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Light Regulation of *Fed-1* mRNA Requires an Element in the 5′ Untranslated Region and Correlates with Differential Polyribosome Association

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Light regulation of *Fed-1* mRNA abundance in the leaves of green plants is primarily a post-transcriptional process. Previously, we have shown that the *Fed-1* mRNA light response requires an open reading frame, indicating that the light regulation of the mRNA depends on its concurrent translation. We now show that light-induced increases in *Fed-1* mRNA abundance are associated with increases in polyribosome association that require both a functional AUG and a normal *Fed-1* translational start context. We also present evidence that light regulation of *Fed-1* mRNA levels requires more than efficient translation per se. Substitution of the efficiently translated tobacco mosaic virus Ω 5′ untranslated region resulted in a loss of *Fed-1* light regulation. In addition, we identified a CATT repeat element located near the 5′ terminus of the *Fed-1* 5′ untranslated region that is essential for light regulation. We introduced two different mutations in the CATT repeat element, but only one of these substitutions blocked the normal light effect on polyribosome association, whereas both altered dark-induced *Fed-1* mRNA disappearance. The element may thus be important for *Fed-1* mRNA stability rather than polyribosome loading. We propose a model in which *Fed-1* mRNA is relatively stable when it is associated with polyribosomes in illuminated plants but in darkness is not polyribosome associated and is thus rapidly degraded by a process involving the CATT repeat element.

INTRODUCTION

Fed-1 is a single-copy, intronless gene from pea that encodes the photosynthetic electron transport protein ferredoxin I. Like other nuclear genes that encode chloroplast proteins, *Fed-1* produces an mRNA that is translated in the cytoplasm. The resulting protein is transported into the chloroplast, where it functions to transfer electrons from photosystem I to $NADP^+$. In both pea and transgenic tobacco plants, *Fed-1* mRNA and protein levels are higher in the light than they are in darkness. Interestingly, however, the light-induced increase in mRNA abundance that occurs in green leaves (as opposed to etiolated seedlings) is mediated mainly by an internal light-regulatory element rather than by the promoter (Elliott et al., 1989a; Gallo-Meagher et al., 1992). We have localized this internal element, referred to as the iLRE, to a fragment that includes the 5′ untranslated region (UTR) and a portion of the coding sequence (Dickey et al., 1992b). This fragment confers positive light responsiveness when fused to chloramphenicol acetyltransferase (*CAT*), β-glucuronidase (*GusA*), or luciferase (*LUC*) reporter mRNAs.

Fed-1 mRNA abundance in the leaves of green plants is regulated by a post-transcriptional process. In dark-adapted transgenic leaves, mRNA from cauliflower mosaic virus 35S::*Fed-1* constructs increases when reilluminated, but there is no accompanying increase in run-on transcription (Dickey et al., 1992a). We have also shown that *Fed-1* mRNA requires an open reading frame (ORF) to be light responsive (Dickey et al., 1994), indicating that the light effect on *Fed-1* mRNA depends on its concurrent translation. In this study, we define sequences in the 5′ portion of *Fed-1* mRNA that are required for iLRE function and further examine the role of translation in altering mRNA abundance. We show that mutations abolishing light regulation also affect translation, as measured by polyribosome association of *Fed-1* mRNA. In addition, we identify a CATT repeat element near the 5′ terminus of the mRNA and show that it is necessary for decreased levels of *Fed-1* mRNA in darkness. These findings lead us to propose a model in which *Fed-1* mRNA stability is modulated by its translational status.

RESULTS

The Minimal *Fed-1* iLRE

Previously, we defined a *Fed-1* light-regulatory fragment that included the 5′ UTR and the first third of the coding

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region (Dickey et al., 1992b). To further define the *Fed-1* iLRE, we tested a series of constructs in which increasing amounts of *Fed-1* coding sequence were fused to the *CAT* coding region (Figure 1). Our standard assay for light regulation (Dickey et al., 1992b) involved dark-adapting clonally identical pairs of transgenic tobacco plants from each independent primary transformant (four to nine plant pairs for each construct) and then reilluminating one plant from each pair. mRNA levels in expanded leaves of light- and dark-treated plants were then compared for each pair. Results of these pairwise comparisons are expressed as light-to-dark ratios (L/D) to avoid complications associated with variations in absolute expression levels among independent transformants. A ratio of one indicates no light response, whereas normally responsive *Fed-1* control constructs give ratios between three and five.

The 35S::*CAT* gene alone showed only a slight light response (L/D = 1.8 ± 0.3), and no further effect was seen when the *CAT* 5′ UTR was replaced by the *Fed-1* 5′ UTR alone (L/D = 1.6 ± 0.3). However, Figure 1 shows that increasingly strong light responses were seen when increasing amounts of the *Fed-1* coding sequence were included along with the *Fed-1* 5′ UTR. The *Fed-1* 5′ UTR together with the first 13 *Fed-1* codons conferred a significant light response in F136::*CAT* mRNA (2.7 ± 0.3), whereas the addition of more coding sequence in F158::*CAT* and F199::*CAT* (containing 21 and 34 codons, respectively) resulted in a gradual increase in light responsiveness. A response similar to that of wild-type *Fed-1* mRNA was observed with the F236::*CAT* transgene, which contains 47 codons.

