found for the chlorophyll A/B binding protein (Cab) gene family (also can be referred to as either Light harvesting complex A [lhca] or light harvesting complex b [lhcb] depending on the primary association of the gene family member with a particular light harvesting complex). Transcription of nuclear-encoded *Cab* genes in pea is mediated through phytochrome and other light receptors [reviewed in (Kuno and Furuya, 2000)]. Further, some members of the *Lhcb* family are also positively photo-regulated at the translational level (Flachmann and Kuhlbrandt, 1995; Petracek et al., 1997). Other examples of translationally regulated nuclear encoded mRNAs include the spinach photosystem I subunits D, F and L (*PsaD*, *PsaF* and *PsaL* respectively) (Sherameti et al., 2002). In soybean and petunia, light controls both transcription and mRNA stability of members of the Rubisco small subunit gene family (*rbcS*) (Thompson and Meagher, 1990). Light effects on gene expression have also been well characterized for the ferredoxin encoding *Fed-1* from pea (Elliott et al., 1989b; Dickey et al., 1992; Gallo-Meagher et al., 1992), *FedA* from *Arabidopsis* (Bovy et al., 1995) and plastocyanin-encoding *PetE* (Helliwell et al., 1997) mRNAs.

Light-regulated *Fed-1* mRNA stability and translation

Efforts to identify light-regulated genes expressed in pea led to the isolation of *Ferredoxin-1* mRNA (*Fed-1*) (Dobres et al., 1987). In etiolated pea seedlings exposed to light, *Fed-1* mRNA accumulated rapidly in higher amounts than many other light-regulated mRNAs and hence was chosen for further characterization (Kaufman et al., 1985). The intronless, single copy, nuclear-encoded *Fed-1* mRNA is translated in the cytoplasm, the protein is transported into the chloroplast, where it functions to transfer electrons from photosystem I to $NADP^+$ (Elliott et al., 1989b).

Depending on the developmental stages of pea, light regulates *Fed-1* gene expression positively at either transcriptional or post-transcriptional levels. Phytochrome-mediated transcriptional regulation occurs when the transgenic etiolated tobacco seedlings are exposed to light. In contrast, post-transcriptional regulation becomes more significant in mature green plants (Gallo-Meagher et al., 1992). The promoter is minimally light responsive at this stage and the regulation is conferred by elements located within the transcribed region of the gene (Gallo-Meagher et al., 1992). When fused to the constitutive cauliflower mosaic virus 35S promoter, the transcribed region of *Fed-1* gene was light responsive in transgenic tobacco plants (Elliott et al., 1989a).

Analysis of cis-elements conferring light responsiveness led to the elucidation of an internal light responsive element (iLRE) constituting 95 nucleotides of the 5' untranslated region (UTR) plus the first 143 nt of the *Fed-1* coding region (Dickey et al., 1992). Mutations in the *Fed-1* mRNA coding sequence inhibit the light response, suggesting that light regulation requires an open reading frame and thus translation of the

mRNA (Dickey et al., 1994). Polyribosome analyses in transgenic tobacco plants revealed that *Fed-1* mRNA is highly associated with the polyribosomes in light, but not in the dark (Petracek et al., 1997) and *in vivo* labeling data confirm that *Fed-1* translation is enhanced in the light (Hansen et al., 2001). In addition, treating transgenic tobacco seedlings with photosynthetic electron transport inhibitor, 3-(3,4 -dichlorophenyl)-1,1-dimethylurea (DCMU) in light or with reduced levels of photosynthetically active radiation resulted in the dissociation of *Fed-1* mRNA from polyribosomes, suggesting translation of *Fed-1* is controlled by photosynthesis (Petracek et al., 1997). Recently, it has been shown that liverwort NADPH: protochlorophyllide oxidoreductase (*por*) (Eguchi et al., 2002) and pea *PetE* mRNAs are also destabilized when the photosynthetic electron transport is inhibited (Sullivan and Gray, 2002).

Replacing *Fed-1* 5' UTR with the TMV Ω-5' UTR ("translational enhancer") results in the loss of light regulation at the level of translation and mRNA abundance. These observations reveal that *Fed-1* 5' UTR does not simply promote efficient translation, but is necessary for differential translation and stability of the mRNA in the light and dark (Dickey et al., 1998). When progressive 3' deletions of the *Fed-1* iLRE are fused to the *CAT* coding region, a minimal sequence of *Fed-1* 5' UTR plus 13 codons of the coding region is sufficient for light-regulated mRNA accumulation (Dickey et al., 1998). In addition, mutation of the $(CAUU)_4$ repeat element (present near the 5' UTR terminus, nucleotides 16-31) to a 37% GC sequence (16.1 CAUU) results in disruption of light-regulated mRNA abundance, but not alteration of *Fed-1* mRNA polyribosome association (Dickey et al., 1998). These observations demonstrated that the $(CAUU)_4$ element is important for differential *Fed-1* mRNA stability and not translation. These

data also suggest that at least two separate cis-elements are present in the *Fed-1* iLRE: one required for rapid polyribosome dissociation, another for decay of *Fed-1* mRNA in the dark (Dickey et al., 1998). Direct measurements of *Fed-1* mRNA half-life shows approximately 2-fold longer half-life in the light than in the dark or in the presence of DCMU, confirming that light regulation of *Fed-1* mRNA abundance occurs at the level of mRNA stability (Petracek et al., 1998).

Light influences multiple levels of chloroplast-encoded gene expression

Models have been put forth suggesting light primarily influences chloroplast-encoded genes at the post-transcriptional level [reviewed in (Bruick and Mayfield, 1999)]. The chloroplast gene *psbA*, encoding PSII D1 protein is one of the well-characterized light-responsive genes. Light affects translation of *psbA* mRNA through its 5' UTR (Danon and Mayfield, 1991). Many nuclear-encoded, *trans*-acting factors have been identified that associate with the 5' UTR of *psbA*, *psbD* (encoding D2 protein) and *psbC* (encoding CP47) mRNAs in a light dependent manner (Danon and Mayfield, 1991; Nickelsen et al., 1994; Zerges and Rochaix, 1998). These proteins have been shown to influence the translation and stability of the mRNAs to which they bind. Interestingly, light-regulated binding of a complex of proteins [RB38 (RNA binding 38 kDa protein), RB47, RB55 and RB60] to the *psbA* mRNA is modulated via two regulatory switches, redox potential and ADP levels of the chloroplasts (Danon and Mayfield, 1991; Danon and Mayfield, 1994a, 1994b). Cyanobacterial chloroplast mRNA that encodes a PS II D1 protein homolog (*psbA2*) is controlled post-transcriptionally by light through a light-responsive element present in the 5' UTR (Agrawal et al., 2001).

Determinants of mRNA stability

Post-transcriptional regulation at the level of mRNA stability involves both *cis*-acting elements present within the mRNAs and their cognate *trans*-acting factors. Various mRNA features conferring differential mRNA stability have been elucidated. (i) AU-Rich Elements (AREs): AREs (adenylate- and uridylate-rich elements) are well-characterized mRNA stability determinants that are present in the 3' untranslated regions of various mammalian mRNAs from early response genes and also in cytokine mRNAs [reviewed in (Chen and Shyu, 1995)]. AREs contain multiple copies of the pentanucleotide sequence, AUUUA, that vary in length from ~50 to 150 nucleotides. These AU-rich sequences direct rapid decay of the mRNAs that contain them in the 3' UTR [reviewed in (Chen and Shyu, 1995)]. In addition to mRNA degradation, ARE sequences in the 3' UTR regulate translation of many mRNAs [reviewed in (Espel, 2005)]. Many ARE binding proteins (AUBPs) including AUF1, HuR and Tristetraprolin have been identified to interact with the ARE sequences and affect the stability and translation of the mRNA [reviewed in (Bevilacqua et al., 2003)].

AREs can be grouped into three classes [reviewed in (Xu et al., 1997)]. Class I AREs, present in *c-fos*, interleukin 4 (*IL-4*) and *IL-6* contain 1-3 copies of AUUUA sequence motif scattered in a U-rich region. Class II AREs are overlapping copies of nonamer UUAUUUA(U/A)U/A present in U-rich regions. Human granulocyte-macrophage colony-stimulating factor *(GM-CSF*), tumor necrosis factor alpha (*TNF-α)* and *IL-3* mRNAs have class II AREs in the 3' UTR. Class III AREs do not have the pentanucleotide sequence and are referred to as the Non-AUUUA. *c-jun* mRNA contains class III ARE.

Many plant mRNAs also contain AU motifs. Takahashi et al. (1989) have shown the presence of three AUUUA pentanucleotide sequences in the 3' UTR of a tobacco auxin regulated mRNA (designated as *par*). Spinach chloroplast mRNA that encodes cytochrome b_6/f complex subunit IV (*petD*) contains an 8-nt ARE sequence in the 3' UTR that acts as an mRNA stability determinant (Chen et al., 1995). It has also been shown that reporter mRNAs containing 11 overlapping repeats of the AUUUA motif in the 3' UTR are degraded more rapidly in tobacco cells than those mRNAs lacking the motif (Ohme-Takagi et al., 1993). As a result, these mRNAs accumulate to a lower level in transgenic plants. Thus ARE-mediated destabilization might be similar in animals and plants.

(ii) Iron-Responsive Element (IRE): Ferritin and transferrin receptor proteins are required for iron metabolism in cells. Transferrin receptor protein is required for importing iron into cells and ferretin for iron storage in cells. mRNAs encoding these proteins are regulated post-transcriptionally by iron that involves an iron-responsive element (IRE) and iron-regulatory proteins (IRP) [reviewed in (Theil, 2000)]. The IRE contains a 28- to 30-bp hairpin structure with a conserved CAGUGX sequence forming the hexaloop of the hairpin, and with a stem containing a mismatched C residue [reviewed in (Theil, 2000)]. The transferrin receptor mRNA (*TfR*) contains five IREs in its 3' UTR and the ferritin mRNA (*fer*) contains a single IRE in its 5' UTR. IRE regulates the half-life of the *TfR* and translation of the *fer* mRNA. When intracellular iron is scarce, IRP (iron regulatory protein) binds to the multiple IREs in the 3' UTR of *TfR* and to the single IRE in the 5' UTR of *fer* mRNA, thereby increasing *TfR* stability and repressing *fer* translation initiation. Conversely, high intracellular iron concentrations

results in a loss of IRP binding to IREs, thereby destabilizing the *TfR* mRNA and enhancing the translation of *fer* mRNA [reviewed in (Theil, 2000)]. Thus iron homeostasis is achieved by controlling the gene expression at post-transcriptional level.

(iii) Downstream Element: Downstream element (DST) is ~40 bp long and contains three highly conserved subdomains separated by two variable sequences [reviewed in (Gutiérrez et al., 1999)]. This element was first identified in the 3' UTR of very unstable soybean *SAUR* (small auxin-up RNA) mRNAs that rapidly accumulate in response to auxin treatment (McClure and Guilfoyle, 1987; McClure et al., 1989). In tobacco cells, a synthetic DST dimer sequence was sufficient to destabilize the reporter mRNAs when present in the 3' UTR (Newman et al., 1993). Through mutational analysis, Sullivan and Green (1996) found that ATAGAT and GTA conserved regions of the DST element are important for its functioning as an instability element. Interestingly, a DST element is also present in the 3' UTR of *Arabidopsis thaliana SAUR-AC1* mRNA, where it is suggested to act as an mRNA instability determinant (Gil and Green, 1996). In fibroblasts, soybean DST element increased the decay of rabbit β-globin reporter mRNA by rapid deadenylation (Feldbrugge et al., 2002). Interestingly, however, a mutant of soybean DST element that stabilized the mRNA in tobacco cells, remained effective in destabilizing the mRNA in the mouse fibroblasts, suggesting that although a plant DST element can function in mammalian cells, the mRNA sequences required for destabilization differ between the systems (Feldbrugge et al., 2002).

(iv) Double-stranded RNAs: An important aspect of gene expression control in plants is post-transcriptional gene silencing (PTGS)-related mRNA specific degradation, which is triggered by double-stranded RNA (dsRNA). PTGS, also called as co-suppression, is

similar to the RNA interference (RNAi) in animals and quelling in fungi [reviewed in (Chicas and Macino, 2001)]. Virus (DNA or RNA), sense transgene, antisense transgene or simultaneously expressed sense/antisense transgenes can mediate PTGS in plants [reviewed in (Fagard and Vaucheret, 2000)]. In plants, three pathways of RNA silencing have been elucidated [reviewed in (Baulcombe, 2004). In all the three pathways, the RdRP (RNA dependent RNA polymerase) uses aberrant RNAs (abRNAs) as a template to synthesize dsRNA. These dsRNAs are degraded into short interfering RNAs (siRNAs) and microRNAs (miRNAs) by an enzyme DICER (consisting RNase III domain). The siRNAs and miRNAs further direct the mRNA-degradation ribonuclease complex (similar to RNA induced silencing complex, RISC in RNAi) to homologous mRNAs, thereby allowing homologous mRNA degradation [reviewed in (Baulcombe, 2004)]. In plants, PTGS has been reported as a defense mechanism against viral infections, although some viruses circumvent PTGS by encoding proteins that suppress PTGS [reviewed in (Vaucheret et al., 2001)].

(v) Stem-loop structures: Stem-loop structures in the 3' UTRs can also act as stability determinants (Klaff et al., 1996). Mammalian insulin-like growth factor II (*IGF-II*) mRNA has a stem-loop structure in its 3' UTR and this structure is the site for endonucleolytic cleavage of *IGF-II* mRNA (Nielsen and Christiansen, 1992). The 3' UTR of rice α-amylase mRNA (*αAmy3*) contains sugar-responsive domains that are putatively folded into extensive stem-loop structures (Chan and Yu, 1998). Analyses of the 3' UTR revealed the presence of I, II and III subdomains that contain AU-rich sequences in the loop regions of each domain. The domains I or III, by themselves were sufficient to confer the sugar-dependent differential stability of *αAmy3* mRNA (Chan and

Yu, 1998). Stem-loop structures in the 3' UTR are also important stability determinants for chloroplast mRNAs (Drager et al., 1996). A stem-loop in the 3' UTR of *petD* mRNA is bound and stabilized by a protein complex termed as chloroplast stem-loop binding proteins (CSPs) (Chen et al., 1995; Yang and Stern, 1997). However, tobacco *psbA* mRNA has a stem-loop in the 5' UTR that is important for its stability and translation (Zou et al., 2003).

(vi) Riboswitches: Recently, it has been shown that bacterial mRNAs contain a complex folded structure (known as Riboswitches) that bind metabolites and regulate their own expression [reviewed in (Lai, 2003; Mandal and Breaker, 2004)]. These riboswitches are present in the untranslated regions of bacterial mRNAs and contain two domains; an aptamer binding domain and an expression domain, which is proposed to transfer the metabolite binding signal into genetic control [reviewed in (Winkler and Breaker, 2003)]. Binding of the metabolite to the aptamer domain results in a conformational change of the expression domain, which in turn leads to transcriptional termination or translational inhibition [reviewed in (Winkler and Breaker, 2003)]. Metabolites that bind to riboswitches often are either direct or indirect products of a regulated operon: coenzyme B_{12}, thiamine pyrophosphate (TPP), flavin mononucleotide, lysine, guanine, adenine, S-adenosylmethionine and glucosamine-6-phosphate [reviewed in (Mandal and Breaker, 2004)]. Sudarsan et al. (2003) have discovered metabolite binding domains in fungi and plants. Putative thiamine biosynthetic mRNAs (involved in the synthesis of TPP) of *A. thaliana*, rice and bluegrass contain a TPP -binding domain in their 3' UTRs (Sudarsan et al., 2003). Using structure-probing and in-line probing of *in vitro* synthesized TPP-binding RNA domain, the authors have shown that this domain has secondary structure

and TPP binding affinity similar to the known bacterial TPP riboswitch. In addition the TPP-binding domain of bluegrass is present upstream of the poly(A) tail in the 3' UTR, and it is speculated that the TPP metabolite binding to the domain might regulate mRNA stability (Sudarsan et al., 2003).

The mRNA decay mechanisms in eukaryotes:

The decay rates vary significantly from mRNA to mRNA within a cell, ranging from 1 min for the yeast pyrimidine pathway regulatory protein 1 (*PPR1*) mRNA [reviewed in (Caponigro and Parker, 1996)] to 60 hrs for human globin mRNAs (Kiledjian et al., 1999). Recent studies in yeast and mammals have localized the occurrence of mRNA decay to cytoplasmic foci (P bodies in yeast) (Sheth and Parker, 2003; Cougot et al., 2004). Most studies on eukaryotic mRNA degradation have been carried out using yeast and mammals. However, several research groups are focusing on the study of plant mRNA decay pathways (Higgs and Colbert, 1994; Kastenmayer and Green, 2000; Baginsky and Gruissem, 2002).

In eukaryotes, mRNAs can be degraded by two general pathways:
Deadenylation-dependent decay pathways: The deadenylation-dependent 5' to 3' decay pathway is the primary pathway by which many yeast mRNAs are degraded (Caponigro and Parker, 1996). This pathway is initiated by deadenylation of the mRNA, wherein the poly(A) tail is first shortened to a length of ~5 to 15 residues by deadenylases [reviewed in (Wilusz and Wilusz, 2004)]. Three mRNA deadenylases have been well characterized in eukaryotes including, CcR4p and Pop2p nuclease complex with many other accessory proteins, poly(A) nuclease (PAN) complex and poly(A) ribonuclease (PARN) [reviewed

in (Parker and Song, 2004)]. Following the shortening of poly(A) tail, the mRNA is decapped by the Dcp1p and Dcp2p enzyme complex [reviewed in (Coller and Parker, 2004)], thereby making the mRNA susceptible to 5' to 3' degradation by the Xrn1p enzyme [reviewed in (Parker and Song, 2004; Wilusz and Wilusz, 2004)].

The second common pathway in yeast is the deadenylation-dependent 3' to 5' decay. It has been reported that this secondary pathway involves degradation of many different yeast mRNAs by the "exosome", a multienzyme ribonuclease complex (Mitchell et al., 1997; Anderson and Parker, 1998; van Hoof and Parker, 1999; van Hoof et al., 2002). The yeast exosome contains at least ten 3' to 5' exoribonucleases and interacts with another protein complex (related to RNA helicase family) to recognize and degrade its substrate mRNA (Guhaniyogi and Brewer, 2001). In yeast, the exosome also functions in non-stop mRNA decay, characteristic of mRNAs lacking a translation termination codon. In this decay pathway, rather than deadenylation by polyA nuclease, the exosome degrades the poly(A) tail along with the rest of the mRNA (Chen et al., 2001; Wilusz et al., 2001).

Many mammalian mRNAs are degraded by exosome-mediated deadenylation-dependent 3' to 5' pathway. However some mammalian mRNAs are also suggested to be decayed by a deadenylation-dependent 5' to 3' pathway [reviewed in (Guhaniyogi and Brewer, 2001)]. AU-rich elements (AREs), present in some mRNA 3' UTRs initiate rapid decay of the mRNAs through AU binding protein recruitment of the exosome complex and PARN to the 3' end and the decapping enzyme to the 5' end of the mRNA (Chen et al., 2001). A similar model has been put forth by Mukherjee et al. (2002) to explain the sequential recruitment of the degradative machinery in mammals.

The exosome structure and function are also being analyzed in plants using *Arabidopsis thaliana*. AtRrp41p, plant homolog of yeast Rrp41p has been shown to interact *in vitro* with two yeast exosomal proteins (Chekanova et al., 2002). Another *A. thaliana* exosome subunit AtRrp4p is a 3' to 5' exoribonuclease, like its yeast counterpart and contains two RNA binding domains (Chekanova et al., 2002). This suggests that the exosome structure and function might be conserved among the eukaryotes.

Deadenylation-independent decay pathways: mRNA degradation may also occur via endonucleolytic cleavage and deadenylation-independent decapping pathway [reviewed in (Caponigro and Parker, 1996)].

Yeast *L2A* mRNA (encoding ribosomal L2 protein) [reviewed in (Caponigro and Parker, 1996)], soybean *SRS4* mRNA (encoding an RbcS gene family member) (Tanzer and Meagher, 1995) and spinach *psbA* mRNA [reviewed in (Monde et al., 2000) begin decay via endonucleolytic cleavage in the coding region of the mRNA . This initial step is succeeded by either 5' to 3' or 3' to 5' decay of the cleaved mRNA. Similarly, some mammalian mRNAs such as, *IGF-II* and *TfR* are degraded by endonucleolytic cleavage [reviewed in (Guhaniyogi and Brewer, 2001)]. Advances in PTGS studies have further increased our understanding of plant mRNA decay pathways. RNase III ribonucleases (Dicer like dsRNases) have been shown to cleave petunia chalcone synthase A (*chsA*) mRNA at a specific site [reviewed in (Gutiérrez et al., 1999)]. Intra- or inter-molecular base pairing within the RNA molecule triggers this cleavage.