Translation Must Initiate at the *Fed-1* AUG Codon

Previously, we found that *Fed-1* light regulation required an ORF (Dickey et al., 1994). When the *Fed-1* translation start codon was mutated to a missense mutation (AUA) or a nonsense mutation (UAA), *Fed-1* mRNA levels were no longer light regulated. To determine whether light regulation requires translation initiation specifically at the *Fed-1* start or whether initiation at a downstream ORF would suffice, we mutated the *Fed-1* start codon to a missense codon (AUA) in the F158::*CAT* and F199::*CAT* constructs. As shown in Figure 1, the light response of the Fed::*CAT* mRNA was abolished in plants containing either the F158MS1::*CAT* or F199MS1::*CAT* transgene.

We next asked whether the chimeric mRNAs with a mutated (missense) *Fed-1* start codon were translated as well as their unmutated counterparts. As shown in Figure 2, however, these missense mRNAs were not efficiently loaded onto polyribosomes. The control mRNAs (F158::*CAT* and F199::*CAT*) were found primarily in polyribosomal fractions in extracts from illuminated plants but not in extracts from dark-treated plants. This pattern is very similar to that of unmodified *Fed-1* mRNA (Petracek et al., 1997; Figure 3). In contrast, only a small fraction of the *Fed-1* missense mRNA (either F158MS1::*CAT* or F199MS1::*CAT*) was associated with polyribosomes, even in the light. Thus, even though CAT protein synthesis could be detected, the loss of light regulation in the missense-containing constructs was accompanied by a dramatic decrease in polyribosome association. These results are consistent with the hypothesis that

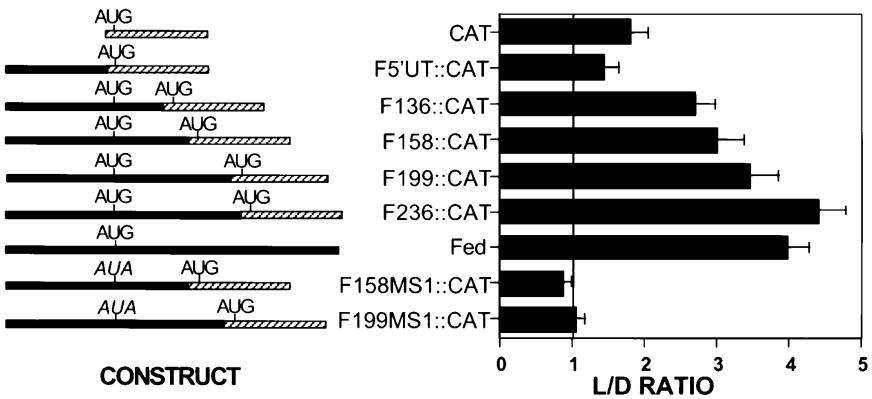

Figure 1. Light Response Data for *Fed-1*::*CAT* Fusions.

The *Fed-1* 5′ UTR (90 bp) plus increasing segments of the coding region, shown as filled boxes, were fused in-frame with the *CAT* coding region, shown as hatched bars, and the nopaline synthase 3′ UTR under the control of the cauliflower mosaic virus 35S promoter. The length (in base pairs) of the *Fed-1* region is denoted by the number after F in the construct name. The light response is shown as the ratio of mRNA levels in the light to mRNA levels in the dark (L/D Ratio). 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. From four to nine independent transformants were analyzed for each construct.

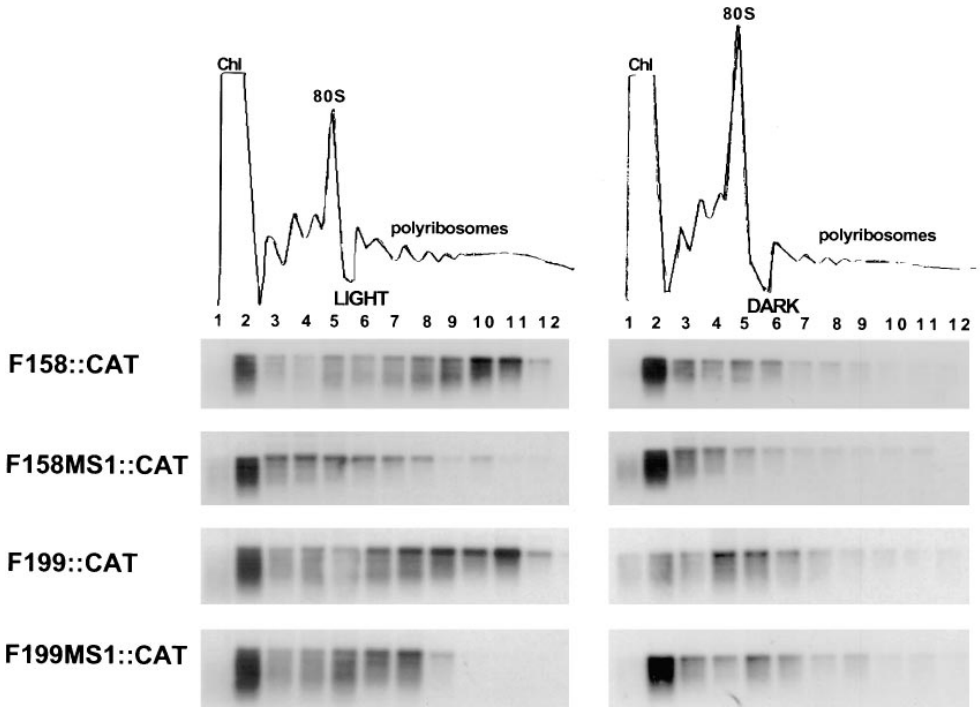

Figure 2. Polyribosome Profiles of Fed::*CAT* mRNAs in Light- and Dark-Treated Plants.