The deadenylation-independent decapping and 5' to 3' decay pathway is characteristic of mRNAs containing premature translation termination codons (PTCs). This decay mechanism is also referred to as Nonsense Mediated Decay (NMD) and

occurs in fungi, plants, nematodes and vertebrates. Current models suggest ribosome associated, RNA surveillance proteins monitor mRNAs with either PTCs, mutations or defects (that occurred during pre-mRNA processing) and subject the aberrant mRNAs to rapid degradation by NMD [reviewed in (Culbertson, 1999; Baker and Parker, 2004)]. In *S. cerevisiae* Upf1p, Upf2p and Upf3p proteins catalyze the initial steps of the NMD pathway. These proteins recruit aberrant mRNAs into the NMD pathway wherein mRNAs are decapped by Dcp1p and degraded 5' to 3' by the Xrn1p exonuclease [reviewed in (Culbertson, 1999)]. However, these aberrant mRNAs are also degraded through the 3' to 5' exonucleolytic decay when the 5' to 3' decay is blocked (Mitchell and Tollervey, 2003). In addition to these two pathways, aberrant mRNAs in *Drosophila* are subjected to NMD through the endonucleolytic decay pathway (Gatfield and Izaurralde, 2004). Mammalian mRNAs with premature termination codons are also targeted to NMD and various models have been proposed to illustrate the decay mechanism [reviewed in (Hentze and Kulozik, 1999; Byers, 2002)]. NMD requires downstream elements in yeasts and introns in vertebrates as primary markers [reviewed in (Culbertson, 1999)]. Nonsense mutations have also been shown to reduce the abundance of bean *PHA* (encoding phytohemagglutinin) (van Hoof and Green, 1996) and pea *Fed-1* (Petracek et al., 2000) mRNAs, suggesting the occurrence of NMD in plants. Deadenylation-independent 5' to 3' degradation is also characteristic of *petD* mRNA (encoding subunit IV of $cytb_6$/f complex) in *Chlamydomonas* chloroplasts (Drager et al., 1999).

As mentioned earlier, light coordinates the expression of many photosynthetic mRNAs in plants. Our laboratory is interested in the light regulation of gene expression

at the level of translation and mRNA stability. We are using pea *Fed-1* mRNA as a model to study the post-transcriptional regulation by light. In this work, I have characterized the *cis*-elements involved in light-regulated *Fed-1* mRNA stability. I have also identified the putative *trans*-acting protein factors that might play a role in *Fed-1* mRNA regulation. In addition I have identified other plant mRNAs that are regulated post-transcriptionally like *Fed-1* in response to the photosynthetic changes.

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CHAPTER 2

THE *FED-1* $(CAUU)_4$ ELEMENT IS A 5' UTR DARK-RESPONSIVE mRNA INSTABILITY ELEMENT THAT FUNCTIONS INDEPENDENTLY OF DARK-INDUCED POLYRIBOSOME DISSOCIATION

This chapter is a published article (Bhat et al., 2004). Writing of the original manuscript and major part of the work described in this chapter were done by me. The polyribosome analysis in Figure 6 was done by Dr. Marie Petracek. Dr. Li Tang did the half-life measurements of P_{Top10}*:F-16.1* construct. RNA isolation of *F5'-NLTP cod*, *USr(CAUU)*$_4$*:NLTP* and *USr:NLTP* constructs were done by Angela Krueger. *F-(CAUU)*$_4$*DSmut* construct and RNA isolations were done by Dr. Sharon Ford. *In vitro* structure analysis was done by Chris Smith. Statistical analysis presented in this work was done by Dr. Larry Claypool.

INTRODUCTION

mRNA stability is controlled by *cis*-acting sequences and *trans*-acting factors. A number of *cis*-acting sequences that destabilize mRNAs in plants have been characterized including the AU-rich element (ARE) which destabilizes mammalian and plant mRNAs in a translation-dependent manner (Ohme-Takagi et al., 1993; Chen et al., 2001; Sarkar et al., 2003) and the downstream element (Carter et al.), which is present in the 3' UTR of unstable plant mRNAs including the SAUR mRNAs in soybean (Gil and Green, 1996; Perez-Amador et al., 2001). RNA secondary structure also may regulate mRNA stability for RNAs such as rice α-amylase (*αAmy3*). Three potential stem-loop forming subdomains within the *αAmy3* 3' UTR confer sugar-dependent differential stability to the mRNA (Chan and Yu, 1998). Finally, the Nonsense Mediated Decay (NMD) system which targets mRNAs that contain a premature termination codon for rapid degradation via a translation-dependent mechanism is also active in plants (van Hoof and Green, 1996; Petracek et al., 2000).

Light regulation of plant mRNA stability has been described for a number of nuclear and chloroplast mRNAs [reviewed in (Petracek and Thompson, 2000)]. Specific *cis*-sequence elements have been implicated in regulation of stability for a number of chloroplast mRNAs, including *psbA* (Eibl et al., 1999; Zou et al., 2003), *petD* (Higgs et al., 1999; Baginsky and Gruissem, 2002), and *atpA* (Drapier et al., 2002). Similarly, *cis*-sequences controlling light-responsive nuclear-encoded mRNA stability have been shown to be located within the 5' UTR and/or adjacent coding sequences. For example, blue light perceived by the phototropin receptor, destabilizes the Lhcb1*4 mRNA through sequences within the 5' UTR (Anderson et al., 1999; Folta and Kaufman, 2003).

Photosynthetic signals control the stability of the pea *Ferredoxin-1* (*Fed-1*) and *plastocyanin* (*PetE*) mRNAs. In transgenic tobacco, constitutively transcribed *Fed-1* and *PetE* mRNAs accumulate more during active photosynthesis than when photosynthetic electron transport is inhibited (Petracek et al., 1997; Sullivan and Gray, 2002). Direct measurement of the stability of *Fed-1* mRNA using the repressible promoter, P_{Top10} showed that *Fed-1* mRNA is destabilized when photosynthesis is inhibited (Gatz, 1995; Petracek et al., 1998). For both *PetE* and *Fed-1*, elements controlling light-regulated mRNA accumulation require the 5' UTR and some of the adjacent coding sequences of the mRNAs when fused to bacterial reporters (Helliwell et al., 1997; Dickey et al., 1998). Site-directed mutagenesis of the *Fed-1* 5' UTR revealed the importance of a $(CAUU)_4$ repeat element (present near the 5' UTR terminus, nt 16-31) for light-regulated mRNA accumulation (Dickey et al., 1998).

Fed-1 mRNA is polyribosome associated in the light but dissociates from polyribosomes in the dark during the same time-frame in which the mRNA is destabilized (Petracek et al., 1998; Hansen et al., 2001). Mutation of the $(CAUU)_4$ sequence to a 37% GC sequence (16.1 mutation) disrupts light-regulated mRNA accumulation but not dark-induced *Fed-1* mRNA polyribosome dissociation suggesting that apparent changes in translation of *Fed-1* mRNA is not due to the destabilization of polyribosome associated mRNA (Dickey et al., 1998). However, these data do not address if polyribosome dissociation is required for *Fed-1* mRNA instability in the dark.

Here we show through direct half-life measurement that a $(CAUU)_4$ repeat mutation stabilizes *Fed-1* mRNA in the dark. Furthermore, a 26 nt region of the *Fed-1* 5' UTR that includes the $(CAUU)_4$ repeat is sufficient to confer an ~ 2.5 fold change in light

induced accumulation to a non-light regulated plant mRNA. Finally, we find that *Fed-1* mRNA shows reduced mRNA accumulation in the dark even for mutants that are retained on polyribosomes in the dark. Together, these data suggest that a small 5' localized $(CAUU)_4$ element is the target for specific mRNA degradation that is active in the dark.

MATERIALS AND METHODS

Plasmid constructs

The plasmids illustrated in Figure 1 were all derived from a plasmid carrying the pea ferredoxin-1 (*Fed-1*) cDNA (Elliott et al., 1989) and transcribed from P_{35S} in pBI121 (Jefferson, 1987; Jefferson et al., 1987). Mutant constructs, *F-(CAUU)$_1$*,*F-(CAUU)$_2$*, *F-(CAUU)$_3$*, *F-(CAUU)$_6$*, *F-ΔCAUU*, *F-(CAUU)$_4$5'DS*, *F-34ntdeln* and *F-(CAUU)$_4$DSmut* were generated by two sequential Polymerase Chain Reactions (PCR), as described previously (Dickey et al., 1994). Mutant clones, *F-(CAUU)$_4$USmut*, *F-US/DSmut, F-(CAUA)$_4$*, *F-(AAUU)$_4$* and *F-51ntdeln* were generated by single PCR reactions using mutated sense primers with *BamH1* site at the 5' end and M13 –20 primer. The PCR-amplified regions of the plasmids were sequenced by the Oklahoma State University DNA/Protein Core facility. All the mutant *Fed-1* genes were inserted in pBI121 by flanking *XbaI* or *BamHI* and *SstI* restriction enzyme sites, replacing the *GUS* gene. P_{Top10}*:F-16.1* and P_{Top10}*:F-16.2* were made similar to P_{Top10}*:Fed-1* construct as described previously (Petracek et al., 1998).

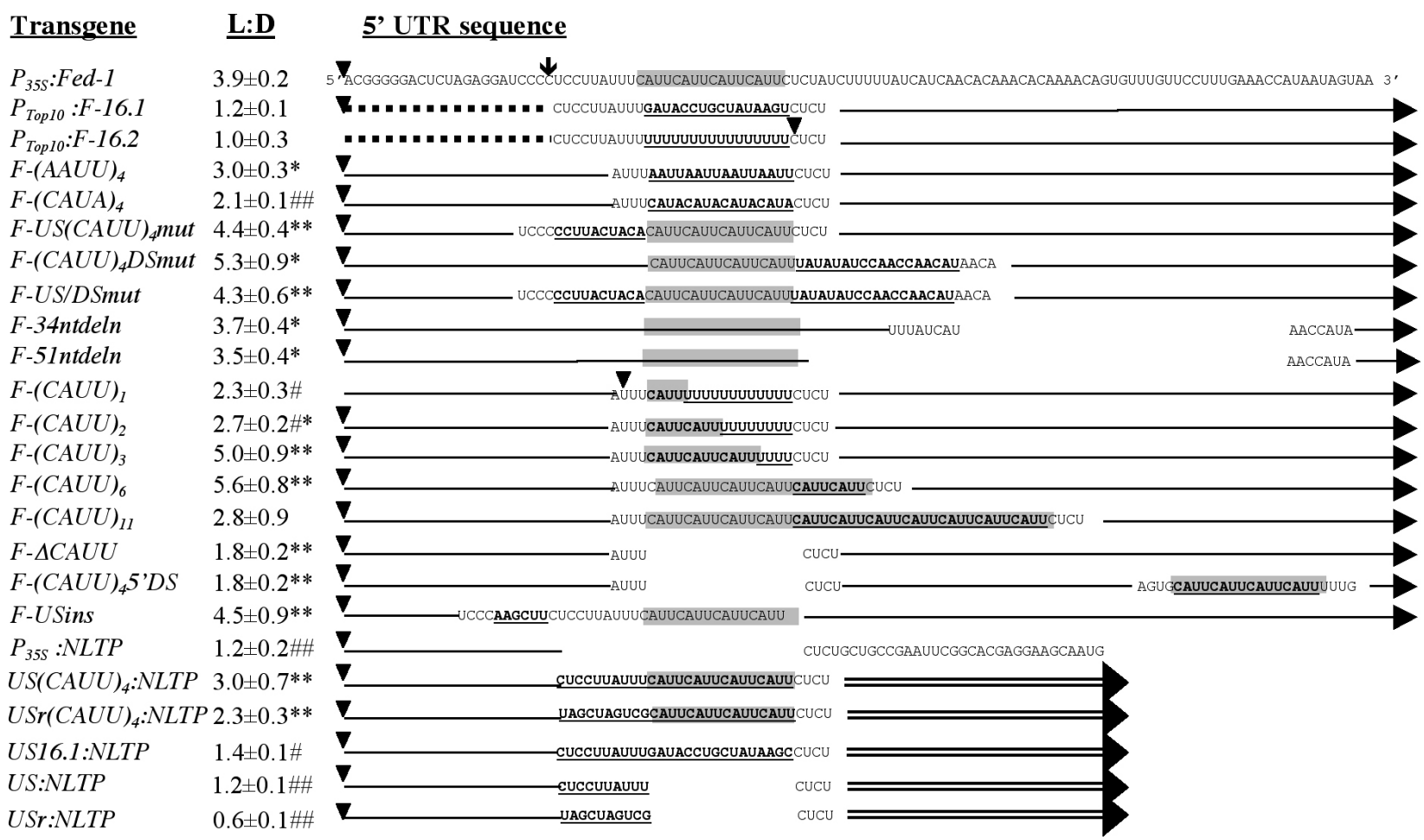
Transgene
L:D
5' UTR sequence
P35S:Fed-1 3.9±0.2
PTop10 :F-16.1 1.2±0.1
PTop10:F-16.2 1.0±0.3
F-(AAUU)4 3.0±0.3*
F-(CAUA)4 2.1±0.1##
F-US(CAUU)4mut 4.4±0.4**
F-(CAUU)4DSmut 5.3±0.9*
F-US/DSmut 4.3±0.6**
F-34ntdeln 3.7±0.4*
F-51ntdeln 3.5±0.4*
F-(CAUU)1 2.3±0.3#
F-(CAUU)2 2.7±0.2#*
F-(CAUU)3 5.0±0.9**
F-(CAUU)6 5.6±0.8**
F-(CAUU)11 2.8±0.9
F-ΔCAUU 1.8±0.2**
F-(CAUU)45'DS 1.8±0.2**
F-USins 4.5±0.9**
P35S :NLTP 1.2±0.2##
US(CAUU)4:NLTP 3.0±0.7**
USr(CAUU)4:NLTP 2.3±0.3**
US16.1:NLTP 1.4±0.1#
US:NLTP 1.2±0.1##
USr:NLTP 0.6±0.1##

Figure 1: **Light:dark mRNA accumulation of transgenes in tobacco**

L:D mRNA accumulation ratios of transgenes and the standard error of the mean were derived from Northern analysis of total RNA isolated from at least 4 independent transgenic tobacco lines treated with 3 day dark + 6 hr light or + 6 hr dark on at least 2 separate days. We used a one-tailed t test to determine if the L:D ratio was significantly increased compared to a control mRNA containing same coding sequence but no $(CAUU)_4$: *F-ΔCAUU* for P_{35S} and *Fed-1cod* transgenes or P_{35S}: *NLTP* for P_{35S} and *NLTPcod* transgenes (*, P< 0.05 and **, P<0.01). Similarly we asked if the L:D was significantly decreased compared to a control mRNA containing the entire *Fed-1* 5' UTR (P_{35S}*:Fed-1* and *F5':NLTP* for *Fed-1* coding and *NLTP* coding transgenes) (#, P<0.05, ##, P<0.01). The WT sequence of the 5' UTR of the *Fed-1* transgene mRNA is presented at the top of the figure. Identity with that sequence in the chimeric or mutant mRNAs is indicated by a solid horizontal line except that WT nt that flank mutation sites are shown in non-bold, non-underlined font to provide sequence junctions. *NLTP* sequences are similarly indicated by double horizontal lines. CAUU sequences are indicated by a gray box. Mutated sequences are indicated by bold font and underlining. Deletion or the absence of sequences is indicated by a gap. P_{35S} and the nopaline synthase terminator were used for all chimeric transgenes shown except where indicated (P_{Top10}). A bold dashed line represents the P_{Top10} polylinker sequence (5'atatcgaattcctgcagcccgggggatccac tagttctagaggatccc3') that is at the 5' end of P_{Top10} driven mRNAs. Transcriptional start sites mapped by 5' RACE are indicated by triangular arrowheads (▼). The transcriptional start site of *Native Fed-1* containing no polylinker sequence was confirmed by 5' RACE, and is marked with an arrowhead (⬇).

Full length *NLTP* cDNA (Genbank accession Y14560) was obtained from pea (*Pisum sativum*) leaf total RNA by 3' RACE (Gibco-BRL) and cloned into pPCR-Script Amp SK+. The resulting pSK-NLTP was used as a template for PCR to generate *F 5'-NLTPcod, US(CAUU)$_4$:NLTP*, *US16.1:NLTP, USr(CAUU)$_4$:NLTP* and *USr:NLTP* plasmid constructs. All PCR amplifications mentioned above were carried out using 5 min at 95ºC, followed by 30 cycles of: 95ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min 30 sec.

Plant transformation and growth

All the constructs in pBI121 were first transferred into *Agrobacterium tumefaciens* LBA 4404 by electroporation and then transformed into *Nicotiana tabacum* (SR-1, Petite Havana) as described previously (Dickey et al., 1998). Transgenic plants were grown in growth chambers (Percival Scientific, Inc.) at 22ºC, with a 12 h light/12 h dark cycle and under a light intensity of 250-300 μmol m $^{-2}$ sec $^{-1}$. For mRNA abundance experiments, clonally -paired plants were transferred to the dark for 3 days and then either brought back to the light or left in the dark for an additional 6 h. The youngest, fully expanded leaf was harvested into liquid N_2 as described previously (Dickey et al., 1998). For polyribosome analyses, transgenic tobacco plants were grown in soil for at least 1-2 weeks on a 12 h L:D cycle. ~8 h into the light cycle plants were transferred to the dark for 15 min or left in the light for an equivalent length of time, then harvested into liquid N_2.

RNA and polyribosome analysis

Total RNA was extracted as described by (Tang et al., 2003) and Northern blot analyses were done as described by (Petracek et al., 1997). Blots were exposed to Kodak X-OMAT Blue XB-1 films with intensifying screens for 1-24 hr at -80°C. Phosphorimager (BioRad, Hercules, CA) analyses were used to detect the amount of mRNA in Northern blot hybridization. Polyribosome analyses, Northern blot analyses and mRNA quantification was done as described previously (Petracek et al., 1997).

5' and 3' RACE

MicroPoly(A)Pure™ Kit (Ambion) was used to isolate mRNA from ~100-200 μg total RNA extracted from the appropriate transgenic tobacco lines. 5' RACE was performed with FirstChoice™ RLM-RACE Kit (Ambion) according to manufacturer's protocol. About 100 ng of wild type and mutated *Fed-1* Poly(A) RNA was used to produce single-stranded cDNA with a gene specific antisense primer 5'-TGTGGTGGTGACTGACATTG-3'. The target cDNA was amplified by PCR with 5' RACE adapter inner primer and gene-specific antisense primer 5'-GCATTGGCTGAG TCTGAGG-3' using either *Taq* DNA polymerase (Invitrogen) or Expand High Fidelity system (Roche Applied Science, Manheim Germany). PCR amplification was carried out using 3 min at 94ºC, 35 cycles of denaturation at 94ºC for 30 sec, hybridization at 55ºC for 30 sec and extension at 72ºC for 30 sec. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Inc.) and sequenced by the Oklahoma State University DNA/Protein Core facility.

Half-life measurements

T2 seeds from transgenic lines P_{Top10}*:Fed-1*, P_{Top10}*:F-16.1* and P_{Top10}*:F-16.2* were sterilized and grown on nylon membranes in magenta boxes as described previously (Petracek et al., 1998). Plants were grown at 22ºC in a growth chamber for 3-4 weeks. Seedlings at the four-leaf stage were treated in the boxes with 10 mg/L tetracycline in Hoagland's solution. The lids of the boxes were propped open for one hour to allow the uptake of tetracycline by transpiration. After one hour, the time zero samples were taken and the remaining boxes were closed and left in light (L) or wrapped in aluminum foil (D). At each time point duplicate or triplicate samples were harvested into liquid nitrogen followed by storage at -80ºC.

***In vitro* structure analysis**

Template DNA for *in vitro* transcription was generated from the constructs *F-5':Pcod*, *F-(AAUU)*$_4$. and *F-(CAUA)*$_4$. *F-5':Pcod* was generated by PCR-based overlap extension method with *Fed-1* and pea plastocyanin coding sequence (pUC LD4) as templates. The 5' primer is homologous to the upstream polylinker sequence and contains the T7 promoter (5'-CGCGCGTAATACGACTCACTATAGGGACGG GGGACTCTAGAGGATCCC-3'). With the 3' primer (5'-CATTACTATTATGG TTTC-3'), a 138 bp PCR product was generated. RNAs were prepared by *in vitro* transcription using the T7-MEGAshortscript kit (Ambion) following the manufacturer's protocol. The transcripts were purified by 6% denaturing PAGE and eluted in Tris-EDTA (pH 8.0) at 65ºC for 2 h and then ethanol precipitated at –80ºC overnight. 5' ^{32}P end-labeled transcripts were made using the KinaseMax kit (Ambion) following the

manufacturer's protocol. Labeled transcripts were re-purified as above. Before RNase digestion, RNAs were denatured at 95ºC for 5 min and then cooled to room temperature for 30 min. RNase A and V_1 digestions were conducted as per the manufacturer's (Ambion) protocol with the following concentrations: RNase A (0.001 and 0.01 μg/ml) and RNase V_1 (0.0001 and 0.001 U/μl). Digested products were separated by a 6% sequencing gel, dried, and visualized by autoradiography.

RESULTS

The $(CAUU)_4$ repeat is necessary for destabilizing the *Fed-1* mRNA in the dark

Previously, using the $P_{Top\ 10}$ system (Gatz, 1995), we showed an ~ 2-fold difference in wild-type *Fed-1* mRNA half-life in light-treated versus dark-treated tobacco ($t_{1/2}$ = 2.4 hr and $t_{1/2}$ = 1.2 hr, respectively) (Petracek et al., 1998). Furthermore, site-specific mutations of the $(CAUU)_4$ repeat located near the 5' end of the *Fed-1* 5' UTR (see Figure 1) abolished light-regulated *Fed-1* mRNA accumulation (Dickey et al., 1998), suggesting a role for $(CAUU)_4$ in light-regulated mRNA stability. When RNA from several of the mutant *Fed-1* expressing transgenic plant lines were combined, the apparent average abundance of the mutated *Fed-1* mRNA appeared to be increased following three days in the dark compared to wild-type *Fed-1* mRNA (Dickey et al., 1998), suggesting that the $(CAUU)_4$ repeat may be necessary for destabilization of the *Fed-1* mRNA in response to dark. To verify the role of $(CAUU)_4$ in *Fed-1* mRNA dark destabilization, we used the P_{Top10} system to directly determine if mutation of the $(CAUU)_4$ repeat increased the half-life of the *Fed-1* mRNA in the dark (Weinmann et al.,

1994; Petracek et al., 1998). We fused the P_{Top10} to two different mutations in the *Fed-1* transgene. The transcriptional start site of the previously described $(CAUU)_4$ substitution mutation *F-16.1* (Dickey et al., 1998) occurs within the polylinker sequence of the Top10 promoter (Figure 1) and the $(CATT)_4$ substituted with polyT in the transgene produces an mRNA with a deletion of the $(CAUU)_4$ along with the upstream sequences (*F-16.2*, Figure 1). Light-regulated mRNA accumulation was abolished for both mutations [steady state L:D mRNA accumulation ratios of 1.2 ± 0.1 and 1.0 ± 0.3, respectively (Figure 1)]. Tetracycline was applied to the roots of intact transgenic tobacco seedlings containing the P_{Top10} driven wild-type or mutated *Fed-1* mRNAs. Following uptake of tetracycline, plants retained in the light or placed in the dark at time 0 were harvested at times shown in Figure 2. *Fed-1* mRNA half-lives were calculated as described previously (Petracek et al., 1998). The half-life measurement of wild-type (WT) P_{Top10}*:Fed-1* mRNA served as a control for each of four biological repetitions (Figure 2A), and repeated previous results showing that *Fed-1* mRNA is destabilized in the dark (light $t_{1/2}$ = 2.1 h and dark $t_{1/2}$ = 1.2 h) (Petracek et al., 1998). Compared to WT *Fed-1* mRNA, mutation of the $(CAUU)_4$ element increased the mRNA half-life in the dark (P_{Top10}*:F-16.1* $t_{1/2}$ = 2.1 hr and P_{Top10}*:F-16.2* $t_{1/2}$ =2.7 h). In contrast, the half-lives of mutant mRNAs were similar in the light (P_{Top10}*:F-16.1* $t_{1/2}$ = 2.1 and P_{Top10}*:F-16.2* $t_{1/2}$ = 2.3 hr) relative to WT mRNA half-life (Figure 2B and 2C). These data indicate that the $(CAUU)_4$ repeat is necessary for destabilization of *Fed-1* mRNA in the dark.

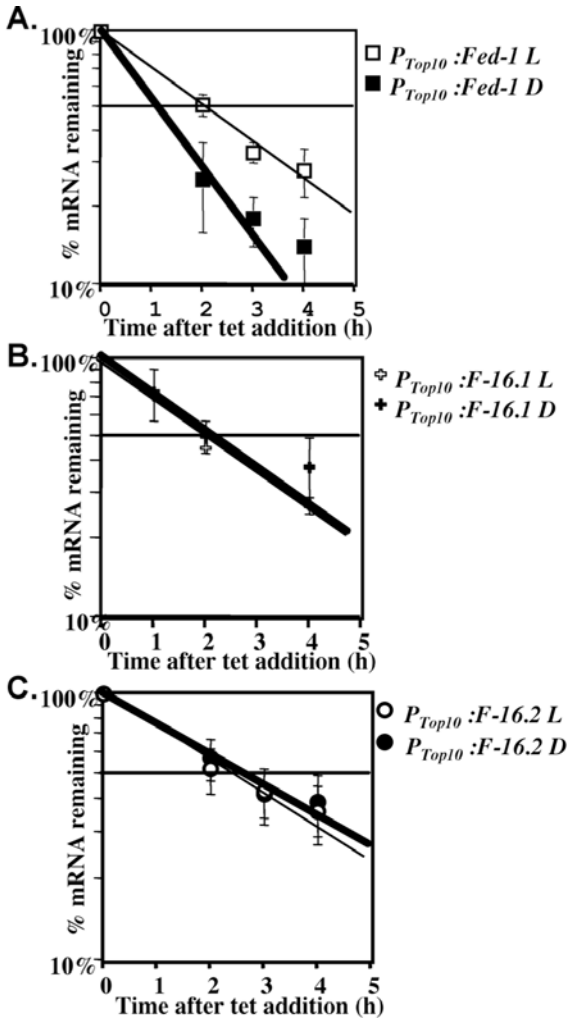

Figure 2: **Effect of two different substitutions of $(CAUU)_4$ sequence on *Fed-1* mRNA half-life in transgenic tobacco plants.**

Half lives of (**A**) wild-type *Fed-1* mRNA **(B)** *F-16.1* mRNA, and **(C)** *F-16.2* mRNA T2 transgenic seedlings from P_{Top10}*:Fed-1*, P_{Top10}*:F-16.1* and P_{Top10}*:F-16.2* (10-15 seedlings per sample) were grown on nylon membranes, and treated with 10mg/L tetracycline for 1 hr and then, at T_0, left in the light (L) or covered with foil for darkness (D) for 1 h, 2 h, 3 h, or 4 h prior to RNA isolation. 10 μg total RNA was separated by gel electrophoresis, transferred to nylon membrane, hybridized with an antisense ^{32}P-labeled *Fed-1* RNA and exposed to X-Ray film. Hybridizing mRNA was quantified by Phosphoimager analysis. The RNA remaining at each time point was determined relative to the amount at the zero time point and plotted on a semi-log graph. Best-fit lines are shown with standard error for each transgene. Each time point is derived from at least three separate experiments. The horizontal line indicates 50% remaining mRNA. L, light and D, dark.

Sequences adjacent to $(CAUU)_4$ are not a part of the *Fed-1* mRNA instability element

Previously, most of the *Fed-1* 5' UTR was mutated in 10 nt increments and showed wild type L:D mRNA accumulation (Dickey et al., 1998). However, it is possible that larger regions, perhaps forming a stem-loop structure or containing redundant sequences contribute to light regulated mRNA accumulation. MFOLD predicts the possible formation of a 34 nt stem-loop structure downstream of the $(CAUU)_4$ repeat, (ΔG, -10.0) (Zuker et al., 1999). We deleted the 34 nt predicted stem loop from the full-length *Fed-1* transcribed sequence and found that light-regulated mRNA accumulation of *F-34ntdeln* does not significantly differ from the wild-type. (L:D ratio of 3.7 $\pm$ 0.4, Figure 1). Furthermore, mutation of the 18 nucleotides immediately downstream of the $(CAUU)_4$ (*F-(CAUU)$_4$DSmut*) or deletion of the downstream sequence and the stem-loop (*F-51ntdeln*) are also light responsive (L:D ratio of 5.3 ± 0.9 and 3.5 ± 0.4 respectively, Figure 1). These data suggest that neither the predicted 34 nt stem-loop structure nor the adjacent stem-loop sequence immediately downstream of the $(CAUU)_4$ element contribute to light-regulated accumulation of *Fed-1* mRNA.

Mapping of the 5' end of the transgene mRNAs has shown that most contain 22 nt derived from the binary vector polylinker (Figure 1). Removal of the polylinker sequence (*Native Fed-1,* transcription start site indicated by arrow in Figure 1) showed that the sequence does not contribute to light regulated mRNA accumulation (L:D ratio of 4.0 $\pm$ 0.5). Furthermore, transgene mRNAs containing either mutation of the 10 nt CU-rich region upstream of the $(CAUU)_4$ element [*F-US(CAUU)$_4$mut*] or a combination

of mutations in the CU-rich upstream and downstream regions of the $(CAUU)_4$ element (*F-US/DSmut*) display normal L:D regulation of mRNA accumulation (L:D ratio of 4.4 ± 0.4 and 4.3 ± 0.6, respectively, Figure 1) suggesting these sequences do not contribute to light regulated mRNA accumulation.

26 nt at the 5' end of the *Fed-1* 5' UTR including the *(CAUU)$_4$* repeat is sufficient to confer at least 2.5 fold change in light-regulated mRNA accumulation

The *(CAUU)$_4$* repeat is clearly necessary for dark-induced mRNA instability of *Fed-1* mRNA. To fully delimit the instability element, we asked what sequences are sufficient to confer light-regulated mRNA accumulation to a plant-derived mRNA that does not show light-regulated mRNA accumulation. We tested a portion of the pea non-specific lipid transfer protein (*NLTP*) cDNA under the control of the CaMV 35S (P_{35S}) promoter for light regulated mRNA accumulation in transgenic tobacco. The resulting mRNA showed a L:D ratio of 1.2 ± 0.2, fitting our criteria of an mRNA that does not show light regulated mRNA accumulation (Figure 1). Replacement of the *NLTP* 5' UTR with the *Fed-1* 5' UTR showed that *Fed-1* 5' UTR is sufficient to confer a 3-fold change in light-regulated mRNA accumulation (L:D ratio of 3.0 ± 0.6, Figure 3). We asked if 26 nt of the *Fed-1* 5' UTR that includes the *(CAUU)$_4$* and the 10 upstream (US) nt was sufficient for dark-induced mRNA instability. To test this, we fused these 26 nt or 26 nt with the 16.1 mutation to the 5' terminus of *NLTP* [underlined and bold for *US(CAUU)$_4$:NLTP* and *US16.1:NLTP* respectively in Figure 1]. Using our standard light-dark mRNA accumulation assay (Dickey et al., 1998), we determined that, similar to the *NLTP* mRNA with no *Fed-1* sequences, the *US16.1:NLTP* mRNA showed a L:D

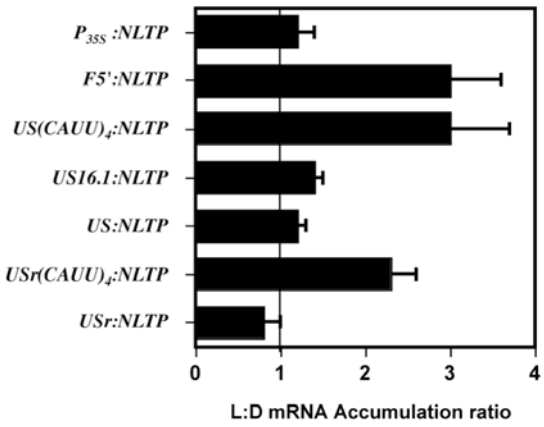

Figure 3: **$(CAUU)_4$ element is sufficient to confer L:D regulated mRNA accumulation on light insensitive *NLTP*.**

Portions of the *Fed-1* mRNA were fused to the *NLTP* mRNA as shown in Figure 1. The light response is shown as a ratio of mRNA levels in the light to mRNA levels in the dark (L:D). There is zero light induction when L:D = 1 (Indicated by the vertical line drawn at 1.) Standard error of the mean is indicated by the error bars. 8-16 independent transformants were analyzed for each construct.

ratio of 1.4 ± 0.1 (Figure 1 and 3). In contrast, *US(CAUU)$_4$:NLTP* mRNA significantly increased the L:D ratio to 3.0 ± 0.7 (Figure 1 and 3, $P < 0.05$ when compared to *NLTP* mRNA). These data confirm that the terminal 26 nt of the *Fed-1* 5' UTR including the $(CAUU)_4$ repeat and 10 nt upstream are sufficient to confer significant light regulation to a non-light-regulated mRNA. We asked if US sequences significantly contributed to the observed L:D regulation of the 26 nt element and found that randomization of the US sequences [*USr(CAUU)$_4$:NLTP*] did not significantly reduce the L:D ratio in the presence of the $(CAUU)_4$ sequence (L:D ratio of 2.3 ± 0.3, Figure 1 and 3), suggesting that the sequences upstream of the $(CAUU)_4$ contribute little to light regulated mRNA accumulation. However, removal of the $(CAUU)_4$ sequence (*USr:NLTP*) significantly reduced (and inverted) L:D response to 0.6 ± 0.1 (Figure 1 and 3, $P< 0.05$). Together these data strongly suggest that a 26 nt element is sufficient for a significant change in L:D mRNA accumulation on a heterologous mRNA.

The primary sequence of $(CAUU)_4$ is essential for its function

We asked what particular feature(s) of the $(CAUU)_4$ element confers dark instability to *Fed-1* mRNA. One hypothesis is that the secondary structure of the CAUU rich region as predicted by MFOLD at 38°C (Zuker et al., 1999), may be either unbase-paired ($\Delta G = -10.0$) or contain a base-pairing between the last CAUU and the AUG ($\Delta G = -9.6$) (M.P unpublished). This relatively unstructured region may make the mRNA vulnerable to decay. To test this idea, we constructed mutations of the $(CAUU)_4$ repeat that changed only a single nucleotide of each repeat and analyzed the L:D mRNA accumulation of each transgene. L:D mRNA accumulation of *F-(AAUU)$_4$*, which

predicted to form a hairpin structure of the AAUU region (ΔG = -11.3) was significantly reduced from wild-type *Fed- 1* mRNA but also significantly greater than *F- CAUU* (P<0.05 and P<0.01 respectively, Figure 4A). In contrast, L:D mRNA accumulation of *F-(CAUA)$_4$*, which was predicted to adopt either an unbase-paired (ΔG = -10.0) or base-paired structure (ΔG = -9.2) similar to the native $(CAUU)_4$ element, was significantly less than wild-type *Fed-1* and not significantly greater than *F- CAUU* (P<0.01 and P>0.05 respectively, Figure 4A). These results suggest that primary sequence or perhaps the repetitive nature of the sequence, rather than base-pairing of the CAUU with other regions, may be the important determinant of the $(CAUU)_4$ element in inducing mRNA degradation in the dark.

To test the possibility that the geometry of the $(CAUU)_4$ region itself may be important for instability (Soukup and Breaker, 1999), we determined the in vitro structure of the wild-type *Fed-1* 5' UTR compared to the *F-(AAUU)$_4$,* and *F-(CAUA)$_4$* mutants. In vitro transcribed, ^{32}P labeled mRNA was treated with RNase A and RNase V_1 and resolved by denaturing polyacrylamide gel electrophoresis. As shown in Figure 5, both the wild-type $(CAUU)_4$ region and the mutated *F-(AAUU)$_4$* form a repeating structure which is cleaved by RNase A following the last U of each repeat although RNase A has the potential to cleave at both ssC and ssU. The position of these cleavages was confirmed by cleavage with RNase T2, which cleaves at the first ssA of each repeat, and alkaline hydrolysis which produces a ladder, (C. S., data not shown). The vulnerability of the *F-(AAUU)$_4$* RNA to single-stranded nucleases and the similarity of these cleavages to those seen for the wild *Fed-1* mRNA suggests that the region does not form the hairpin

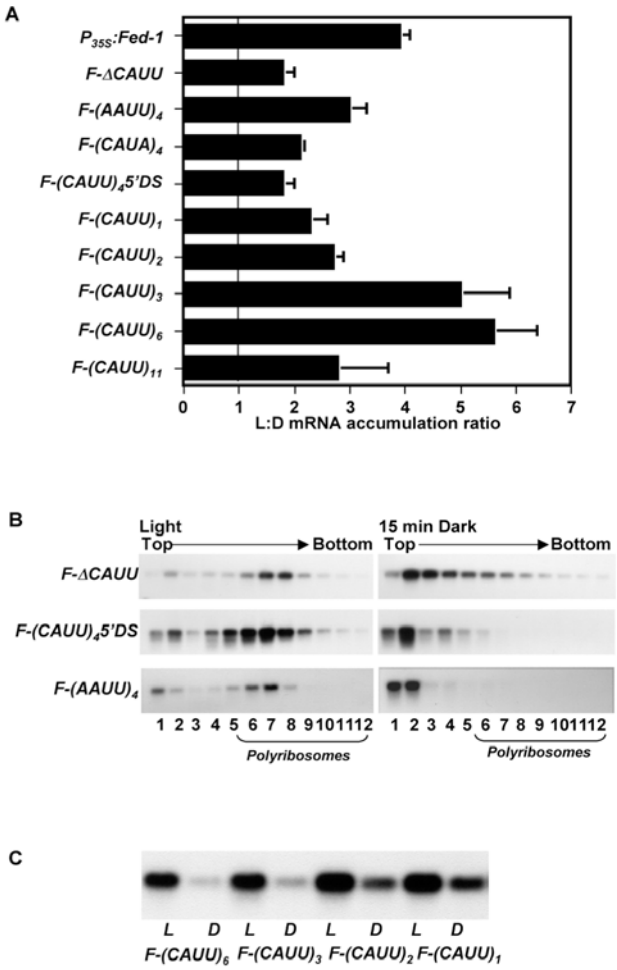

Figure 4: **Mutation of the $(CAUU)_4$ repeat.**

(A) The light response is shown as a ratio of mRNA levels in the light to mRNA levels in the dark (L:D). There is zero light induction when L:D = 1 (Indicated by the vertical line drawn at 1.) Standard error of the mean is indicated by the error bars. 4-23 independent transformants were analyzed for each construct. **(B)** Polyribosome analysis of transgene mutants in the $(CAUU)_4$ repeat. mRNA fractions from sucrose gradients were resolved by gel electrophoresis, blotted and hybridized with ^{32}P-labeled *Fed-1* antisense RNA. Each analyses presented is representative of at least two separate experiments. Fractions indicated as 1 through 12, represent the top to the bottom of the gradient and fractions 6 to 12 are polyribosomal fractions. **(C)** L:D mRNA accumulation of mutant *Fed-1* mRNA. For each different transgene, 5μg of total mRNA from each of nine clonally paired transgenic tobacco lines that had been treated with 3 d dark + 6 h light or 3d dark + 6 h dark was pooled. 5 μg of the light (L) and dark (D) treated pooled RNAs were resolved on the same gel, blotted, and the transgene mRNA was detected following hybridization with ^{32}P-labeled *Fed-1* antisense RNA and exposure to X-ray film.