RNAs from each fraction of a sucrose gradient were resolved by gel electrophoresis, blotted, and probed with antisense ^{32}P-labeled RNA to the *CAT* coding region. Each polyribosome hybridization profile is representative of at least three separate experiments. The polyribosome 254-nm UV tracings at the top were collected from light- and dark-treated samples, respectively. Chl indicates a UV absorbance peak resulting from the chlorophyll layer at the top of the gradient; 80S and polyribosomes refer to the position of monoribosomes and polyribosomes in the gradient. The UV tracings are positioned to indicate the gradient positions of the various fractions analyzed on RNA gel blots. Fractions are numbered from the top to the bottom of the gradient. Note that the amount of RNA between gradients cannot be quantitatively compared because of variations in the quantity of tissue and grinding efficiency.

efficient translation and a high degree of polyribosome association are required for light regulation of *Fed-1* mRNA abundance.

The *Fed-1* 5′ UTR Is Not Simply a Translational Enhancer

The preceding results raise the possibility that the *Fed-1* 5′ UTR may function in light regulation by facilitating ribosome loading in light-treated plants rather than by providing specific light-regulatory elements. If so, it should be possible to replace the *Fed-1* 5′ UTR with another 5′ UTR that supports efficient translation without affecting the ability of the mRNA to respond to light treatments.

We previously observed that light regulation was lost when the *Fed-1* 5′ UTR was replaced by the bacterial *GusA* 5′ UTR (Dickey et al., 1992b). However, the *GusA* 5′ UTR does not support efficient translation in plants (Marcotte et al., 1989; Helliwell and Gray, 1995). To test whether light regulation is retained when the *Fed-1* 5′ UTR is replaced by an efficiently translated 5′ UTR, we substituted a 5′ UTR from the tobacco mosaic virus RNA, commonly referred to as Ω, for the *Fed-1* 5′ UTR. The Ω 5′ UTR is often used as a "translational enhancer" in overexpression studies (Gallie et al., 1987, 1989; Gallie and Walbot, 1992). However, Ω did not confer light responsiveness to the Ω::*Fed* transcript in transgenic plants (Figure 3).

To assess the translational status of Ω::*Fed* mRNA, we examined polyribosome association. The Ω::*Fed* mRNA was polyribosome associated in light-treated plants, as it was in wild-type *Fed-1* (Figure 3). However, in dark-treated plants, the majority of Ω::*Fed* mRNA was still associated with polyribosomes, even though the bulk of total polyribosomes, as assessed by the absorbance of the fractions at 254 nm, was reduced in 40-hr dark-treated plants (Petracek et al., 1997). This distribution of Ω::*Fed* mRNA contrasts sharply with that of wild-type *Fed-1* mRNA, most of which was not polyribosome associated in the dark. We conclude that the

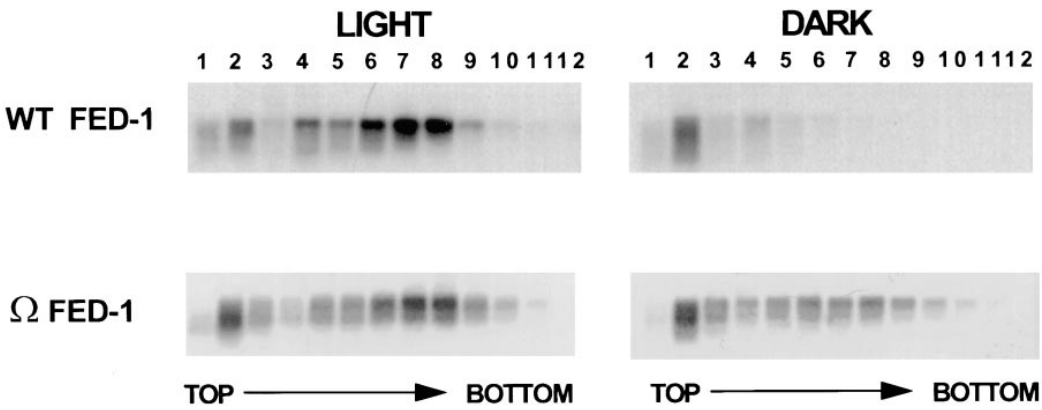

Figure 3. Polyribosome Association of Ω::*Fed* mRNA in Light- and Dark-Treated Plants.

RNAs were collected from a sucrose gradient, as described in Figure 2 and Methods, except that the gel blots were probed with ^{32}P-labeled *Fed-1* antisense RNA. Each polyribosome hybridization profile presented is representative of at least three separate experiments. Note that the amount of RNA between gradients cannot be quantitatively compared because of variations in the quantity of tissue and grinding efficiency. WT, wild type. Fractions are numbered from top to bottom of the gradient.

Fed-1 5′ UTR must function as more than just a simple translational enhancer because it confers differential rather than constitutive polyribosome association.

Mutational Analysis

To locate discrete *Fed-1* elements that may be involved in light regulation, we made a series of site-specific mutations. These mutations were made in the context of the intact *Fed-1* mRNA, which covers almost the entire sequence represented in F136, the minimal sequence required for light regulation (see Figure 1). This region includes the 5′ UTR plus the first 14 codons of the *Fed-1* ORF (Figure 4). Our previous work shows that nucleotides 1 to 18 of the 5′ UTR sequence are not necessary for light responsiveness (Dickey et al., 1992b). Thus, we made site-specific mutations beginning at nucleotide position +14 (+1 being the presumptive start of transcription, as shown in Elliott et al. [1989b]).

Between positions +16 and +31 of the 5′ UTR (64 nucleotides from the start of translation), the tetranucleotide sequence CATT is repeated four times, creating a 16-nucleotide element that we refer to as the CATT repeat. We mutated this region in two different ways. The 14.1 mutation substituted 20 bp of an 85% GC sequence for the CATT repeat, whereas the 16.1 mutation substituted 16 bp of a 37% GC sequence. In both cases, *Fed-1* mRNA levels were no longer light regulated (Figure 4, constructs 14.1 and 16.1). Thus, the CATT repeat region of the *Fed-1* 5′ UTR is necessary for light regulation.

We next asked whether these CATT repeat mutations also block the light effect on polyribosome loading. Figure 5 shows that the GC-rich mutation (14.1) largely prevented loading of the *Fed-1* mRNA onto ribosomes. The majority of this mRNA was found in the nonpolyribosomal fractions of both light- and dark-treated plants, perhaps because the relatively GC-rich sequence of this substitution forms secondary structures that block ribosome binding (Laso et al., 1993; Linz et al., 1997). The inability of this mRNA to load onto polyribosomes distinguishes it from wild-type *Fed-1* mRNA, which is located predominantly in the polyribosomal fraction in extracts from light-grown plants.

In sharp contrast, mRNA from plants transformed with transgenes containing the 16.1 mutation was found mostly in polyribosomes in light-treated plants. Much less was in the polyribosomes of dark-treated plants, as is the case for wild-type *Fed-1* mRNA (Figure 5). Thus, the 16.1 mutation (in which the substituted sequence has a GC content of 37%) prevented the light effect on mRNA abundance without affecting ribosomal loading patterns. We conclude that differential polyribosome loading per se is not sufficient to cause changes in mRNA abundance. In addition, because alterations in the CATT repeat can block light effects on mRNA abundance without altering its polyribosome association, we suggest the CATT region has an independent function distinct from any effects it might have on polyribosome formation.

We asked whether the mutations of the CATT repeat changed the absolute levels of *Fed-1* mRNA from wild-type levels in the light or dark. Seedlings from independent transformants containing the wild-type *Fed-1*, 14.1, and 16.1 constructs were dark adapted for 40 hr and either illuminated or kept in the dark for an additional 6 hr. Because the absolute expression of *Fed-1* mRNA varies among independent transformants, we pooled RNA from at least four inde-

pendent transformants per construct. Figure 5 shows that the relative levels of *Fed-1* mRNA in the light are constant in the wild-type and CATT mutant constructs (14.1 and 16.1). In contrast, the relative levels of CATT mutant *Fed-1* mRNA in the dark are significantly elevated by at least twofold, suggesting that the CATT repeat is important for reduction of *Fed-1* mRNA levels in the dark.

The rest of the *Fed-1* 5′ UTR was examined for discrete light-regulatory elements by using linker scanning mutagenesis, as shown in Figure 4. In construct 32, a 14-bp PstI linker was substituted for the *Fed-1* sequence starting at position +32, a region that is homologous to a sequence in the Arabidopsis *FedA* 5′ UTR (Caspar and Quail, 1993). In the remainder of the 5′ UTR and coding region, 10-bp PstI linker substitutions were made at the positions indicated in Figure 4. It should be noted that because these are substitution mutations, the reading frame was not changed in any of these constructs. None of the substitutions in the 5′ UTR had an effect on light responsiveness except for a slight reduction in construct 86 (substituted from positions +86 to +95). Because this portion of the 5′ UTR is immediately adjacent to the *Fed-1* translation start at position +96, construct 86 alters the translational start context.

Mutations of the *Fed-1* start codon are known to abolish light regulation of *Fed-1* mRNA levels (Dickey et al., 1994). As predicted, light regulation was lost when the *Fed-1* start codon was altered in construct 96, which contains a 10-bp linker substitution beginning at the start codon. Unexpectedly, however, light regulation was abolished in construct 106, which contains a substitution altering codons 5 through 7. Substitutions after the seventh codon (constructs 116 and 126, altering codons 8 through 14) had no effect on the light response. Thus, only very early in the coding region (codons 1 through 7) did the linker substitutions inhibit the light response.