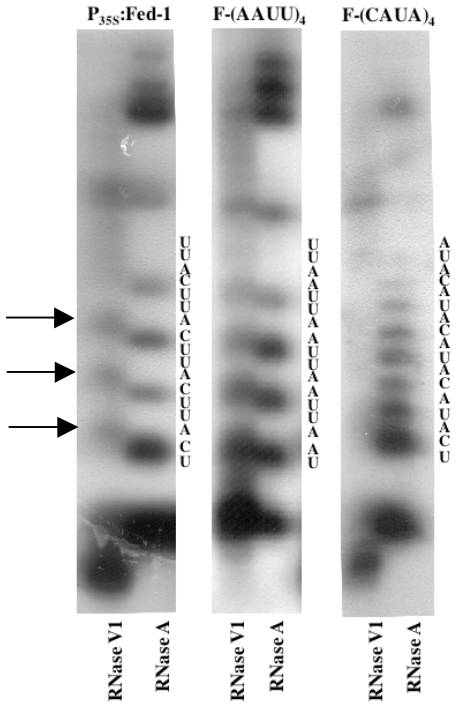

Figure 5: **A $(CAUA)_4$ mutation of the $(CAUU)_4$ region has altered sensitivity to RNase V_1 and RNase A in vitro.**

In vitro transcribed wild-type $(CAUU)_4$, $(AAUU)_4$ mutant, and $(CAUA)_4$ mutant *Fed-1* 5' UTRs was labeled with ^{32}P, subjected to RNase V_1 or RNase A cleavage as indicated, and resolved by denaturing PAGE, and exposed to X-RAY film for 1-4 days. Nucleotides within the wild-type or mutant $(CAUU)_4$ region are as indicated. Arrows show the region of RNase V_1 sensitivity in each wild-type repeat that is absent in the mutant mRNA.

structure predicted by MFOLD. RNase V_1, which cleaves primarily at helical and often double-stranded structures (Lowman and Draper, 1986), appeared to cleave both the wild-type and the mutated *F-(AAUU)$_4$* sequences with regularity in the AU junction of each repeat. Because the region is sensitive to single-stranded nucleases RNase A and RNase T2, we conclude that RNase V_1 cleavages of wild-type and *F-(AAUU)$_4$* are more likely due to a helical rather than base-paired structure. In contrast, the *F-(CAUA)$_4$* mutant repeat did not appear to be sensitive to RNase V_1 and the first C and the third U in each repeat was accessible to RNase A cleavage in the mutant. Thus the data indicate that the *F-(CAUA)$_4$* mutation changes the geometry of the repeat in vitro. These changes in vitro structure correlate with the relative sensitivity of the CAUU mutations to light:dark mRNA accumulation in vivo. Therefore, it is possible that these structural changes take place in vivo, either altering the sensitivity to endonuclease attack, or changing an inherent ability for self-cleavage (Soukup and Breaker, 1999).

The $(CAUU)_4$ repeat does not function in a downstream location within the *Fed-1* 5' UTR

Although the $(CAUU)_4$ element is clearly necessary for *Fed-1* mRNA destabilization in the dark, it remains unclear how this element functions in the *Fed-1* mRNA decay. We hypothesized that the position of the $(CAUU)_4$ repeat near the 5' end of the mRNA targets the *Fed-1* mRNA for decapping, thus promoting mRNA decay (Higgs and Colbert, 1994). If so, its position at the 5' end should be critical for its function. To test this idea, we inserted the $(CAUU)_4$ repeat 38 nt downstream of its original position in the 5' UTR with simultaneous deletion of the $(CAUU)_4$ repeat from its

normal location (*F-(CAUU)$_4$5'DS*, Figure 1) and compared the L:D mRNA accumulation to L:D accumulation of a *Fed-1* transgene containing a deleted $(CAUU)_4$ sequence (*F-ΔCAUU*, Figure 1). In both cases, the mutated transgene mRNA showed a significantly reduced differential mRNA accumulation in response to light (Figure 4A) suggesting that the position of the *(CAUU)$_4$* repeat within the *Fed-1* 5' UTR is important for its function. Furthermore, both mutated mRNAs showed wild type dissociation from polyribosomes in the dark (Figure 4B) supporting the idea that the *(CAUU)$_4$* repeat is not required for polyribsome dissociation of full-length *Fed-1* mRNA in the dark.

Three CAUU repeats are required for full light regulation of mRNA abundance

From a comparison of 14 5' UTR sequences of mRNAs that accumulate in the light but not in the presence of DCMU (Tang et al., 2003), we noted that in a total of 749 nt, 5 of the 5' UTRs contained a total of 7 dispersed (CAUU)s, a frequency greater than expected for a random nt distribution (M.P unpublished). Thus, we hypothesized that just one CAUU repeat or two repeats could be sufficient for at least partial destabilization of *Fed-1* mRNA in the dark. We re-introduced 1, 2 or 3 CAUU repeats into the *F-16.2* to form *F-(CAUU)$_1$*, *F-(CAUU)$_2$* and *F-(CAUU)$_3$* respectively (Figure 1). In transgenic tobacco, the presence of either 1, 2, or 3 CAUU units in the 5' UTR of *Fed-1* resulted in L:D mRNA accumulation ratios of 2.3 ± 0.3, 2.7 ± 0.2, and 5.0 ± 0.9, respectively (Figure 4A). These results suggest that one or two (CAUU)s can confer partial light-regulated mRNA accumulation and that an increased number of CAUU repeats correlates with an increased difference in light-responsive mRNA accumulation.

For AU-rich mRNA instability elements (see Introduction) it has been demonstrated that increasing the number of AUUUA motifs in a reporter mRNA increases the instability of the mRNA (DeMaria and Brewer, 1996). Therefore, we asked if adding additional CAUU units would further increase the L:D mRNA accumulation ratio. A *Fed-1* mRNA with six CAUU repeats [*F-(CAUU)*$_6$, Figure 1) showed a slight, but significant increase in the L:D mRNA accumulation compared to *WT* P_{35S}*:Fed-1* mRNA ($P<0.05$, Figure 4A) and *F-(CAUU)*$_3$ mRNA whereas eleven CAUU repeats decreased the differential mRNA accumulation (*F-(CAUU)*$_{11}$, Figure 4A). Thus the maximal L:D mRNA accumulation conferred by the CAUU element appears to be approximately 5-fold.

We hypothesized that the increased differential accumulation of *Fed-1* mRNA with increased numbers of (CAUU)s [up to six (CAUU)] resulted from decreased mRNA stability in the dark. This predicts that overall abundance between the transgenic lines in the dark should decline, but should remain relatively the same in the light. Since the abundance of a transgene in individual transgenic lines can vary greatly, depending upon the individual genomic insertion, individual transgenic lines cannot be used to compare light and dark mRNA abundances for different transgenes. However, a good estimate of relative mRNA abundance (and mRNA stability if transgenes are driven by the same promoter) can be achieved by combining total RNA from several lines containing the same transgene to get an average abundance (e.g. compare the results in (Dickey et al., 1998) and direct half-life measurements in Figure 2). Thus, we pooled 5 μg of total RNA from clonal pairs of nine independent T-DNA insertion lines treated with the light or dark (Dickey et al., 1998). 5 μg of each pooled total RNA was resolved on gels, blotted to

nylon membrane, hybridized with ^{32}P-labeled antisense *Fed-1* mRNA and quantitated the resulting bands using Phosphorimager analysis. Comparison of the relative light and dark abundances of the *F-(CAUU)$_1$, F-(CAUU)$_2$, F-(CAUU)$_3$,* and *F-(CAUU)$_6$* mRNAs shown in Figure 4C suggests that, as predicted, increasing the numbers of CAUU units most dramatically decreased levels of *Fed-1* mRNA in the dark.

Light/dark regulation of *Fed-1* mRNA abundance is retained in the absence of dark induced polyribosome dissociation

Mutation of the $(CAUU)_4$ repeat abolishes light-regulated mRNA stability of *Fed-1* mRNA but not light-regulated polyribosome association ((Dickey et al., 1998), Figure 1 and Figure 4). Thus, we hypothesized previously that *Fed-1* mRNA stability in the light is a direct consequence of polyribosome protection of the mRNA in the light and that, in the dark, polyribosome dissociation left the *Fed-1* mRNA vulnerable to nuclease digestion mediated through the $(CAUU)_4$ repeat (Dickey et al., 1998). This hypothesis predicted that a mutation that abolishes polyribosome dissociation in the dark should also abolish the dark-induced *Fed-1* mRNA instability. We assayed a number of *Fed-1* 5' UTR mutations for loss of light-regulated polyribosome loading, including several mutations that do not alter light-regulated mRNA accumulation (Bhat, S. and Petracek, M.E. manuscript in prep). In Figure 6, we show that L:D accumulation of *Fed-1* mRNA containing either an insertion of 5 nt between the CaMV P_{35S} polylinker derived sequence and the *Fed-1* 5' UTR sequence (*F-USins,* Figure 1) or the 51 nt deletion did not significantly differ from wild-type *Fed-1* mRNA (L:D ratio of 4.5 $\pm$ 0.9 and 3.5 $\pm$ 0.4 respectively, Figure 1). However, in contrast to WT *P_{35S}:Fed-1* mRNA, *F-USins* and *F-*

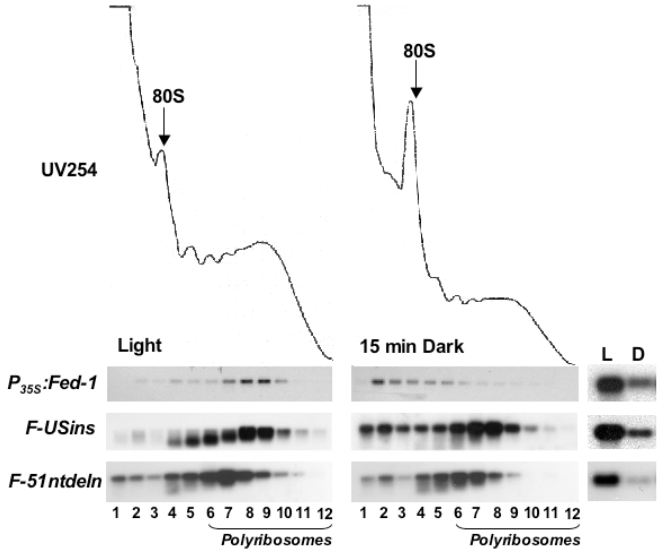

Figure 6: **Light regulated mRNA accumulation of *Fed-1* mutant with dark-insensitive polyribosome association.**

Polyribosome 254-nm UV tracings were recorded for light and 15 min dark-treated plant samples resolved on 15% to 60% sucrose gradients and presented left to right from the top to the bottom of the gradient. The 80S monosome peak is as indicated. RNA fractions from gradients were resolved by gel electrophoresis, blotted and hybridized with ^{32}P-labeled *Fed-1* antisense RNA. Each analyses presented is representative of at least three separate experiments. Northern-blot analyses of mRNA abundance for 3 day dark + 6 h light (L) versus 3 day dark-treated + 6 h dark (D) mRNA is presented to the right of the polyribosome data for a representative clonal pair containing WT *P$_{35S}$:Fed-1*, *F-USins* or *F-51ntdeln* transgene in tobacco. 5 μg total RNA from the indicated transgenic line was separated by gel electrophoresis, transferred to nylon membrane, probed with an antisense ^{32}P-labeled *Fed-1* RNA probe, and exposed to X-RAY film.

51ntdeln mRNA showed little dissociation from polyribosomes following 15 min dark. We also tested if mRNAs containing the *F-USins* are retained on polyribosomes following an extended dark treatment and found the mRNA on polyribosomes following 40 h dark (M.P. data not shown). These data suggest that *Fed-1* mRNA can be destabilized in the dark even if most of the mRNA remains in polyribosomal fractions supporting the notion that the element(s) controlling light-responsive translation and mRNA stability are separate. These data, combined with those in Figure 2, suggest that a dark-active factor destabilizes *Fed-1* mRNA through the $(CAUU)_4$ repeat.

DISCUSSION

Here we present evidence for an mRNA instability element that is completely contained within the *Fed-1* 5' UTR and is centered around a $(CAUU)_4$ repeat. The $(CAUU)_4$ element is necessary and sufficient for significant induction of dark-induced mRNA degradation and is active in the dark whether or not the mRNA is retained primarily on polyribosomes. These data strongly suggest that light regulation of *Fed-1* mRNA decay and translation are two separate dark-induced events that require separate trans-acting factors. Previously, data obtained from site-directed mutants of *Fed-1* mRNA was consistent with the idea that *Fed-1* mRNA decay requires active translation. Introduction of a missense codon in place of the *Fed-1* AUG or nonsense (NS) codons in the *Fed-1* mRNA abolishes light-regulated mRNA accumulation suggesting that light regulated accumulation of *Fed-1* mRNA requires translation (Dickey et al., 1994). However, introduction of NS codons leads to nonsense-mediated decay (NMD) of *Fed-1* mRNA (Petracek et al., 2000). This decay pathway is reported to be active during the

pioneer round of translation and thus may be epistatic to the *Fed-1* mRNA decay pathway (reviewed in (Maquat, 2004)). Similarly, introduction of a missense codon in place of the AUG is likely to lead to translation initiation at an out-of-frame downstream AUG and end in premature termination, thus triggering NMD. We present evidence here showing that mutant *Fed-1* mRNA retained on polyribosomes in the dark appears to be subject to dark-responsive mRNA decay mechanisms. When combined with our observation that disruption of dark-responsive mRNA decay does not block decreased polyribosome association in the dark (Dickey et al., 1998), the data strongly suggest that regulation of *Fed-1* mRNA stability and translation act through independent processes.

Analysis of mRNA abundance, polyribosome association and transcription initiation showed that a number of photosynthetically regulated mRNAs may be regulated through the combined contribution of transcriptional, post-transcriptional, and translational mechanisms (Tang et al., 2003). A single CAUU within the *Fed-1* 5' UTR confers slight light-responsive mRNA stability and increasing the number to two is significantly greater than the control. Similarly, a strict CAUU may not be required since mutations of a single nucleotide within each repeat can significantly reduce the response below the WT control, but is also significantly greater than an mRNA with no CAUU sequences (Figure 4). This intermediate effect may indicate a mechanism for varying sensitivities of endogenous mRNAs to dark-induced degradation. We found significant enrichment of G-poor sequences in the 5' UTRs of genes preferentially expressed during active photosynthesis (M.P., unpublished). It is possible that, if located near the 5' terminus, CAUU (or similar) motifs may contribute to overall light-regulated mRNA accumulation by a destabilization of these mRNA in the dark. If, as with the single

CAUU mutant of *Fed-1* mRNA, the contribution is < two-fold, the presence of a much larger transcriptional contribution may obscure the smaller post-transcriptional regulation.

The mechanism by which $(CAUU)_4$ repeat triggers *Fed-1* mRNA decay is unclear. Plant mRNA decay pathways that are initiated by endonucleolytic cleavage, decapping or deadenylation have all been identified (Gutiérrez et al., 1999). The $(CAUU)_4$ repeat may be bound by factors that direct light-regulated decapping, endonucleolytic cleavage, or exonucleolytic degradation. It is also possible that the $(CAUU)_4$ repeat itself may be inherently unstable due to the alignment of the nucleotides and self-hydrolysis (Soukup and Breaker, 1999). The possibility that RNA cleavage results from "in line cleavage" then raises the question of why the mRNA is less stable in the dark than in the light. In this case, it is possible that a light-regulated interacting factor may interact to enhance or disrupt the inherent geometry of the $(CAUU)_4$ region that promotes self-hydrolysis without directly catalyzing cleavage of the mRNA. The $(CAUU)_4$ element's location near the 5' end of the 5' UTR is important, as it is non-functional when moved further downstream within the 5' UTR. It is possible that the "downstream" $(CAUU)_4$ repeat is not functional due to loss of important sequence context. However, we think this is unlikely, since the $(CAUU)_4$ repeat confers light-regulated mRNA abundance on heterologous mRNAs (*NLTP*, Figure 1, 3 and *CAT*, S.B. unpublished). Also, mutation of all other sequences within the *Fed-1* 5' UTR including those immediately adjacent to the $(CAUU)_4$ repeat have little to no effect on light regulation of mRNA abundance. Thus, we prefer the idea that the location-effect on $(CAUU)_4$ function in mRNA degradation reflects its interaction with factors localized to

the 5' end of the mRNA. Competing complexes formed by $(CAUU)_4$ interacting factor(s) and cap-binding proteins, and decapping enzymes may initiate regulated degradation of the *Fed-1* mRNA.

Because the *Fed-1* 5' UTR alone is sufficient to confer light-regulated mRNA stability to heterologous mRNAs (*NLTP*, Figure 1, 3 and *CP12* and *PetE,* S.B., unpublished), it seems reasonable to assume that any secondary structures affecting RNA stability are between regions within the 5' UTR. Thus, we favor the possibility that by sequence-specific binding, a factor such as a protein or metabolite interacts with the $(CAUU)_4$ element, possibly in combination with proteins at the 5' end of the mRNA to control light-responsive mRNA stability. HSP101 binds *Fed-1* 5' UTR in vitro and may be necessary for enhancing *Fed-1* mRNA translation (Ling et al., 2000), although whether or not HSP101 interacts with the $(CAUU)_4$ repeat or affects mRNA stability is yet unclear. It is interesting to note that mammalian HSP70 and HSP110 also have been shown to bind AU-rich RNAs in vitro (Henics et al., 1999). We are currently attempting to identify a protein(s) that binds to the CAUU repeat and participates in dark-induced *Fed-1* mRNA degradation.

Why does a 26 nt region of the *Fed-1* 5' UTR show a significant sufficiency to confer light regulated mRNA accumulation when previous work suggested that the *Fed-1* 5' UTR was <u>not</u> sufficient to confer light-responsive mRNA accumulation when fused to bacterial reporter mRNAs (Dickey et al., 1992; Dickey et al., 1998; Hansen et al., 2001)? Although we observed no L:D change for in vivo protein production or mRNA abundance of the *Fed-1 5':CAT* mRNA (Hansen et al., 2001), *Fed-1 5':CAT* mRNA dissociates from polyribosomes in the dark (M. P., data not shown). Thus, it is possible

that the *Fed-1* 5' UTR decreased mRNA stability in the dark and increased ribosome recruitment of the *CAT* mRNA in light. The light-increased translation of bacterially-derived *CAT* coding sequences (with non-optimum codon usage (Lemm and Ross, 2002)) could accelerate decay of the mRNA in the light, and the *Fed-1* 26 nt 5' UTR element could accelerate decay of the mRNA in the dark. Such opposing mechanisms may lead to no apparent light-induced change in mRNA or protein abundance. Indeed, fusion of the *Fed-1* 26 nt does not confer regulated polyribosome association to *CAT* mRNA, but does increase the ratio of L:D mRNA accumulation from 1.8 fold to 3.2 fold (Dickey et al., 1998 and S.B., unpublished). These results are consistent with the results obtained for the *Fed-1* 5' UTR + 21 *Fed-1* codons fused to *CAT* and support the idea that rare codons, particularly in the first part of the coding region may accelerate mRNA decay during translation (Dickey et al., 1998). It has been noted that changing the codons of foreign transgene mRNAs to contain fewer A's or U's in the third position enhances the abundance of the transgene mRNA and protein (Koziel et al., 1996). It has further been shown that elimination of these rare codons also eliminates instability elements, and is likely the principle reason for increased mRNA abundance (van Hoof and Green, 1997). Our data support this idea and suggest that these instability elements are more active during translation.

Although the *Fed-1* 5' UTR confers significant light regulated mRNA accumulation (3-fold) on to the *NLTP* coding sequence and 3' UTR, these data appear to be slightly lower than the 4-fold change observed for the full-length *Fed-1* mRNA (Elliott et al., 1989). Thus it is possible that other sequences within the *Fed-1* mRNA contribute to small extent to the light response. In the case of the 26 nt $(CAUU)_4$ element,

spacing from the AUG, removal of redundant helper *Fed-1* sequences, or the presence of inhibitory *NLTP* 5' UTR sequences may reduce the effectiveness of the element.

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CHAPTER 3

LIGHT CONTROL OF NUCLEAR GENE mRNA ABUNDANCE IN TOBACCO

This chapter is a part of the published article (Tang et al., 2003). Dr. Marie Petracek wrote the major part of this manuscript. I performed the nuclear run-on experiments and wrote the experimental methods. Dr. Marie Petracek made the PCR-select cDNA subtraction library and Dr. Li Tang and Dr. Marie Petracek performed polyribosome analyses which appear in the appendix. The introduction, materials and methods and a section of the results are taken directly from the paper.

INTRODUCTION

Plant fitness is enhanced by the ability to rapidly change light harvesting capabilities in response to fluctuating light conditions (Kulheim et al., 2002). The genes for mRNAs encoding chloroplast proteins are divided between the nuclear and the chloroplast genomes and are coordinately regulated in response to light, maximizing energy storage while minimizing photo-oxidative damage [reviewed in (Goldschmidt-Clermont, 1998; Barkan and Goldschmidt-Clermont, 2000; Brown et al., 2001; Rochaix, 2001)]. Photosynthetic control of the abundance of chloroplast-encoded mRNAs occurs at the levels of transcription, mRNA processing and mRNA stability (Kubicki et al., 1994; Nickelsen et al., 1994; Alexciev and Tullberg, 1997; Deshpande et al., 1997; Yamamoto et al., 1997; Pfannschmidt et al., 1999; Tullberg et al., 2000). In addition, in depth study of many *Chlamydomonas reinhardtii* chloroplast-encoded mRNAs has revealed the importance of photosynthetic regulation of translation, often via binding of the encoded protein to the 5' or 3' UTR of the corresponding chloroplast mRNA [reviewed in (Bruick and Mayfield, 1999)]. Light-regulated binding of proteins to mRNAs may be regulated, at least in part, by changes in redox state generated by photosynthesis (Kim and Mayfield, 1997; Yohn et al., 1998; Fong et al., 2000; Shen et al., 2001). Photosynthetic inhibitors have been used widely to distinguish redox regulators of chloroplastic gene expression, implicating the ferredoxin/thioredoxin, glutathione, and PRK/CP12/GAPDH complexes in the signal transduction chain within chloroplasts (Wedel et al., 1997; Vener et al., 1998; Irihimovitch and Shapira, 2000; Trebitsh and Danon, 2001).

Regulation of gene expression between the chloroplast and nucleus includes cross-signaling between the organelles. In addition to the complex effects of photoreceptor signals on nuclear mRNA transcription [reviewed in (Quail, 2002)], signals from the developing plastid are essential for mature chloroplast formation (Oelmuller, 1989) and for accumulation of nuclear-encoded chloroplast proteins [e.g.(Zubko and Day, 2002)]. A series of *Arabidopsis thaliana* mutants have been isolated that uncouple nuclear and plastid gene expression, supporting the idea that signals between the plastid and chloroplast are essential for establishing and maintaining the appropriate balance of chloroplast- and nuclear-encoded proteins (Susek et al., 1993; Mochizuki et al., 1996; Vinti et al., 2000; Mochizuki et al., 2001). The plastid signals include, but may not be limited to, redox signaling and signaling through chlorophyll biosynthetic intermediates or carotenoids [(Surpin et al., 2002) and reviewed in (Rodermel, 2001)], and they may regulate gene expression at the transcriptional, post-transcriptional, and translational levels [reviewed in (Petracek and Thompson, 2000)].

To maintain appropriate stoichiometry between photosystems in response to light quality, promoters for a number of nuclear-encoded genes encoding chloroplast-localized proteins have differential responsiveness to oxidative and reductive signals (Pfannschmidt et al., 2001). For example, in *Dunaliella tertiolecta*, *LHCII* mRNA levels are sensitive to the redox state of the plastoquinone pool. Oxidation of plastoquinone using the photosynthetic electron transport inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), or low light levels, increases *LHCII* mRNA levels while reduction of plastoquinone using 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) or high light levels decreases *LHCII* mRNA levels (Escoubas et al., 1995).

Furthermore, in Arabidopsis, blockage of plastoquinone pool reduction with DCMU dominantly suppressed sucrose-induced transcriptional activation from the chlorophyll a/b-binding protein 2 and plastocyanin promoters. These results suggest a redox-regulated coordination of signals that measures sugar stores and ongoing photosynthesis (Oswald et al., 2001).

As with chloroplast-encoded mRNAs, photosynthetic signals have been shown to regulate mRNA stability of a few nuclear-encoded mRNAs. Constitutively transcribed *Pisum sativum Fed-1* mRNA in transgenic *Nicotiana tabacum* (tobacco) accumulates in the light. However, in the dark or in the presence of DCMU in the light, *Fed-1* mRNA is destabilized (Petracek et al., 1998). Mutation of a $(CAUU)_4$ repeat element in the *Fed-1* 5' UTR abolishes light-regulated *Fed-1* mRNA accumulation, suggesting a role of this repeat in mRNA stability (Dickey et al., 1998). Nuclear run-on and transgene analysis in tobacco of the pea plastocyanin (*PetE*) gene suggest light regulates *PetE* abundance at the level of mRNA stability (Helliwell et al., 1997). Like *Fed-1* mRNA, *PetE* and protochlorophyllide oxidoreductase mRNAs are destabilized in the presence of DCMU (Eguchi et al., 2002; Sullivan and Gray, 2002). Interestingly, the DCMU-induced destabilization of *PetE* mRNA is counterbalanced by a DCMU-induced increase in the rate of *PetE* transcription (Sullivan and Gray, 2002). Opposing effects of light regulation of transcription and mRNA stability have also been observed for pea and Arabidopsis *LHCB1* mRNAs. Transcription of these mRNAs increases following a pulse of blue light but the mRNA abundance is unchanged, suggesting a blue-light induced destabilization of *Lhcb1*4* mRNA (Warpeha et al., 1989; Marrs and Kaufman, 1991; Anderson et al.,

1999). This destabilization is likely induced through the *Lhcb1*4* 5' UTR (Anderson et al., 1999).

In a few cases, photosynthetic signals have been shown to have profound effects on the translational regulation of nuclear-encoded genes. Darkness or treatment with DCMU induces a rapid (within 20 min) and reversible dissociation of *Fed-1* mRNA from polyribosomes, with a concomitant decline in the in vivo translation rates of *Fed-1* mRNA (Petracek et al., 1998; Hansen et al., 2001). This regulation is conferred by sequences within the 5' UTR and the first one-third of the *Fed-1* coding sequence (Petracek et al., 1997). Similarly, in spinach polyribosome association of photosynthetic *PsaD, PsaF,* and *PsaL* mRNAs all decline in response to DCMU or dark treatment (Sherameti et al., 2002), suggesting regulation of translation by light. Mutational analysis of the *PsaD* mRNA showed a critical role of the 5' UTR in this response (Sherameti et al., 2002). Dark treatment of maize or amaranth results in the translational inhibition of *Cat2* and *RbcS*, respectively (Berry et al., 1986; Skadsen and Scandalios, 1987; Berry et al., 1988). However, unlike *Fed-1, PsaD, PsaF,* and *PsaL* mRNAs, neither *Cat2* nor *RbcS* mRNAs dissociate from polyribosomes, suggesting an inhibition of translational elongation rather than initiation. Dark-induced expression of the *ATB2* transcription factor mRNA is inhibited, probably at the level of translation, in the presence of 25 mM sucrose (Rook et al., 1998). Finally, light-regulated translation has been implicated in the coordination of gene expression between mesophyll and bundle sheath cells in C4 plants (Giglioli-Guivarc'h et al., 1996; McCormac et al., 1997).

Little is known about the mechanisms by which light regulates cytoplasmic mRNA translation. However, recent results suggest the striking possibility that

translation of the rye *Cat1* mRNA may occur via direct modification of the mRNA. Rye *Cat1* mRNA isolated from light-exposed leaves was translated more efficiently in vitro than *Cat1* mRNA isolated from dark-exposed leaves (Schmidt et al., 2002). The presence of the methylation inhibitors cycloleucine and phosphinothricin prevented the enhanced translation of *Cat1* mRNA isolated from light-exposed leaves, suggesting that methylation of the *Cat1* mRNA may be involved.

In this study we used PCR suppressive-subtractive hybridization to identify *Fed-1* like regulated endogenous tobacco genes. This screen identified 14 nuclear-encoded tobacco mRNAs whose light-induced increase in abundance is suppressed in the presence of DCMU. Strikingly, many of these mRNAs identified encode proteins involved in photosynthesis, suggesting that photosynthesis may regulate the mRNA levels of many genes involved in photosynthesis. In addition, we asked if the abundance of these mRNAs was regulated transcriptionally or post-transcriptionally. Of the five mRNAs with sufficient abundance to detect using nuclear run-on assays, we observed both transcriptional and post-transcriptional control of mRNA abundance. For example, Photosystem A subunit L (*PSAL*) and, to a lesser extent, α-tubulin (*TUBA3*) and *pSKA10* (an unknown gene) may be post-transcriptionally regulated by light. In contrast, Rubisco small subunit (*RBCS*) mRNA abundance appears to be transcriptionally up-regulated, but post-transcriptionally down-regulated, in the light.

MATERIALS AND METHODS

Plant growth

Tobacco seedlings were germinated in sterile magenta boxes with membrane rafts (Sigma V8380 and Sigma M1917, St. Louis, MO) in sterile MS media (Life Technologies, Grand Island, NY) for the PCR-select library, or on 0.5 X MS/ 1.5 % agarose petri plates for subsequent DCMU or light/dark mRNA abundance analysis, as described (Petracek et al., 1997; Petracek et al., 1998). Seedlings were grown for three weeks in a growth chamber at 22°C using a 12 h light/12 h dark cycle with 12 fluorescent and six incandescent lamps to give a light fluence of approximately 240 μmol m^{-2} sec^{-1} between 380 and 780 nm. For dark treatments, plants grown in Magenta boxes or petri plates were wrapped in foil for the indicated length of time before harvest or after being unwrapped and re-exposed to the light.

DCMU treatment

Plants were treated with DCMU as described (Petracek et al., 1997). Briefly, a 100 mM DCMU stock solution in 100% ethanol was diluted to 1 mM in sterile H_2O and 3 mL was pipetted onto the roots of tobacco seedlings on membrane rafts or on 1.5% agar plates. Control plants were treated with 3 mL of a 1% ethanol solution. Uptake was allowed with the covers propped open for 1 h before they were wrapped in foil for a 40 h dark treatment. Plants were then brought to the light for 6 h before harvesting the leaves into liquid nitrogen and storage at -80°C.

RNA isolation and cDNA subtraction library generation

Total RNA was prepared as described (Thompson et al., 1983) with the following modifications. Briefly, plant samples were homogenized with a polytron in phenol:chloroform:isoamyl alcohol (25:24:1) (US Biochemical, Cleveland, OH) and RNA extraction buffer [1% sodium dodecyl sulfate (Sigma), 1% (w/v) tri-isopropylnaphthalene-sulfonic acid (Kodak) (This chemical is no longer commercially available; we now omit this without problem), 4% (w/v) *p*-aminosalicyclic acid (Sigma), 10 mM Tris pH 7.5, 1 mM EDTA, and 2% (v/v) ß-mercaptoethanol, 1 mM aurin tricarboxylic acid (ATA, Sigma)]. ATA was omitted from samples used for cDNA preparation. Samples were centrifuged at 11,000 x g for 30 min at 4°C, the supernatant was removed, and the pellet resuspended in 100 µM ATA in water or, in samples used to produce cDNA, in water alone. The resuspended RNA was then precipitated with ammonium acetate and ethanol at -80°C overnight and collected at 11,000 x g for 30 min at 4°C. The resulting pellet was resuspended in 100 µM ATA water or water alone. All water used was treated with DEPC.

For cDNA library preparation, mRNA was isolated from 400 µg total tobacco leaf RNA using the MicroPoly(A)Pure kit (Ambion, Austin, TX) following the manufacturer's instructions. Approximately 2 µg of the resulting PolyA mRNA was then used to create a PCR-select cDNA library (Clontech, Palo Alto, CA) following the manufacturer's instructions, using light-treated samples as the tester cDNA and DCMU/light-treated samples as the driver cDNA. The resulting PCR samples were cloned into pGEM-T (Promega Biotech, Madison, WI) or PCR-Script Amp (Stratagene, La Jolla, CA). The resulting cDNAs were screened with the reverse-subtracted cDNAs

using the PCR-Select Differential Screening Kit (Clontech) to identify cDNAs likely to represent mRNAs differentially expressed in light versus DCMU/light-treated plants. Positive candidate cDNAs were then used as probes in northern blot hybridization analysis (Dickey et al., 1992), and cDNAs were sequenced by the North Carolina State University sequencing facility and the Oklahoma State University Recombinant DNA/Protein Resource Facility.

Nuclei isolation and RNA run-on assays

Nuclei isolation was as described (Folta and Kaufman, 2000) except nylon meshes of sizes 500, 300, 100, 50 and 20 μm were used for filtering the homogenate. Run-on transcription assays used 60 μg of the isolated nuclei incubated in 50 μl of buffer containing 50 mM Tris pH 7.9, 10 mM $MgCl_2$, 10% (v/v) glycerol, 500 μM each ATP, CTP, GTP, 75 mM NH_4Cl, 0.8 mM DTT, 125 μCi of [α ^{32}P] UTP (800 Ci/mmol) and 37.5 units of RNasin (Promega). Transcription reactions were incubated for 15 min at 27°C and the reaction stopped with 3 μl of a solution containing 3.3 μg/μl *E. coli* tRNA and 17 mM UTP. After 2 min, 1 μl RNase-free DNase Q (Promega) was added and the mixture was incubated an additional 5 min. 100 μl of 7.5 M urea, 5% SDS, 20 mM EDTA, 100 mM $LiCl_2$ and 10 mM ATA was added and the mixture extracted with an equal volume (150 μL) of phenol:chloroform:isoamyl alcohol (25:24:1). The ^{32}P-labeled RNA was centrifuged through a Sephadex G-50 column equilibrated with 10 mM Tris pH 8.0, 1 mM EDTA and 100 μM ATA and added to 900 μl of 1.1x hybridization buffer [1x= 50% formamide, 50 mM $NaPO_4$ pH 7.0, 5x SSC, 0.2%SDS, 5x Denhardt's solution

(100x Denhardt's is 2% each of BSA, Ficoll and polyvinyl pyrrolidone), 100 μg/mL *E. coli* tRNA, 100 μg/mL of poly A].

Two μg of each denatured cDNA-containing plasmid was dot blotted onto Gene Screen nylon membranes using a Hybri-Dot Manifold (Life Technologies). The membranes were prehybridized overnight at 42°C in 1 mL 1.1x hybridization buffer and then probed with ^{32}P-labeled run-on RNA for 72 h at 42°C. Membranes were washed twice in 2x SSC, 0.1% SDS at 42°C for 15 min each, followed by three washes in 0.5x SSC, 0.1% SDS at 68°C for 30 min each. Blots were exposed to Kodak XB-1 film with intensifying screens for 24 to 48 hrs at -70°C and to phosphorimager screens for quantitation.

RESULTS

Previously, we showed that the abundance of *Fed-1* mRNA in transgenic tobacco is regulated by photosynthesis. Upon shift from dark to light, *Fed-1* mRNA abundance increases 3- to 4-fold, and this increase is blocked by the addition of DCMU (Petracek et al., 1997). To identify similarly regulated endogenous tobacco mRNAs, we constructed a suppressive-subtractive cDNA library. For isolation of tester and driver cDNAs, we treated tobacco plants with 1% ethanol or with DCMU in 1% ethanol respectively, wrapped the plant containers in foil for a 40 h dark treatment, then re-exposed the plants to light for 6 h. PCR was used to amplify mRNAs enriched in the light versus DCMU-light, yielding plasmids containing 146 partial cDNAs. These 146 cDNAs were transferred to a nylon membrane and hybridized with ^{32}P-labeled light-enriched (forward-subtracted) or DCMU-enriched (reverse-subtracted) PCR-amplified cDNA. This screen

identified 26 cDNAs that showed greater hybridization to the forward-subtracted than the reverse-subtracted probe, suggesting that these mRNAs display increased accumulation in light versus light plus DCMU. Sequence analysis of these 26 cDNAs identified 22 different cDNAs. Thiazole biosynthetic enzyme (*THI1*) was isolated three times and *TUBA3* and light harvesting complex (*LHC-I*) were each isolated twice. The remaining 19 mRNAs were identified as unique cDNAs, suggesting that this screen was not saturated.

Light-regulation of mRNA abundance can occur either via regulation of promoter activity (transcriptional regulation) and/or regulation of mRNA stability (post-transcriptional regulation). We used nuclear run-on assays to determine if light altered the rates of transcription of the genes we identified in our screen. Five-week-old *35S:Fed-1* transgenic tobacco plants were transferred to the dark for 40 h and then either brought to the light or left in the dark for an additional 6 h. Nuclei were isolated and run-on nuclear RNAs were labeled with ^{32}P and hybridized to each of the differentially expressed cDNAs. Run-on analysis of many of these RNAs was not possible as the signals were not sufficiently above background levels of hybridization to vector only (pKS-, Figure 1) and salmon sperm DNA (S.B., data not shown). However, five endogenous tobacco mRNAs and the *Fed-1* RNA expressed from the transgene were clearly detectable above background (Figure 1). These assays indicate that *Fed-1* transcription rates are the same in the light and the dark (Figure 1), while *Fed-1* mRNA shows a four-fold increase in the light compared to the dark (Figure 1), consistent with our observation that dark results in a decreased half-life of *Fed-1* mRNA

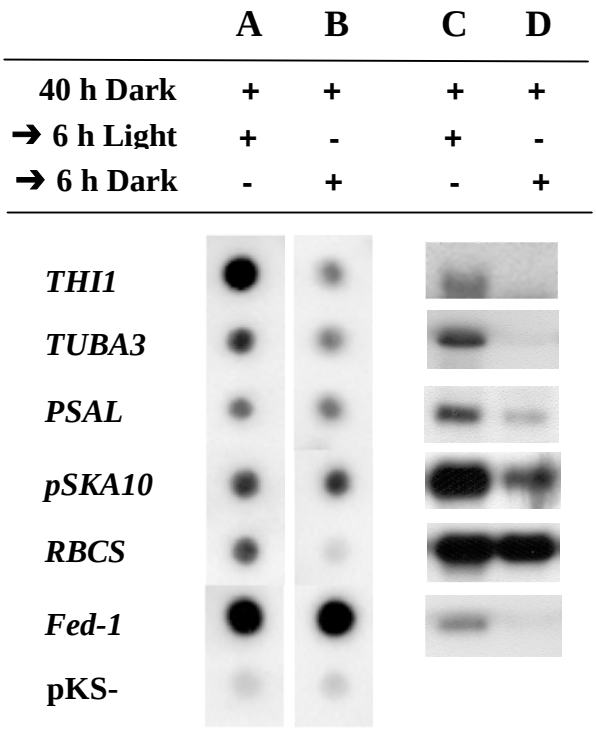

Figure 1**: Nuclear run-on analysis and mRNA abundance of differentially selected cDNAs.**

Nuclei were isolated from a pair of soil-grown plants treated with 46 or 40 h of darkness followed by 6 h light. ^{32}P-UTP nuclear run-on RNAs were then hybridized to dot blots of plasmids carrying the indicated differentially selected cDNAs. Each hybridization was repeated at least three independent times and the data presented are the results from one representative blot (A and B). Tobacco seedlings grown on plates containing 1.5% (w/v) agar were placed in the dark for 40 h, and then re-exposed to the light for an additional 6 h (C) or kept in the dark for an additional 6 h (D). Five micrograms of total RNA were isolated from a pooled sample containing at least 10 plants, resolved by gel electrophoresis, blotted and probed with ^{32}P-labeled antisense probes to the indicated mRNAs. A summary of the analysis for all differentially selected mRNAs, repeated with at least three separate hybridization experiments, is presented in Table 1.

Putative gene name	40D➞6L: 40D➞6L +DCMU	40D➞6L: 40D➞6D	Run-on 40D➞6L: 40D➞6D	Chloroplast Signal Peptide
LHC-I Light harvesting complex I	6.8 ± 1.2	6.2 ± 1.3		Yes
THII Thiazole biosynthetic enzyme	6.1 ± 0.7	6.2 ± 0.4	4.3 ± 0.8	Yes
TUBA3 Alpha tubulin	3.5 ± 0.4	4.7 ± 0.9	2.2 ± 0.4	No
pSKA10 unknown	3.1 ± 1.4	2.8 ± 0.3	1.6 ± 0.4	ND
RPL29 Ribosomal protein L29	3.0 ± 0.4	8.0 ± 2.0		Yes
PER Peroxidase	2.6 ± 0.8	3.6 ± 0.2		No
CP12 Chloroplast Protein 12	2.4 ± 0.9	6.9 ± 1.0 *		Yes
PSII-X Photosystem II subunit X	2.1 ± 0.3	6.2 ± 0.7 *		Yes
RBCS Small subunit Rubisco	1.8 ± 0.5	2.0 ± 0.0	12.3 ± 1.7 *	Yes
PSAL Photosystem A subunit L	1.7 ± 0.4	2.9 ± 0.8	1.1 ± 0.1	Yes
PSAK Photosystem A subunit K	1.7 ± 0.2	3.0 ± 0.5		Yes
PSAF Photosystem A subunit F	1.7 ± 0.3	2.1 ± 0.3		Yes
OEC 33 kDa Oxygen evolving complex 33kDa subunit	1.4 ± 0.1	1.2 ±.0.1		Yes
CAT-1 Catalase I	1.4 ± 0.2	1.3 ± 0.1		No
Annexin	1.2 ± 0.1	2.5 ± 0.2 *		No
CAB16 Light harvesting complex B	1.1 ± 0.1	5.9 ± 0.2 **		Yes
CAB4 Light harvesting complex B	0.9 ± 0.3	3.8 ± 1.0		Yes
CAB10-like Light harvesting complex B	0.8 ± 0.1	1.8 ± 0.8		Yes
CAB36-like Light harvesting complex B	0.7 ± 0.0	3.8 ± 1.0		Yes

Table I. **The effects of light, dark, and inhibition of photosynthesis by DCMU on tobacco mRNA accumulation.**

Tobacco plants were put in the dark for 40 h, then re-exposed to light for 6 h in the absence (40D➞6L) or presence (40D➞6L+DCMU) of DCMU or left in the dark for an additional 6 h (40D➞6D). Ratios for mRNA abundance for 40D➞6L: 40D➞6L+DCMU and 40D➞6L: 40D➞6D are followed by standard errors and were calculated by combining phosphorimager results from at least three separate experiments. The statistical significance of the differences in the ratios of 40D➞6L: 40D➞6L+DCMU and 40D➞6L: 40D➞6D and for the differences in the ratios of 40D➞6L: 40D➞6D and run-on 40D➞6L: 40D➞6D were determined by using the two-tailed two independent samplesT-test (*p<0.05, **p<0.01). Chloroplast signal peptides were predicted using TargetP (**http://www.cbs.dtu.dk/services/TargetP/**).