DISCUSSION

The *Fed-1* system is the only example to date of a plant nuclear gene in which a regulated change in mRNA abundance requires translation of the affected mRNA. Our data are consistent with a model in which *Fed-1* mRNA is relatively stable when it is associated with polyribosomes, as is normal in illuminated plants, but rapidly degraded once it loses its association with polyribosomes, as it normally does in darkness. Recent data suggest a significantly longer half-life of *Fed-1* mRNA in light- versus dark-treated plants (M.E. Petracek, unpublished data). We propose that increased turnover in the dark is facilitated by the primary sequence or unstructured character of the CATT repeat near the terminus of the 5′ UTR. Decreased translation initiation in the dark could expose the CATT repeat region to nucleases, resulting in more rapid degradation of *Fed-1* mRNA. Increased polyribosome association in the light might provide direct protection for

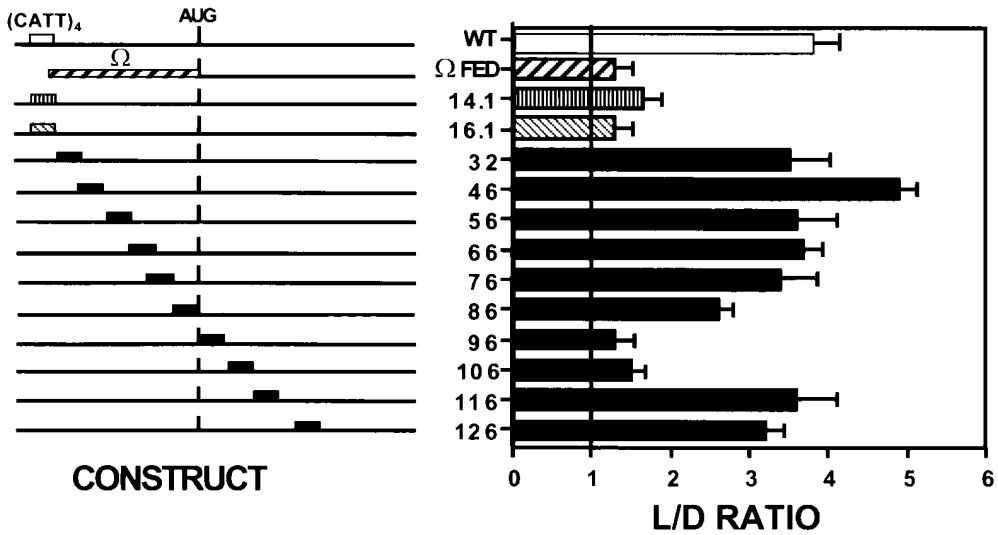

Figure 4. Effect of Various Mutations in the 5′ UTR and Early Coding Region on Light Induction of *Fed-1* mRNA.

Light responses of constructs containing the various mutations are plotted as light induction ratios (L/D). Included are the wild-type *Fed-1* (WT; see message construct in Elliott et al. [1989a]), a construct in which the tobacco mosaic virus Ω 5′ UTR replaces the *Fed-1* 5′ UTR (ΩFED), and two site-specific mutations of the CATT repeat region (14.1 and 16.1). Linker scanning mutations are indicated by the number of the first altered base (32 to 126). The vertical line at L/D = 1 represents zero light induction. Four to nine independent transformants were analyzed for each construct. All constructs are under the control of the cauliflower mosaic virus 35S promoter. Standard error of the mean is indicated by the error bars.

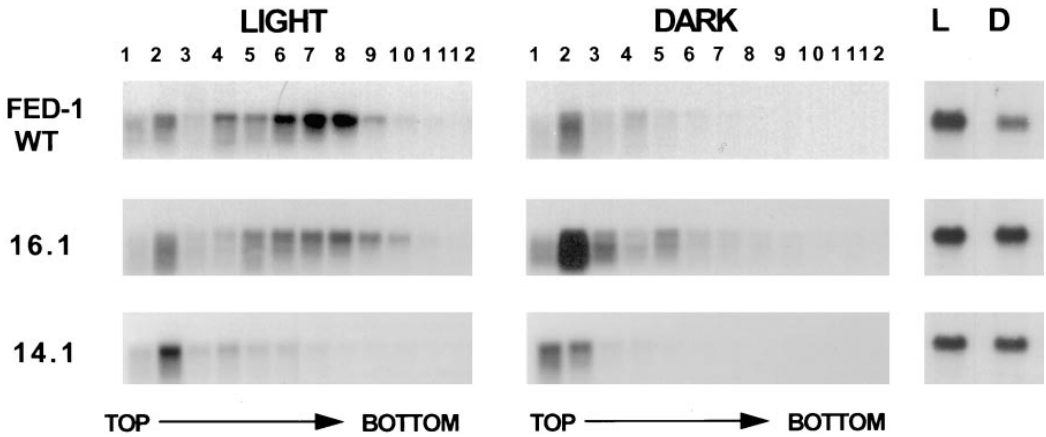

Figure 5. Polyribosome Association of *Fed-1* mRNA with Two Different Substitutions for the CATT Repeat Region in the 5′ UTR.