(Petracek et al., 1998). Of the five experimental mRNAs with hybridization levels above background, *TUBA3*, *PSAL* (and perhaps *pSKA10*) showed a slightly greater difference in steady-state mRNA accumulation than in transcriptional run-on assays following a 40 h dark + 6 h light treatment compared to a 46 h dark treatment (Table I and Figure 1). Thus, the differential accumulation of these mRNAs is, at least in part, post-transcriptionally regulated.

As expected, we also identified genes that appear to be mainly regulated at the level of transcription by light. *THI1* mRNA showed similar differences in run-on assays compared to steady state mRNA accumulation between the light and dark, suggesting transcriptional control of *THI1*. In strong contrast to the other mRNAs, *RBCS* mRNA showed a dramatic 13-fold higher rate of transcription in the light compared to in the dark, but only a two-fold difference in steady-state mRNA accumulation ($P < 0.05$, Table I and Figure 1). These results suggest transcription of *RBCS* mRNA is higher in the light, but that the mRNA is destabilized in the light or stabilized in the dark, resulting in counterbalancing effects on *RBCS* mRNA accumulation.

DISCUSSION

Here we have identified tobacco cytoplasmic mRNAs that are, like *Fed-1*, affected by changes in photosynthetic electron transport. Using nuclear run-on assays we observed both transcriptional and post-transcriptional regulation of the differentially selected cDNAs. We were able to detect only five of the most abundant mRNAs in our nuclear run-on assay, of which one (*PSAL*) mRNA appears to be regulated at the post-transcriptional level. The regulatory element(s) of *PSAL* mRNA may be present

anywhere within the transcribed sequence. Like pea plastocyanin (*PetE*) mRNA, light-regulation might be mediated by elements present both in the 5' UTR and within coding sequence (Helliwell et al., 1997). In contrast, like *Fed-1*, the regulatory element(s) of *PSAL* might solely be confined to the 5' UTR. Sequence comparison between the 5' UTRs of *Fed-1* and *PSAL* shows that, *PSAL* 5' UTR does not contain any CAUU sequences (M.P. unpublished data). However, since we cannot recognize the regulatory elements by simple sequence comparisons, further experimental analysis of the *PSAL* mRNA, by fusing portions of *PSAL* sequences to reporter genes and by measuring the mRNA decay *in vivo*, such as we have done for the *Fed-1* mRNA (Bhat et al., 2004) could be important in understanding its regulation.

Although two of the cDNAs (*TUBA3* and *pSKA10*), show only a two fold difference in run-on assays, the difference between the run-on results and steady state mRNA accumulation was not statistically significant, suggesting primarily transcriptional regulation of these two mRNAs. In addition, *THI1* was also not regulated at the level of mRNA stability, suggesting that the light regulation is mediated by the promoter sequence. Many nuclear-encoded photosynthetic genes (e.g. *Lhcb*) studied in detail, are regulated at the level of transcription which is mediated by light-responsive promoters [(Folta and Kaufman, 1999) and reviewed in (Thompson and White, 1991)].

From our nuclear run-on assays, we observed dramatically increased transcription of *RBCS* in the light. However, the slight difference in steady state mRNA accumulation of *RBCS* between the light and dark suggests the mRNA is either destabilized in the light or stabilized in the dark. Such opposing effects have been previously observed with pea *PetE* mRNA in transgenic tobacco plants. Sullivan and Gray (2002) showed that DCMU

treatment increased the transcription rate of *PetE* from its native promoter in nuclear run-on assays, but decreased the *PetE* mRNA stability. Light regulation at both transcriptional and post-transcriptional levels has been previously described for *RBCS* mRNA in soybean seedlings and petunia (Shirley and Meagher, 1990; Thompson and Meagher, 1990). In similarity to *RBCS* instability in light, Anderson et al. (1999) show that pea *Lhcb1*4* mRNA is destabilized in the presence of blue light. In addition they have identified the blue light-induced instability element to be present in the 5' UTR of *Lhcb1*4* mRNA. It is possible that both, *Lhcb1*4* and *RBCS* might contain similar instability sequence elements. However, the presence of multiple opposing regulatory mechanisms hinders the recognition of common regulatory elements. In such cases further analyses, through mutational studies become important for identifying sequences that may be responsible for light-regulated mRNA stability.

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CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE *FED-1* mRNA BINDING PROTEINS

The work described in this chapter is ongoing. Additional experiments will be done prior to preparation of a manuscript. Dr. Sharon Ford helped with plasmid constructs *F-(-55C)* and p3'Fed-1 for yeast three-hybrid assays. Affinity purification studies were done by Angela Krueger.

INTRODUCTION

Regulation of plant gene expression by various signals occurs at transcriptional and post-transcriptional levels. RNA-binding proteins interacting with various mRNA features can play an essential role in post-transcriptional regulation. These proteins contain different types of RNA-binding domains, such as the RNA-recognition motif (RRM, also referred to as RBD or RNP motif), K-homology (KH) motif (similar to human hnRNP K protein domain), Arg-Gly-Gly (RGG) boxes, zinc fingers, oligonucleotide/oligosaccharide binding domain (OB-fold domain), tryptophan RNA binding attenuation protein (TRAP) like domain and Pumilio-homology domain (PUM-HD) and the double-stranded RNA-binding domains (dsRBDS) [reviewed in (Siomi and Dreyfuss, 1997; Messias and Sattler, 2004)]. The tertiary structures and the functions of these RNA-binding motifs have been reviewed in detail (Messias and Sattler, 2004; Stefl et al., 2005).

AREs (adenylate- and uridylate-rich elements) are well-characterized mRNA stability determinants that are present in the 3' untranslated regions of various mammalian mRNAs. Several proteins that bind mammalian ARE-containing mRNAs have been studied in depth and have been identified to play either direct or indirect roles in mRNA decay [reviewed in (Bevilacqua et al., 2003)]. It is hypothesized that ARE-binding proteins (AUBPs) such as AUF-1, TPP and KSRP proteins might recruit the exosome complex to ARE-containing mRNAs and destabilize them (Bevilacqua et al., 2003). However, AUF-1, like AUBPs HuR, HuD and HuC, can also act as a stabilizing factor and protects the mRNA from degradation (Bevilacqua et al., 2003). It has been observed that AUF1 stabilizes Interleukin-3 (*IL-3*) mRNA in one cell line and

destabilizes it in another (Loflin et al., 1999; Ming et al., 2001). Ming et al. (2001) have also shown that class II ARE-containing mRNAs with overlapping copies of nonamer UUAUUUA(U/A)U/A in U-rich regions are also stabilized by AUF1 proteins.

Although RNA-binding proteins have been well characterized in yeast and mammals, knowledge of these proteins in plant post-transcriptional gene regulation is still rudimentary. A recent computational survey of the *Arabidopsis* genome has revealed nearly 200 RNA-binding proteins with RRM domains and 26 with KH domains (Lorkovic and Barta, 2002). However, this survey does not include the proteins with RGG, zinc finger and other known RNA-binding motifs.

Pea *Fed-1* is regulated by light through the process of photosynthesis at the level of both translation and stability in transgenic tobacco (Petracek et al., 1997; Petracek et al., 1998). Like *Fed-1*, several other endogenous tobacco mRNAs are light-regulated at the level of translation initiation and mRNA stability (Tang et al., 2003). Previous work revealed the importance of a $(CAUU)_4$ repeat (present near the 5' UTR terminus, nucleotides 16-31) in light-regulated *Fed-1* mRNA abundance (Dickey et al., 1998). Recent analyses of *Fed-1* mRNA *cis*-instability element(s) from our laboratory, revealed that the *Fed-1* 5' UTR and particularly the $(CAUU)_4$ element is necessary and sufficient for dark-induced mRNA degradation [Chapter II or (Bhat et al., 2004)]. In addition, our work also suggested that *Fed-1* mRNA can be destabilized in the dark even if most of the mRNA remains in polyribosomal fractions, supporting the notion that *cis*- and *trans*-acting factors controlling light-responsive translation and mRNA stability are independent [Chapter II (Bhat et al., 2004)].

To understand the mechanism of *Fed-1* light-regulation, it is important to identify factors that might interact with the $(CAUU)_4$ instability element. Identification of proteins involved in *Fed-1* mRNA translation has been attempted first by Ling et al. (2000). They have shown that plant heat shock protein HSP101, a member of the ClpB family, binds to *Fed-1* 5' UTR *in vitro* and may be necessary for enhancing *Fed-1* translation in a heterologous yeast system.

In this study, we have utilized *in vivo* and *in vitro* methods to identify putative protein factors that bind to *Fed-1* mRNA 5' UTR. Using a yeast three-hybrid assay, we identified tobacco ribosomal protein S2 (RP S2, formerly called p40) as a protein that strongly interacts with the *Fed-1* 5' UTR. In addition, we describe the isolation of other proteins that might interact with *Fed-1* 5' UTR using affinity purification of *Arabidopsis* proteins. Three affinity-purified proteins were identified using MALDI-TOF mass spectrometry, including a ~26 kDa probable RNA binding protein (pRNAbp). UV-crosslinking and *in vitro* binding of the over-expressed proteins to various RNAs suggests different specificities of binding for RP S2 and pRNAbp proteins to sense and anti-sense *Fed-1* mRNA.

MATERIALS AND METHODS

Plant materials and growth conditions

Nicotiana tabacum (SR-1, Petite Havana) and *Arabidopsis thaliana* ecotype Columbia plants were grown in growth chambers (Percival Scientific, Inc.) at 22ºC, with a 12 h light/12 h dark cycle and under a light intensity of 250-300 μmol m^{-2} sec^{-1}. For

cDNA library generation, tobacco plants were transferred to the dark for 1 hour or left in the light for equivalent length of time, then harvested into liquid N_2. For affinity purification, *Arabidopsis* plants were harvested into liquid N_2 after ~8 hours into the light cycle and stored at -80°C.

Plasmid constructs

Plasmid constructs for three-hybrid assay: Plasmid construct *F-(-55C)*, with mutated *Fed-1* 5' UTR wherein -55 T is mutated to a C, was generated by a single PCR reaction using plasmid template containing pea *Ferredoxin-1* (*Fed-1*) cDNA (Elliott et al., 1989), a sense primer (5'CGGGGATCCCTCCTTATTTCATTCATTCATTCATTCTCTATCT TCTTATCATCAACAC3') with *Bam*HI site at the 5' end and a M13 -20 primer. The PCR-amplified product was ligated into pBluescript vector (Stratagene, La Jolla, CA) and sequenced at Oklahoma State University Recombinant DNA/Protein Resource facility. The mutant *Fed-1* flanked with *Bam*HI and *Sst* I sites was inserted in pBI121 (Jefferson, 1987) through replacement of the *GUS* gene to form plasmid *35S::F-(-55C)*. The *35S::F-(-55C)* plasmid was used as a template in PCR to amplify *Fed-1* 5' UTR bait for the three-hybrid assay. Primers used for the PCR amplification were: Bait 5'primer containing *Avr* II restriction enzyme site, 5'-ACACCTAGGGGCCGCCCGGCGCTA TACGGGGGACT CTAGAGGATCCCTC-3' and Bait 3'primer with *Sma* I site, 5'-ACACCCGGGGGCCGGGCGGCCTAACATT ACTATTATGGTTTCAAAG-3'. p3'Fed-1 was obtained by ligating *Avr* II/*Sma* I digested PCR product into *Avr* II/*Sma* I digested pRH3' plasmid (Invitrogen, Carlsbad, CA) using Rapid DNA ligation kit (Roche, Indianapolis, IN). Similarly p3'ΔCAUUFed-1 and p3'USmutFed-1 bait plasmids were

obtained with the respective mutated Bait 5'primers and with template plasmids *F-ΔCAUU* and *F-(CAUU)$_4$USmut* respectively [template plasmids were constructed as described in Chapter II (Bhat et al., 2004)].

Plasmid constructs for *in vitro* transcription and affinity purification: For *in vitro* transcription, the *Fed-1* 5'UTR region was used fused to a T7 promoter (underlined in the primer sequence) by PCR with the sense primer (5' TAATACGACTCACTATAGGGC GAATTGGAGCTCGGGGACTCTCTAGAGGATCCCCTCCTTATTTCATTCATTCA TT 3') and gene-specific anti-sense primer (5' GCATTGGCTGAGTCTGAGG 3'). The resulting PCR fragment was cloned into pPCR-Script Amp SK(+) plasmid (Stratagene, La Jolla, CA) to generate pSKT7-F-(CAUU)$_6$. The F-(CAUU)$_6$Poly(A) plasmid for affinity purification was synthesized by PCR amplification from a pSKT7-F-(CAUU)$_6$ template. Reverse primer (5'GGAAACAGCTATGACCATG3') and 5'-TTTTTTTTTT TTTTTTTGGTGTGGTTGCCATTACTATTATGGTTT-3' primer were used for the PCR. Above mentioned PCR amplifications were carried out as described in Chapter II (Bhat et al., 2004).

Plasmids constructs for glutathione S-transferase (GST) fusion proteins: Coding regions of tobacco *RP S2* and *Arabidopsis pRNAbp* were fused in frame with GST in the vector pEGKT (Mitchell et al., 1993). The coding region of tobacco *RP S2* was PCR amplified with the primers: Forward 5'-TCTGGTGGTGGTGGTGGTCTGGTTCGGGGT GGATCCGGATCCATGGCGGCTACACATGAAG-3'; and reverse 5'-TGAAT TAAGCTTGAGCTCGAGTCGACCCATGGAGTCTAGATACTAAGAGCCAGAAAA GAA-3'. The coding region of *Arabidopsis pRNAbp* was amplified with the primers: Forward 5'-TCTGGTGGTGGTGGTGGTCTGGTTCGGGGTGGATCCGGAT

CCATGGCTGCTTCAGCTTCGT-3'; and reverse 5'-TGAATTAAGCTTGAG TCGACCCATGGAGTCTAGATATCAATATTGGCGCCTTGGAGGC-3'. The PCR products were transformed along with *Bam*HI and *Xba* I cut pEGKT into the yeast strain, YML145 (*α ura3 lys2 leu2 his3 trp1Δ pep4Δ::HIS3MX6*). Through homologous *in vivo* recombination (Guthrie and Fink, 1991; Gietz et al., 1992), plasmids containing plant cDNAs were circularized and maintained as separate plasmids in the yeast strain, YML145 (provided by Dr. Mark Longtine).

RNA isolation and cDNA library generation

Total RNA for cDNA library generation was prepared as described by Tang et al. (2003). Total RNAs from light- and dark-treated tobacco leaves were combined and 200 μg of total RNA was used to isolate mRNA by MicroPoly(A)Pure™ Kit (Ambion, Austin, TX). Approximately 200 ng of the resulting Poly(A) mRNA was used to generate a cDNA library as described by the commercial protocol (BD Biosciences Clontech, Palo Alto, CA). Poly(A) mRNA in a total volume of 4 μL was incubated at 72°C for 2 min with the primer 5'-CAGAATTCCAGCACACTGGCGGCCGTTTT TTTTTTTTTTTTTTTTTTTTTTTTTVN-3' . MMLV Reverse Transcriptase (Ambion) was added to the mRNA/primer mixture along with the First Strand 10x Buffer and 10 mM dNTPs. The resulting mixture was incubated at 42°C for 10 min, followed by the addition of primer 5'-GCTCAATTGCGGCCGGCCGCTATATACATAGAAGCTAAG CTTGGG-3'. The reverse transcriptase reaction was carried out for 1 h at 42°C and terminated at 72°C for 10 min. 1 U of RNase H was added, followed by incubation at 37°C for 20 min to degrade the residual mRNA. 1-2 μL of first-strand cDNA was used

for LD-PCR with the Advantage cDNA PCR Kit (BD Biosciences). Primers used for the PCR were: Hyb5'PCRII, 5'- GACGAGTACGGTGGGATCGATTGGATCCCCGGGT ACCGAGCTCAATTGCGGCCGGCCGCTAGAT-3' and Hyb3'PCRII, 5'-CATGATG CGGCCCTCTAGATCCATGCCTCGAGCGGCCGCCAGTGTGATGGATATCTGCA GAATTCCAGCACACTGGCGGC-3'. PCR amplification was done as per the manufacturer's instruction. The resulting cDNA product was purified with PCR purification kit (Qiagen, Valencia, CA) and used for subsequent library transformation into the yeast strain, L40-*ura3*.

cDNA library transformation into yeast for three-hybrid selection and screening

The yeast three-hybrid assay was done using the RNA-Protein Hybrid Hunter Kit (Invitrogen) and as described by SenGupta et al. (1996), with the following modifications. The Zeocin resistance gene in the three-hybrid plasmid pHybLex/Zeo-MS2 was replaced by the *LEU2* marker. The *LEU2* marker was PCR amplified from *YIplac128* (Gietz and Sugino, 1988) using primers ML 737, 5'-TATCGGCATAGT ATAATACGACAAGGTGAGGAACTAAACCGCAACCATTATTTTTTTCC-3' and ML 738, 5'-ATCTCCGAGGCCTGGGACCCGTGGGCCGCCGTCGGACGTGCTA CCCTATGAACATATTC-3'. The resulting PCR product was co-transformed with pHybLex/Zeo-MS2 for homologous recombination in the yeast L40-*ura3* host strain (Invitrogen), followed by the transformation of bait plasmid p3'Fed-1 {containing the hybrid MS2 RNA binding site fused to the *Fed-1* 5' UTR with a C mutation at position -55 [*F-(-55C)*]}. RNA hybrid control pRH3'/IRE, supplied by the manufacturer, was also independently introduced into L40-*ura*HybLex/*Leu* for control assays. Competent yeast

L40-*ura*HybLex/*Leu*p3'Fed-1 strains were prepared by standard yeast protocols and stored at -80°C (Guthrie and Fink, 1991). Competent L40-*ura*HybLex/*Leu*p3'Fed-1 cells were used to co-transform *Hind* III cut prey plasmid pYesTrp3 and the tobacco cDNA library. The library transformants were selected on synthetic YC medium (Leu^-, Ura^-, Trp^-, His^-). pYESTrp2/IRP (Invitrogen) control plasmid was transformed into L40-*ura*HybLex/*Leu*pRH3'/IRE yeast strain separately. Primary screening for three-hybrid interactions was carried out through the His selectable marker. 10 mM 3-amino-1,2,4-triazole (3-AT) was added to the media to suppress background colonies resulting from leaky selection through the His marker. A β-Galactosidase overlay assay was done as described by the manufacturer's protocol for secondary screening. All yeast transformations and genetic manipulations mentioned above were performed as described earlier (Guthrie and Fink, 1991; Gietz et al., 1992).

Protein extract preparation, *in vitro* transcription and affinity purification

Arabidopsis leaves were homogenized according to Langland et al. (1995) with 50 mM Tris-Cl, pH7.5, 5 mM EDTA, 5 mM KCl, 5 mM DTT, 2 mM PMSF and 1x Protease inhibitor (Sigma, St.Louis, MO). S-100 fraction was dialyzed at 4°C in 20 mM Tris-Cl, pH 7.5, 10% (v/v) glycerol, 100 mM KCl, 5 mM $MnSO_4$, 5 mM DTT and 2 mM PMSF.

Unlabeled *F-(CAUU)$_6$Poly(A)* was transcribed *in vitro* using the RiboMAX™ kit (Promega, Madison, WI) as per the commercial protocol. *Cla* I cut F-$(CAUU)_6$Poly(A) plasmid template was used for *in vitro* transcription using T7 polymerase. The *in vitro* unlabeled transcript was purified with a Sephadex G-50 (Sigma) column and quantitated

by spectrophotometry.

The unlabeled transcript was covalently linked to CNBR-activated Sepharose 4B beads (Amersham, Piscataway, NJ) as described by the manufacturer. Affinity purification was done as described by Newman et al. (2000) with the following modifications. Three mL of covalently linked Sepharose was packed in columns. *Arabidopsis* protein extract (S-100 fraction) was diluted in an equal volume of buffer containing 40 mM HEPES, pH 7.0, 400 mM KCl, 6 mM $MgCl_2$, 1.5 mM DTT, 0.2 mg/mL tRNA, 10% (v/v) glycerol. The resulting mixture was added to the columns. Columns were washed with 10 mL DLG 200 (20 mM HEPES, pH 7.0, 200 mM KCl, 3 mM $MgCl_2$, 0.5 mM DTT, and 10% glycerol) and DLG 500 (same composition as DLG 200 buffer, but with 500 mM KCl). *Fed-1* RNA-binding proteins were eluted with 3.0 M and 1.0 M LiCl, 1 M urea and further dialyzed overnight at 4°C in dialysis buffer (20 mM Tris, pH 7.5, 10% glycerol 100 mM KCl, 5 mM $MnSO_4$, 5 mM DTT, 2 mM PMSF). Proteins were precipitated with 55% trichloroacetic acid and 2.2 % phosphotungstic acid as described by Yeang et al. (1995). Purified proteins were separated by SDS-PAGE and stained using Coomassie blue stain.

Over-expression and purification of GST fusion proteins

Cultures of YML145 strain with GST fusion plasmids were grown to mid-log phase in SDC(glucose)-Ura. Cells of 0.1 OD_{600}/mL were harvested and added to SDC(raffinose)-Ura and grown to an OD_{600} of 0.9 to 1.5. Galactose was added to a final concentration of 2% and the cultures were allowed to grow for 7 h. Cells were harvested, washed with water and used for protein purification.

Cells (80 mg wet weight) were resuspended in 500 μL of lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 10% glycerol, 1X Protease Inhibitor (Sigma), 1X Phosphatases (Sigma). 250 μL of glass beads was added, and the cells were lysed by vortexing for 2.5 minutes. The mixture was incubated at 4°C for 2 minutes and again vortexed for 2.5 minutes. The lysate was centrifuged for 5 minutes at 15,000 X *g*. The soluble fraction was removed, and the proteins were quantitated by Bio-Rad protein assay (Bio-Rad, Hercules, CA). 2.5 mg of the soluble protein fraction was gently mixed with washed immobilized glutathione beads (Pierce, Rockford, IL) for 1 hour. The beads were collected by centrifugation and washed three times with IP 150 (20 mM PIPES, 150 mM NaCl and 0.5% Tween 20) buffer. The GST fusion proteins were eluted from beads by incubating with 10 mM reduced glutathione in IP 150 buffer for 2 days at 4°C.

UV-crosslinking

In vitro analysis of protein binding by UV-crosslinking was done according to Alexander et al. (1998) with the following modifications. Radiolabeled RNA transcripts for UV-crosslinking were synthesized from pSKT7-F-$(CAUU)_6$ DNA (*Cla* I cut for sense and *Sst* I cut for antisense) template with RiboMAX™ kit (Promega) as per the commercial protocol. 1 to 5 μL of over-expressed purified protein was incubated with $1x10^6$ cpm of radiolabeled RNA in binding buffer (5 mM Tris-Cl, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 5 mM EDTA, 0.1 mM DTT, 1X Protease Inhibitor and 0.02 mM PMSF) for 20 minutes at 25°C. The opened microfuge tubes containing the RNA/protein mixture were covalently cross-linked with UV light for 10 minutes in a Stratalinker

(Stratagene, Heidelberg, Germany). Following RNase A and RNase T_1 treatment, proteins bound to radiolabeled RNA were resolved by 10% SDS-PAGE and electro-blotted onto a PVDF membranes (Amersham). The blotted membranes were exposed to Kodak X-OMAT Blue XB-1 films at -80 °C for 12 to 72 hr.

RESULTS

Identification of tobacco proteins that bind *Fed-1* 5' UTR by yeast three-hybrid assay

To identify proteins that may interact with the *Fed-1* mRNA 5' UTR, we utilized a yeast three-hybrid assay by using the commercially available RNA-Protein Hybrid Hunter Kit (Fig. 1). However, the system was designed for direct cloning and testing of a small number of specific mRNA binding proteins rather than for extensive library screening. Therefore, we modified the system to establish an inexpensive and efficient three-hybrid screen for *Fed-1* mRNA specific binding proteins produced from PCR-generated tobacco cDNA libraries. Advantageous modifications included the replacement of the zeocin selectable marker with a *Leu* selectable marker as well as co-transformation of the restriction enzyme digested prey plasmid with PCR amplified cDNAs generated with primers that allowed homologous recombination of the cDNA into the appropriate site of the prey plasmid. The plasmids for LexA-MS2 coat protein fusion (pHybLex/Leu-MS2) and hybrid *Fed-1* 5' UTR bait RNA (p3'Fed-1) were

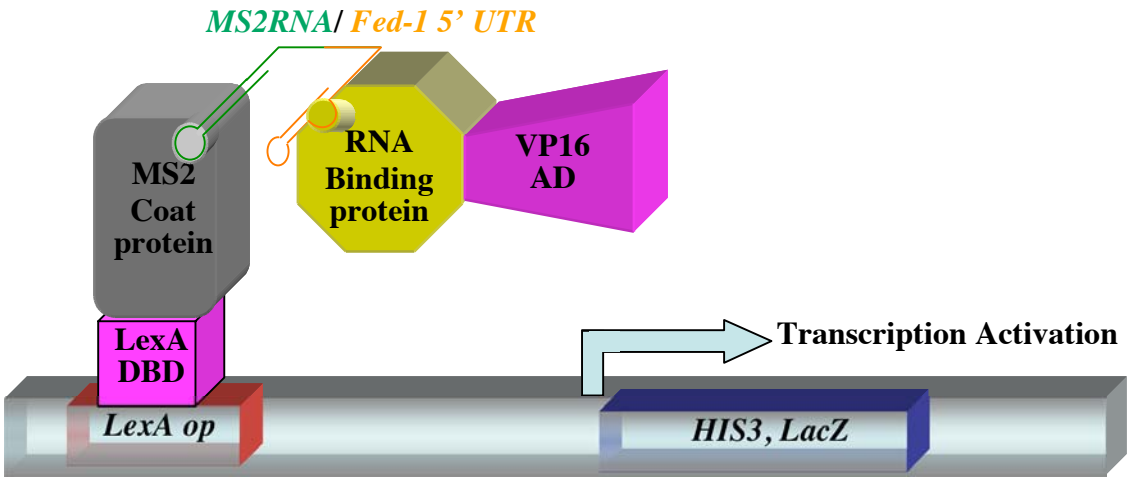

Figure 1. **Yeast Three-hybrid system constructs**

The yeast three-hybrid system (SenGupta et al., 1996) involves interaction between three components, LexA-MS2 coat protein fusion (pHybLex/Leu-MS2), *MS2-Fed-15' UTR* bait RNA (p3'Fed-1) and the VP16-cDNA fusion protein (pYESTrp3 + cDNA library), resulting in the activation of two reporter genes (*HIS3* and *LacZ*). The *HIS3* and *LacZ* reporter genes are integrated in the L40 *S.cerevisiae* strain under the control of LexA operator and the protein and RNA hybrids are encoded by separate plasmids. Adapted from Invitrogen RNA-Protein Hybrid Hunter™ Kit instruction manual.

introduced into L 40 yeast strain in two successive steps as described in the methods. Since the *Fed-1* hybrid RNA used in this system contained a poly U stretch (at nucleotide position -58 to -53), where yeast RNA polymerase III could terminate transcription, we mutated the sequence to UUCUU. The *F-(-55C)* construct containing this mutation showed wild-type light-regulated mRNA stability and polyribosome association when assayed into stably transformed tobacco (SB and MP, data not shown).

cDNA libraries prepared from mRNA isolated from light- and dark-grown tobacco leaves were used to screen for candidate *Fed-1* binding proteins. A total of 8×10^4 recombinant cDNAs were screened, of which 780 produced yeast transformants that grew in the primary selection on histidine deficient media in the presence of 10 mM 3-AT. From these we identified 12 positive clones that activated the expression of a secondary β-galactosidase reporter gene in a secondary screen (Fig. 2A). The cDNA inserts of the positive clones were sequenced and subsequent analysis showed that these encode ribosomal protein S2 (4 of the 12 clones, isolated from multiple cDNA libraries), 2-isopropylmalate synthase, glycine-rich protein, mucin-related protein, and a protein of unknown function. Three of the cDNA clones had no homologous sequences in the Entrez nucleotides database (clones #65, 421 and 734). However these cDNA inserts were much shorter in length, ranging from 200-250 bp, than the other positive cDNAs. Since first-strand synthesis of the cDNAs was primed from the polyA tail, these cDNAs are likely to be primarily the 3' UTR from the associated RNA. Opening reading frame analysis (MacVector) showed short stretches of amino acids (~30-50 aa) that when fused with the VP16 activating domain (AD) may fortuitously bind the bait mRNA.

The ribosomal protein S2 (RP S2) showed strong interaction with the *Fed-1* 5' UTR, similar to the positive control, *IRE*-IRP (iron-responsive element RNA interaction with the iron-responsive protein), in both growth rate on –His media and in the β-galactosidase screen (Fig. 2A). In addition, the RP S2 did not produce the $lacZ^+$ phenotype when transformed into yeast without the *Fed-1* RNA-expressing plasmid and also the *IRE* RNA-expressing plasmid (Fig. 2C and 2D), suggesting that it is a genuine positive which specifically interacts with the *Fed-1* 5' UTR RNA. We have previously shown that a 26 nt element of the *Fed-1* 5' UTR consisting of the $(CAUU)_4$ repeat and 10 nt adjacent upstream sequence is both necessary and sufficient for dark-induced light-regulated mRNA decay. RP S2 cDNA was isolated from the L40 yeast strain, amplified in *Escherichia coli* and re-introduced along with the activation domain (AD) plasmid into a yeast strain carrying *ΔCAUU* mutant (in which the $(CAUU)_4$ is deleted) and *USmut Fed-1* 5' UTR (in which the $(CAUU)_4$ upstream 10 nt are mutated) bait RNA. As shown in Figure 2D, RP S2 interacts with both of the mutant *Fed-1* 5' UTRs, suggesting that the $(CAUU)_4$ sequence or the upstream sequence may not be the binding site for this protein.

Isolation of *Fed-1* 5' UTR binding proteins by affinity purification

In addition to the three-hybrid assay, Angela Krueger utilized affinity purification and mass spectrometry to identify *Fed-1* mRNA binding proteins present in *Arabidopsis*. S-100 fractions from light-grown *Arabidopsis* plants were passed over a column containing *Fed-1* 5' UTR linked to CNBr-activated Sepharose beads via a 3' terminal poly(A) tail. The proteins that bound to *Fed-1* 5' UTR RNA were eluted by high salt washes from the column, separated by SDS-PAGE and revealed by Coomassie staining

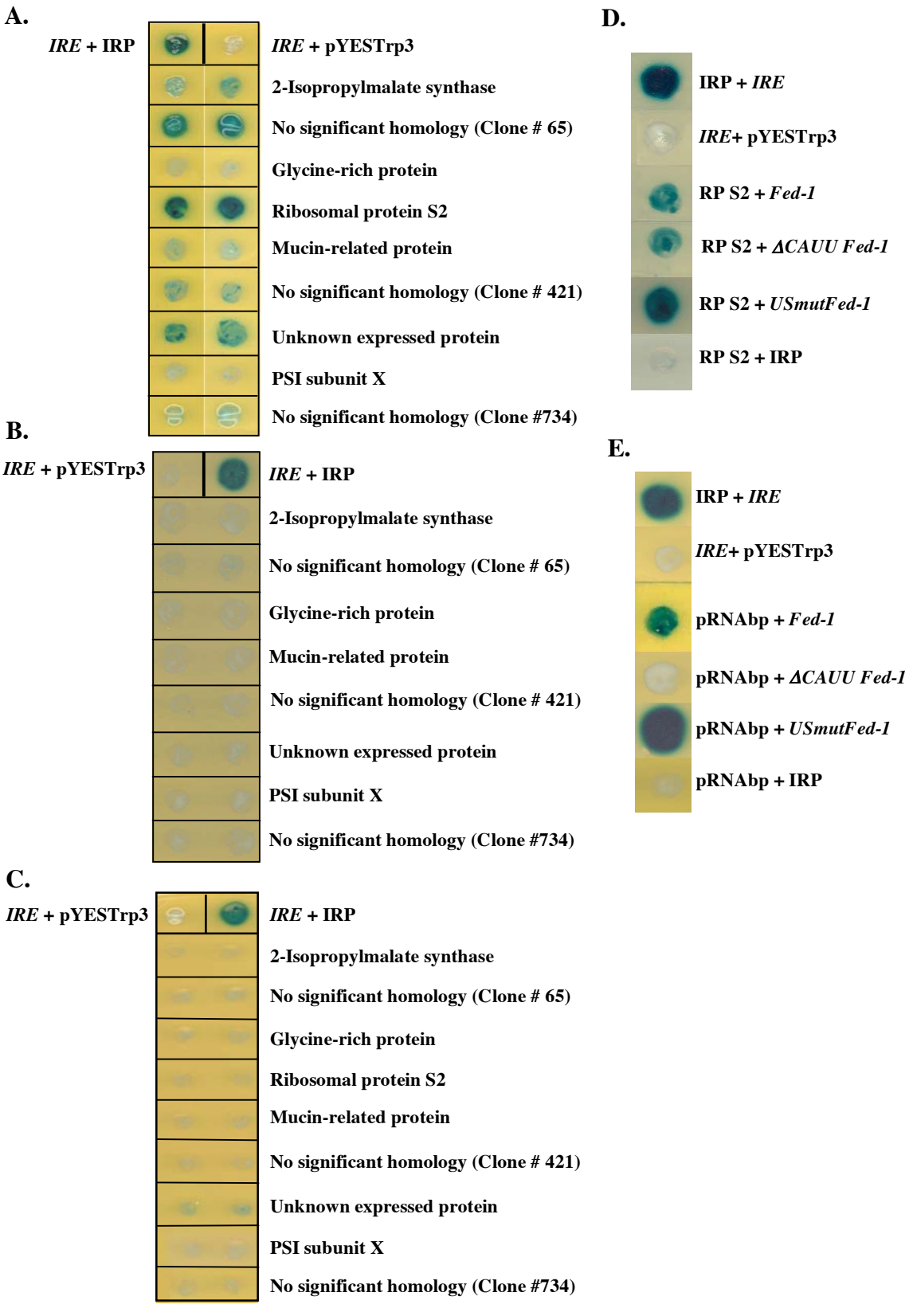
A.
IRE + IRP
IRE + pYESTrp3
2-Isopropylmalate synthase
No significant homology (Clone # 65)
Glycine-rich protein
Ribosomal protein S2
Mucin-related protein
No significant homology (Clone # 421)
Unknown expressed protein
PSI subunit X
No significant homology (Clone #734)
B.
IRE + pYESTrp3
IRE + IRP
2-Isopropylmalate synthase
No significant homology (Clone # 65)
Glycine-rich protein
Mucin-related protein
No significant homology (Clone # 421)
Unknown expressed protein
PSI subunit X
No significant homology (Clone #734)
C.
IRE + pYESTrp3
IRE + IRP
2-Isopropylmalate synthase
No significant homology (Clone # 65)
Glycine-rich protein
Ribosomal protein S2
Mucin-related protein
No significant homology (Clone # 421)
Unknown expressed protein
PSI subunit X
No significant homology (Clone #734)
D.
IRP + IRE
IRE+ pYESTrp3
RP S2 + Fed-1
RP S2 + ΔCAUU Fed-1
RP S2 + USmutFed-1
RP S2 + IRP
E.
IRP + IRE
IRE+ pYESTrp3
pRNAbp + Fed-1
pRNAbp + ΔCAUU Fed-1
pRNAbp + USmutFed-1
pRNAbp + IRP

Figure 2. **Identification of tobacco proteins that interact with *Fed-1* 5' UTR**

(A) Yeast three-hybrid screen (duplicates of X-gal overlay assay are shown) (**B**) Interaction of three-hybrid positives without *Fed-1* RNA (shown in duplicates) (**C**) Interaction of three-hybrid positives with *IRE* RNA (shown in duplicates) **(D)** Interaction of RP S2 with WT, *ΔCAUU* and *US mut Fed-1* 5' UTR **(E)** pRNAbp interaction with WT and *ΔCAUU* and *US mut Fed-1* mRNA 5' UTR in yeast three-hybrid assay. Tobacco cDNA library, RP S2 and pRNAbp cDNA were co-transformed with a plasmid encoding the transcriptional activation domain in a yeast strain (L40) expressing the MS2 RNA binding:LEXA DNA binding domain, *MS2:Fed-1* fusion mRNA or *MS2:ΔCAUU Fed-1* fusion mRNA, two reporter genes, *HIS3* and *LacZ*. Transformants were selected on plates lacking histidine to select for *HIS3* expression. Primary transformants were picked, patched onto plates and overlaid with X-Gal. Figure (A) represents yeast colonies that are positive for β-galactosidase activity at varying intensities. Iron-responsive element RNA (*IRE*) and the iron-responsive element protein (IRP) interaction (+ve control) is shown along with the interaction of *IRE* with only the pYESTrp3 plasmid (-ve control).

(Fig. 3). 1.0 M salt elution yielded three major bands at 50, 27 and 26 kDa. 3.0 M salt elution yielded major bands at 41, 26 and 15 kDa. All of these major bands were subjected to trypsin digestion and identified by MALDI-TOF mass spectrometry. Three bands (Fig. 3, bands1.a, 1.b and 3.e) showed significant sequence similarity to a probable RNA binding protein (pRNAbp) (At2g37220) of *Arabidopsis*. Bands at 41 and 15 kDa showed sequence similarity to an annexin-like protein (At5g10220) and a probable ribosomal protein L9 (At4g10450) respectively (Fig.3, bands 3.b and 3.h respectively). The 50 kDa band present in the 1.0 M salt eluate and not in the higher 3.0 M salt eluate was due to Rubisco contamination.

pRNAbp interacts with *Fed-1* 5' UTR in yeast three-hybrid assay

The open reading frame of the isolated full-length *pRNAbp*, along with the AD-plasmid was introduced into three different three-hybrid yeast strains, harboring *Fed-1*, the mutants *ΔCAUU* and *USmut Fed-1* hybrid bait RNA. The resulting transformants were selected on histidine deficient media for *HIS3* expression and the resulting primary transformants assayed for β-galactosidase expression. pRNAbp interacted with wild-type *Fed-1* 5' UTR resulting in formation of blue colonies in the presence of X-gal (Fig. 2E), suggesting *in vivo* interaction of pRNAbp with *Fed-1* 5' UTR. As shown in Fig 2E, pRNAbp also interacted with *USmut Fed-1* mutant. However, pRNAbp did not bind the *ΔCAUU* mutant *Fed-1* in the three-hybrid assay, suggesting that the $(CAUU)_4$ sequence is required for the binding.