RNA was fractionated from a sucrose gradient, as described in Figure 2 and Methods, except that the RNA gel blots were probed with ^{32}P-labeled *Fed-1* antisense RNA. 14.1 and 16.1 denote the transgenes containing the CATT repeat region mutations. *Fed-1* wild type (WT) is taken from Figure 3 for relative comparison. Each polyribosome hybridization profile is representative of at least three separate experiments. Note that the amount of RNA between gradients cannot be quantitatively compared because of variations in the quantity of tissue and grinding efficiency. RNA gel blots of RNA extracted from plants containing wild-type *Fed-1*, the 14.1 mutation, or the 16.1 mutation are positioned to the right of the polyribosome hybridization profiles. Five micrograms of equally pooled RNAs from four to six independent transformants (T_2 seedlings grown as described for polyribosome experiments) was treated with 40 hr of darkness and 6 hr of light (L) or 46 hr of darkness (D). L/D ratios for the wild type, 16.1, and 14.1 are 3, 1.1, and 1.3, respectively. Fractions are numbered from top to bottom of the gradient.

translated mRNA or sequester it from the machinery that degrades nonpolyribosomal-associated *Fed-1* mRNA. Replacement of the *Fed-1* 5′ UTR with Ω 5′ UTR would result in the loss of differential translation in the light and dark as well as the nuclease-sensitive CATT repeat. Several aspects of this model are discussed in more detail below.

The Role of Translation

Fed-1 mRNA (Figures 3 to 5) or Fed::*CAT* fusion mRNAs (Figures 1 and 2) showing light-regulated changes in abundance also show light-regulated changes in polyribosome association. These mRNAs are mostly associated with the polyribosome fraction in illuminated plants, suggesting that they are being actively translated, whereas in dark-treated plants, mRNA abundance is reduced and the residual transcripts are mostly not associated with ribosomes.

Evidence that light-induced translation may be required for light effects on mRNA abundance comes from previous work in our laboratory as well as several findings presented here (Dickey et al., 1994). First, Fed::*CAT* mRNA levels become light insensitive when the *Fed-1* translation start codon is mutated to a missense codon. Even in the light, these transcripts showed only minimal association with polyribosomes, and neither the mRNA abundance nor the degree of polyribosome association was light regulated (Figures 1 and 2). We can measure significant levels of CAT protein in plants containing these constructs (L.F. Dickey and E.R. Hansen, unpublished data); thus, the *Fed-1* missense mutation does not completely prevent translation. We believe that there is a low level of initiation at the AUG of the *CAT* ORF but that the low efficiency of this process prevents extensive ribosome loading.

Second, we found that changes in the translational initiation context, as in the linker scanning mutation LS86, also affected light regulation (Figure 4). Altering this context, but not the AUG itself, would be expected to reduce translational efficiency. This hypothesis might also explain the fact that light regulation is abolished when the *Fed-1* 5′ UTR was fused directly to the *CAT* reporter gene (Figure 1) or to the *GusA* gene (Dickey et al., 1992b). These results indicate that some important feature of the *Fed-1* ORF involved in light regulation is lacking in these bacterial reporters. Whether this feature involves specific sequences or merely an optimal translation start and/or coding context is not known. According to our model, extensive ribosome loading is required to stabilize these mRNAs; in the absence of efficient loading, we predict the mRNA would be equally unstable in light and darkness.

In addition to showing that reducing translational initiation efficiency abolishes light regulation of the *Fed-1* mRNA, our data suggest that a reduction in translational elongation rates can also abolish light regulation of *Fed-1* mRNA. Constructs carrying decreasing amounts of *Fed-1* coding sequence show decreasing amounts of light responsiveness

(Figure 1). We speculate that constructs with more *Fed-1* codons are translated more efficiently in plants as a result of more optimal codon usage by *Fed-1* than by the bacterial *CAT* gene. In addition, our data suggest that *Fed-1* coding sequences are more important close to the start of translation, where ribosome stalling might have more of an effect on the rate of initiation.

Consistent with the idea that disruption of translational elongation could affect the light response, we found that introduction of rare codons in linker scanning mutation 106 also had an effect on light regulation of *Fed-1.* Both codon 5 (CCA, encoding Pro) and codon 7 (UUG, encoding Leu) were replaced with CUG (Leu), which is a rare codon in tobacco (see Wada et al., 1990). However, although the altered codons (5, 6, and 7) are not normally considered part of the initiation context, they are relatively close to the AUG codon, and it is possible that they influence the efficiency of ribosome loading rather than translational elongation. It is also possible that these two rare codons affect *Fed-1* mRNA levels in much the same way that nonsense mutations do (Dickey et al., 1994). Consistent with this hypothesis is the yeast model, in which rare codons decrease mRNA stability (Parker and Jacobson, 1990; Caponigro et al., 1993; Caponigro and Parker, 1996).

Although little is known about the effect of rare codons on mRNA stability in plants, it is known that codon usage must be optimized to obtain high-level expression of foreign proteins, such as the *Bacillus thuringiensis* toxin (Adang et al., 1993; reviewed in Diehn et al., 1996; Stewart et al., 1996) and the green fluorescent protein (Rouwendal et al., 1997). Recently, Van Hoof and Green (1997) reported that rare codons did not destabilize a transcript when inserted 41 codons away from the translational start codon. However, it remains unclear whether the rare codons would affect mRNA half-life if inserted closer to the start of translation. We are currently investigating whether the rare codons in linker scanning mutation 106 and other rare codons can destabilize plant mRNAs.