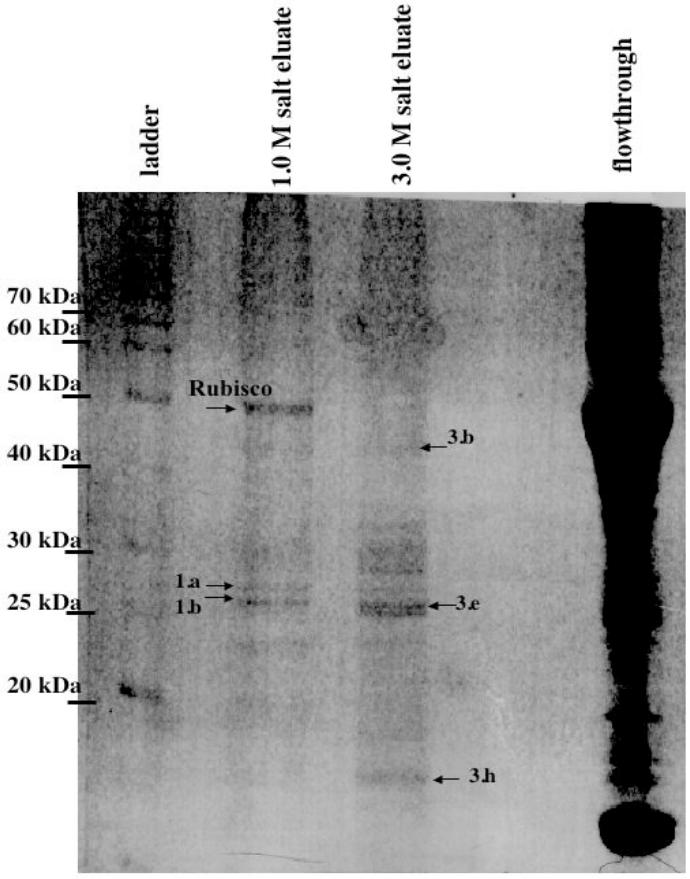

Figure 3. **Affinity purification of *Fed-1* 5' UTR binding proteins from *Arabidopsis***

S-100 fractions from light-grown *Arabidopsis* plants were applied to equilibrated affinity columns containing *Fed-1* 5' UTR covalently linked to Sepharose beads. The columns were thoroughly washed and Fed-1 binding proteins were eluted with 1.0 M and 3.0 M salt, separated by 10% SDS-PAGE and revealed by Coomassie staining. Shown here is a Coomassie-stained gel of the eluted proteins along with the flow-through. Arrows indicate major bands that were excised and identified by MALDI-TOF mass spectrometry. (Experiment performed by Angela Krueger)

RP S2 and pRNAbp interact with *Fed-1* 5' UTR *in vitro*

We have begun testing the *in vitro* binding activities of the putative *Fed-1* RNA binding proteins identified in our screen. Below, I present the most advanced studies detailing the interactions of RP S2 and pRNAbp with the *Fed-1* 5' UTR. To test if RP S2 and pRNAbp interact with the *Fed-1* mRNA 5' UTR *in vitro*, full-length cDNAs for both were isolated by RT-PCR, the proteins were over-expressed and purified from yeast as a GST fusion. The ^{32}P-labeled *Fed-1* 5' UTR transcribed in both sense and anti-sense orientations was UV-crosslinked with GST-fusion RP S2, pRNAbp and yeast septin (control) proteins. As shown in Figure 4, RP S2 and pRNAbp bound to both sense and anti-sense *Fed-1* with different affinities (Fig. 4A lanes 2 and 6 and Fig. 4B lanes 2 and 6 respectively). However, the control protein (yeast septin) did not show binding affinity to both sense and anti-sense *Fed-1* 5' UTR (data not shown). To test the specificity of binding, competition experiments were carried out with 1, 5 and 20x unlabeled sense competitor *Fed-1* mRNA with radiolabeled sense and 5 and 20x unlabeled anti-sense competitor *Fed-1* mRNA with radiolabeled anti-sense *Fed-1* mRNA. As shown by the UV-crosslinking assays with RP S2 (Fig. 4A), the presence of 5x unlabeled sense *Fed-1* resulted in a 15-fold reduction of the binding complex (Fig. 4A, lane 4) as indicated by the phosphoimager analysis. However, 5x unlabeled anti-sense *Fed-1* resulted only in a 2-fold reduction of the binding complex (Fig. 4A, lane 7). Competition assays with unlabeled sense *Fed-1* for pRNAbp (Fig. 4B) gave results similar to that of RP S2-*Fed-1* competition with sense competitor RNA. Presence of 5x unlabeled sense *Fed-1* resulted in a 6-fold reduction of the binding complex (Fig. 4B, lane 4). However, 5x anti-sense

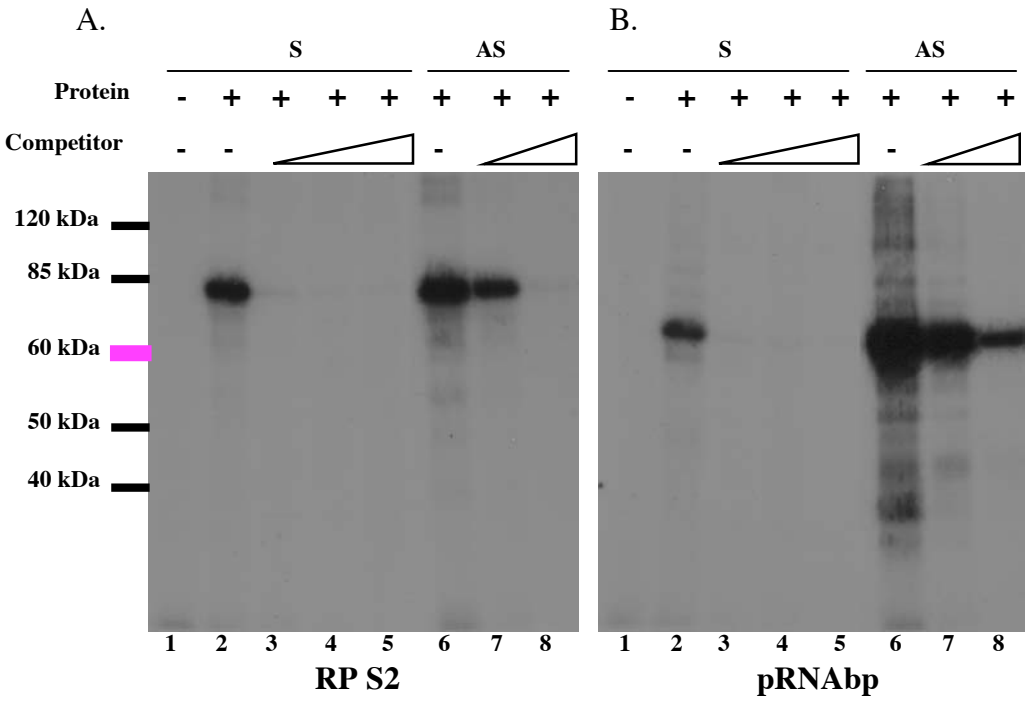

Figure 4. ***In vitro* binding of RP S2 and pRNAbp to *Fed-1* mRNA 5' UTR**

A. RP S2 and **B.** pRNAbp GST-fusion proteins were UV-crosslinked to ^{32}P-labeled *Fed-1* mRNA 5' UTR transcribed in the sense (S) or anti-sense (AS) orientation. Following UV-crosslinking, unlinked mRNA was digested with RNases, labeled proteins were resolved on 10% SDS-PAGE gels, blotted onto PVDF membranes and visualized by autoradiography. Lanes 3-5 and 7-8 in both figures (A) and (B) show competition binding experiments with unlabeled sense and anti-sense competitor *Fed-1* mRNA 5' UTR. Lanes 3-5 in both (A) and (B) are with 1, 5 and 20x sense competitor RNA. Lanes 7-8 in both (A) and (B) are with 5 and 20x anti-sense competitor RNA.

competitor resulted only in a 1-fold reduction of the binding complex (Fig. 4B, lane 7). These results suggest specific binding of RP S2 and pRNAbp to sense *Fed-1* 5' UTR.

DISCUSSION

In transgenic tobacco, pea *Fed-1* is regulated by photosynthetic electron transport at the level of both translation and mRNA stability (Petracek et al., 1997). Furthermore, we identified sequences within the *Fed-1* 5' UTR that are necessary and sufficient for light-regulated mRNA stability [Chapter II (Bhat et al., 2004)]. We hypothesized that along with regulatory *cis*-elements present within the *Fed-1* mRNA 5' UTR, certain protein factors might also be involved in *Fed-1* mRNA post-transcriptional regulation. In this chapter, we utilized yeast three-hybrid and affinity purification methods to identify proteins that interact with *Fed-1* 5' UTR. With the yeast three-hybrid assay we identified several proteins from tobacco that appear to interact with the *Fed-1* mRNA 5' UTR. One of the strongly interacting proteins showed sequence similarity to ribosome-associated protein, RP S2. It has been previously shown that this protein is an accessory component of the ribosomes and is preferentially associated with the ribosomes during plant tissue growth and development (Garcia-Hernandez et al., 1996).

We also identified a glycine-rich protein (GRP) that interacted with *Fed-1* 5' UTR in the three-hybrid assay. This protein contains glycine-rich amino-acid sequence, with seven GGGY repeats and three RGG repeats. Both of these repeats have been documented to be RNA-binding sequences (Sachetto-Martins et al., 2000; Landsberger et al., 2002). In addition the expression of GRP genes have shown to be regulated by various biotic and abiotic factors. Molina et al. (1997) have shown that the barley

glycine rich protein-3 (*grp-3*) expression is also light/dark modulated. We have isolated full-length cDNA for the tobacco GRP protein, over-expressed the protein as a GST-fusion in yeast and are currently performing *in vitro* binding assays with *Fed-1* 5' UTR. Three of the cDNA clones identified to interact with *Fed-1* 5' UTR in the three-hybrid assay, of which one (Fig. 2A, #65) showed a strong interaction, were short inserts and had no similarity to any of the known sequences in the database. This suggests that these cDNA inserts might be parts of novel genes encoding RNA-binding proteins or that fortuitous short AA sequences bound the RNA somewhat. The proteins that showed strong interaction with the *Fed-1* RNA, do not show interaction without the *Fed*-1 RNA and *IRE* RNA in this assay (Fig. 2B and 2C respectively). The rest of the proteins identified in yeast-three hybrid system, excluding RP S2 await further analysis.

Affinity purification of *Fed-1* 5' UTR binding proteins from *Arabidopsis* resulted in the identification of three candidate proteins, including a previously uncharacterized probable RNA-binding protein that contains two RNA recognition motifs. This protein also showed interaction with *Fed-1* 5' UTR in yeast-three hybrid system, suggesting that it specifically binds to *Fed-1* mRNA. A probable ribosomal protein L9 was also identified by affinity purification. It has been previously suggested that ribosomal proteins, in addition to their function as general translation factors, can also regulate expression of specific genes (Garcia-Hernandez et al., 1994; Garcia-Hernandez et al., 1996; Ma and Dooner, 2004)

In our assays we have used effective specific competitors to confirm the binding affinity. Although there is an increased binding complex with the anti-sense *Fed-1* for both proteins, it remains possible that there is a higher specific activity of the

radiolabeled anti-sense *Fed-1* than the sense. Competition with specific competitors suggests that the binding of both proteins is specific to sense *Fed-1* mRNA. However, to further characterize the protein binding, it is important to use mutant *Fed-1* mRNAs, a control RNA sequence and also non-specific competitors such as unlabeled *E. coli* or yeast mRNA. Since *Fed-1* mRNA is differentially regulated by light, we hypothesize that the protein factor might preferentially bind *Fed-1* mRNA under different conditions (light or dark). In such a case, expression of the mRNA encoding the binding protein might also be regulated by light. Northern blot analyses did not show differential accumulation of both tobacco *RP S2* and *Arabidopsis pRNAbp* mRNA in the light and the dark (data not shown), suggesting that the genes for these RNA binding proteins are not light responsive. However, it is possible that these proteins might be a part of a complex or may recruit additional sequence specific protein(s) to *Fed-1* mRNA and these protein(s) may be responsible for light-regulated *Fed-1* mRNA translation and stability.

The data discussed in this chapter are the results of preliminary experiments. Further analyses, specifically to assay for the interactions of three-hybrid positives with mutant *Fed-1* RNAs, UV-crosslinking assays with mutant *Fed-1* mRNAs, control RNA and competition assays with non-specific RNA will be done prior to preparation of a manuscript for publication.

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CHAPTER 5

SUMMARY

Both nuclear- and chloroplast-encoded photosynthetic genes are modulated by light. This modulation occurs at multiple levels, transcriptional or post-transcriptional including mRNA stability, translation and post-translational modifications. Many chloroplast-encoded genes are regulated by light primarily post-transcriptionally [reviewed in (Bruick and Mayfield, 1999; Rochaix, 2001)]. However, post-transcriptional control at the level of mRNA stability and translation initiation also plays an important role for regulating some of the nuclear genes that encode chloroplast proteins [reviewed in (Bailey-Serres, 1999; Petracek and Thompson, 2000)]. Nuclear-encoded pea *Ferredoxin-1* (*Fed-1*) mRNA has been our model to study the post-transcriptional light regulation mechanism. The work presented here starts with the characterization of *cis*-element(s) required for light-regulated *Fed-1*mRNA instability. Other plant mRNAs that could be regulated in response to photosynthetic changes are identified. Putative protein factors that interact with *Fed-1* mRNA are also identified.

Characterization of Fed-1 mRNA instability element

Previously it has been shown that site-specific mutations of the $(CAUU)_4$ repeat abolished light-regulated *Fed-1* mRNA abundance (Dickey et al., 1998). Here we

present evidence that the $(CAUU)_4$ repeat is necessary for the differential stability of *Fed-1* mRNA in light and in the dark. Three CAUU repeats are sufficient for full light regulation of *Fed-1* mRNA accumulation and an increase in the number of CAUU units significantly increases the light:dark mRNA accumulation ratio compared to wild-type *Fed-1* mRNA. Mutation of all other sequences within the *Fed-1* 5' UTR including that are adjacent to the $(CAUU)_4$ repeat do not alter *Fed-1* light-regulated mRNA accumulation significantly, suggesting these sequences are not a part of the *Fed-1* mRNA instability element. Furthermore, 26 nucleotides of the *Fed-1* 5' UTR consisting of the $(CAUU)_4$ repeat and 10 adjacent upstream nucleotides are sufficient to confer three-fold light regulation of mRNA accumulation to a non-light-responsive plant mRNA.

A $(CAUA)_4$ mutation of the $(CAUU)_4$ repeat that significantly changes the light-regulated *Fed-1* mRNA instability also alters the *in vitro* structure of this region. This observation implies that the structural changes in this region of *Fed-1* might be important for the interaction with a *trans*-acting factor. In addition, the position of $(CAUU)_4$ repeat near the 5' end of the *Fed-1* 5' UTR is important for its function, suggesting that the interaction with the *trans*-factors is localized to the 5' end of *Fed-1* mRNA. Most of the eukaryotic mRNA instability elements described until recently are present in the 3' UTRs. Also the major pathway for mRNA degradation in yeast and mammals is initiated by the 3' deadenylation of the mRNA followed by decapping and nucleolytic degradation. However, there is evidence for plant mRNA decay initiated by decapping and endonucleolytic cleavage (Higgs and Colbert, 1994). Since the dark specific $(CAUU)_4$ instability element of *Fed-1* mRNA is present in the 5' UTR, it seems likely that the *Fed-1* mRNA decay involving the $(CAUU)_4$ element, is initiated by a pathway

other than deadenylation such as decapping and 5' to 3' nucleolytic degradation or endonucleolytic cleavage in this region.

Fed-1 mRNA 5' UTR cis-elements for light regulation of mRNA stability and translation are separable.

Dependence of mRNA stability on translation has been shown for many prokaryotic and eukaryotic mRNAs [reviewed in (Ross, 1995; Abler and Green, 1996; Jacobson and Peltz, 1996)]. For *Fed-1* mRNA, it has been previously shown that the mRNA decay requires an active translation (Dickey et al., 1994; Petracek et al., 2000). Mutation of the $(CAUU)_4$ repeat alone significantly disrupts light regulation of *Fed-1* mRNA stability and does not affect the dark-induced polyribosome dissociation (Dickey et al., 1998; Bhat et al., 2004). Conversely, mutation of the sequences upstream and downstream of the $(CAUU)_4$ repeat disrupts the dark-induced polyribosome dissociation but not the differential regulation of *Fed-1* mRNA abundance. First, these data imply that the *Fed-1* mRNA instability and translational control *cis*-elements are separate and the sequences upstream and downstream of the $(CAUU)_4$ repeat play a role in *Fed-1* translational regulation. Second, these mutant *Fed-1* mRNAs may be degraded by a dark-responsive *trans*-acting factor(s) that interacts with the $(CAUU)_4$ element, even though these mRNAs are actively translated in the dark.

Fed-1 mRNA 5' UTR binding proteins

Little is known about the *trans*-acting factors involved in post-transcriptional regulation of plant mRNAs. Much of the work done over the past decade have identified

nucleus-encoded proteins interacting with chloroplast-encoded photosynthetic mRNAs (Danon and Mayfield, 1991; Nickelsen et al., 1994; Alexander et al., 1998; Vaistij et al., 2000). However, the protein factor(s) involved in light-regulation of many nuclear-encoded mRNAs is still unknown and our study is likely to provide some clues about the regulation and possible signaling pathways.

Yeast three-hybrid and affinity purification assays were utilized to identify and isolate proteins that interact with *Fed-1* mRNA 5' UTR. Nine tobacco proteins with the three-hybrid system and three *Arabidopsis* proteins with affinity purification assays were identified to interact with *Fed-1* 5' UTR. Two of these proteins, tobacco ribosomal protein S2 (*RP S2*) and *Arabidopsis* probable RNA binding protein (pRNAbp), were further characterized for their specificity to bind *Fed-1* 5' UTR in vitro. Both RP S2 and pRNAbp bind sense and antisense *Fed-1* mRNA with different affinities. Tobacco glycine-rich protein (GRP) identified to interact with *Fed-1 5'* UTR with the three-hybrid system contains RNA-binding domains and is yet to be further characterized. The identification of ribosomal proteins binding to *Fed-1* 5' UTR provides insights for the role of these proteins in post-transcriptional regulation of gene expression.

Conclusion

To adapt to a changing environment, plants have to carefully regulate the expression of several genes. This regulation occurs at multiple levels in response to various stimuli including light, temperature, sugar and water. We have identified many tobacco endogenous mRNAs that are regulated by photosynthesis through transcriptional, post-transcriptional and/or translational mechanisms (Tang et al., 2003). In addition, the

data presented in this thesis focuses on light regulation of *Fed-1* mRNA. We have thoroughly analyzed the *cis*-elements responsible for dark induced *Fed-1* mRNA instability. The $(CAUU)_4$ element is clearly necessary for *Fed-1* mRNA destabilization in the dark (Bhat et al., 2004). However, the decay mechanism of *Fed-1* mRNA and how this $(CAUU)_4$ element functions in the *Fed-1* mRNA decay still remains unclear. In an effort to understand the light regulated *Fed-1* mRNA decay, we have identified a few proteins that may interact with *Fed-1* 5' UTR. However we do not know if these proteins are involved in light-regulated *Fed-1* mRNA stability and/or translation *in vivo*. Silencing and overexpression studies of the genes for these proteins in plants will be important for a complete understanding of the *Fed-1* light-regulation mechanism.

Our work is likely to enhance the knowledge of plant post-transcriptional gene regulation. This study adds a novel dark-induced instability element to the known array of mRNA stability determinants. Although the presence of the $(CAUU)_4$ repeat is unique to *Fed-1*, my work showing that mutations of the repeat, or shorter versions of the repeat retain some degree of dark-induced mRNA instability opens the possibility that similar sequences may be present in other mRNAs, where they may function as light responsive destabilizing elements.

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VITA

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Light regulates translation and stability of pea *Ferredoxin-1* (*Fed-1*) mRNA in transgenic tobacco plants. Previous work showed that mutation of a $(CAUU)_4$ repeat in the *Fed-1* 5' UTR abolished light regulated *Fed-1* mRNA accumulation in transgenic tobacco. Using the tetracycline repressible promoter system we show that the $(CAUU)_4$ repeat is necessary for dark induced destabilization of *Fed-1* mRNA. Substitution of single nucleotides within this repeat had dramatically different effects on mRNA accumulation. A minimal sequence of $(CAUU)_3$ is required for destabilization of *Fed-1* mRNA in the dark. However, sequences surrounding the $(CAUU)_4$ element are not required for light regulated *Fed-1* mRNA accumulation. Furthermore, 26 nt of the 5' UTR, including the $(CAUU)_4$ repeat and 10 nt upstream sequence are sufficient to confer a significant ~2.5-fold light regulation of mRNA accumulation when fused to a non-light-responsive plant mRNA. Significantly, dark-induced *Fed-1* mRNA instability does not require *Fed-1* mRNA polyribosome dissociation, and thus is an independent dark-regulated event. Finally, we have identified two proteins, ribosomal protein S2 (RP S2) and a probable RNA binding protein (pRNAbp) that bind *Fed-1* 5' UTR. UV-crosslinking assays reveal differential binding affinity of these proteins to sense and antisense *Fed-1* mRNA.

Advisor's Approval Dr. Ulrich. K Melcher