Importance of the CATT Region

Replacement of the *Fed-1* 5′ UTR with the Ω 5′ UTR resulted in loss of light regulation as well as differential polyribosome loading. Extensive polyribosome loading of Ω::*Fed* mRNA in the dark is consistent with our model, in which differential translation in light and darkness is required to produce light effects on *Fed-1* mRNA stability and thus abundance. However, it also indicates that one or more features of the *Fed-1* 5′ UTR other than just its ability to support efficient translational initiation is required for normal light regulation.

One feature of the *Fed-1* 5′ UTR that may be involved in light regulation is the CATT repeat element located between nucleotides 16 and 31. Two different substitution mutations in this region each blocked the normal light effect on mRNA abundance. Only one of these two substitutions, however, blocked the normal light effect on polyribosome formation. Therefore, we conclude that the CATT element does not merely facilitate polyribosome loading in the light or unloading in the dark but that it may be a direct stability determinant.

Many studies have indicated that mRNA translation and turnover are intimately connected (reviewed in Gallie, 1993; Abler and Green, 1996; Jacobson and Peltz, 1996). Many instability elements function only if the mRNA containing them is being translated (Parker and Jacobson, 1990; reviewed in Peltz et al., 1991; Savant-Bhonsale and Cleveland, 1992; reviewed in Ross, 1995), leading to a negative correlation between translational activity and mRNA stability. Virtually all known elements of this type are located in coding or 3′ UTRs (Peltz et al., 1991; Gallie, 1993; Sachs, 1993; Sullivan and Green, 1993; Jacobson and Peltz, 1996).

Although most recent studies have focused on elements that destabilize unstable mRNAs, there is also precedent for translational stabilization. An example is the yeast *PGK1* mRNA, for which it has been shown that inserting a strong secondary structure upstream of the AUG codon reduces both translational initiation efficiency and mRNA half-life (Muhlrad et al., 1995). Thus, for *PGK1* and perhaps many other longer lived messages, translation seems to increase rather than decrease mRNA stability. In these cases, the 5′ sequences and secondary structures that determine translational initiation efficiency (Kozak, 1988, 1994) should also influence mRNA stability.

Our data are consistent with just such a positive relationship between translation and *Fed-1* mRNA stability. Light-induced increases in *Fed-1* mRNA abundance are associated with increases in translational activity (measured by polyribosome association) in illuminated tissues, and both a functional AUG and a normal *Fed-1* translational start context are required for the *Fed-1* iLRE to regulate Fed::*CAT* transcripts. It is formally possible that the CATT element could act to stabilize *Fed-1* mRNA in the light, for example, by inhibiting a polyribosome-based degradation mechanism in a light-dependent manner. However, we prefer the hypothesis that the CATT element somehow acts to increase turnover of the mRNA in darkness. This preference is based on the lack of precedent for small, discrete mRNA stabilizing elements in other mRNAs. In addition, translationally defective *Fed-1* constructs (missense mutation of the AUG start codon, as shown in Dickey et al. [1994]; L.F. Dickey, unpublished data) accumulate less mRNA in the light than do the unmutated controls, which is consistent with the supposition that these CATT-containing RNAs turn over faster when they are not associated with polyribosomes.

Finally, plants containing constructs with mutated CATT elements tend to show relatively high levels of mRNA in both light and darkness (Figure 5). Comparing mRNA levels is potentially confounded by “position effect” variability among transformants with respect to rates of transgene transcription in various transformants; therefore, our conclusion must be regarded as tentative. However, we are currently working

on direct, in vivo measurements of half-lives for mutated and normal mRNAs. These measurements should clearly distinguish between stabilization and destabilization effects.

METHODS

Gene Constructions

All constructs were transcribed from a cauliflower mosiac virus 35S promoter derived from pBI121 (Jefferson, 1987; Jefferson et al., 1987). The chloramphenicol acetyltransferase (*CAT*) gene was obtained from pCM4 (Pharmacia). Chimeric genes were transferred into pBIN19 (Bevin, 1984; Elliott et al., 1989a). The wild-type *Fed-1* construct was the "message" construct, as described by Elliott et al. (1989b). The constructs shown in Figure 1 were made by amplifying the specified *Fed-1* fragments by polymerase chain reaction (PCR) and ligating them to the *CAT* gene at the BamHI site and to the 35S promoter in pBI121 at the XbaI site. All fusions were sequenced by the ICBR/DNA sequencing facility at University of Florida at Gainesville. The message construct was subcloned into pBSM13−. F5′UTR::*CAT* was made by PCR amplifying the 5′ untranslated region (UTR) of the message construct, using an M13 reverse primer and an antisense primer that annealed to the 3′ end of the *Fed-1* 5′ UTR and included a BglII site at the 3′ end (AGATCTTACTATTATGGTTTC). The *Fed-1* 5′ UTR was ligated to the *CAT* gene by a BglII-BamHI fusion 28 bp upstream of the *CAT* coding region, and the 5′ end of the *Fed-1* was ligated to the 35S promoter of pBI121 at the XbaI site.

Similarly, the F136::*CAT*, F158::*CAT*, and F199::*CAT* constructs were made by amplifying *Fed-1* fragments from the 5′ end of the message construct using the antisense *Fed-1* primers 5′-AGATCTGTGCTGACAGCAGTT-3′, 5′-AGATCTGGCTGAGTCCTGAGG-3′, and 5′-AGATCTCCATTGGAAAATGCT-3′, respectively. F236::*CAT* was made by ligating the XbaI-BglII fragment from the message construct to the XbaI site of pBI121 and the BamHI site of the *CAT* gene. The *CAT* gene was followed by the nopaline synthase terminator. F158MS1::*CAT* and F199MS1::*CAT* were made as F158::*CAT* and F199::*CAT*, except that *Fed-1* fragments were amplified from the *Fed-1* template that had a missense mutation, ATA, at the start codon (Dickey et al., 1994).

All of the constructs shown in Figure 4, except for wild-type *Fed-1*, were made by PCR-based overlap extension (Ho et al., 1989) and sequenced by the ICBR/DNA sequencing facility at the University of Florida at Gainesville. The Ω::*Fed* construct was made by fusing the Ω 5′ UTR to the *Fed-1* coding region, using PCR-based overlap extension. The Ω 5′ UTR was amplified from pT7-Ω (kindly provided by D.R. Gallie, University of California at Riverside), using the sense primer 5′-TATTTTTACAACAATTACCAACAACAA-3′, which included a BamHI site at the 5′ end, and the antisense primer 5′-ATTACTATTTACAATTACGTCGACCATGGCAACCACACCAGCTTTGTATGGA-3′, which annealed to the 3′ end of Ω and the 5′ end of the *Fed-1* coding region. Mutation 14.1 was made by substituting AGCGGCCGCTCAAGCGGCCG from positions +14 to +31 in the wild-type 5′ UTR sequence. Mutant 16.1 was made by substituting GATACCTGCTATAAGT for positions +16 to +31. Linker scanning mutation 32 was made by substituting a PstI linker (CTGCAGCTGCAGCT) for positions +32 to +45. The other linker scanning mutations from positions +46 to +136 were made by substituting the PstI linker AGCTGCAGCT at 10-bp intervals.

Transgenic Plant Growth

The various constructs described above were subcloned into pBIN19 and transferred into *Agrobacterium tumefaciens* LBA4404 by triparental mating (Elliott et al., 1989a). *Nicotiana tabacum* plants (SR-1, Petite Havana) were transformed using the leaf disc method (Horsch et al., 1985). Each primary transformant was divided during the regeneration process so that each transformation event was represented by a pair of clonally identical plants (Elliott et al., 1989a). Plants were grown in a growth chamber at 22°C on a 24-hr light cycle with a mixture of fluorescent and incandescent lights (light intensity of ∼240 μmol m^{-2} sec^{-1} between 380 and 780 nm).

Clonally duplicated transgenic plants described above and used for mRNA accumulation experiments shown in Figures 1 and 4 were transferred to soil and grown to midmaturity (height of 15 to 25 cm with approximately six leaves of 4 to 8 cm long). At this stage, plant pairs were dark adapted in total darkness at 22°C for 3 days, at which time one plant from each pair was returned to light for 6 hr (light intensity of ∼240 μmol m^{-2} sec^{-1} between 380 and 780 nm). Developmentally similar leaves (the top two unfolded leaves) were harvested into liquid nitrogen, total RNA was prepared, and 5 μg of total RNA (as determined by absorbance at 260 nm) was analyzed by RNA blot analysis, as described by Thompson et al. (1983).

Fed-1 or *Fed*::*CAT* mRNA was detected by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or radioanalytic imaging (AMBIS Systems, Inc., San Diego, CA) of the radioactive signals resulting from RNA blot hybridization using antisense RNA probes, as described by Dickey et al. (1992b). High-stringency hybridization conditions were used to avoid detection of the endogenous ferredoxin mRNA. Light induction ratios were averaged from separate experiments on clonal pairs representing from four to seven independent transformants.

The plants used for polyribosome analysis shown in Figures 2, 3, and 5 were grown from T_1 seed that were germinated and selected on 100-mm plates containing Murashige and Skoog medium (Gibco), kanamycin (50 μg/mL), and 1.25% Phytagar (Gibco). After selection (∼1 to 2 weeks), seedlings were transferred to the same medium without kanamycin and grown for 3 weeks in a growth chamber set on a 12-hr-light and 12-hr-dark cycle with a mixture of fluorescent and incandescent lights (light intensity of ∼120 μmol m^{-2} sec^{-1} between 380 and 780 nm). At this time, the plants were dark adapted for 48 hr, and half of the plates were reilluminated for 2 hr. Leaves from at least 10 seedlings for each sample were harvested, and RNA was extracted and analyzed as described below.

Polyribosome Analysis

Sucrose gradients (15 to 60%) were prepared by layering 7 mL of 15% sucrose on top of an equal volume of 60% sucrose in Sorvall 17-mL polyallomer tubes (Du Pont, Wilmington, DE). Gradients were formed by placing stoppered tubes in a horizontal position for 5.5 hr (Davies and Abe, 1995). Approximately 0.25 g of leaf tissue previously frozen in liquid nitrogen was ground in liquid nitrogen with a mortar and pestle and then in 2 mL of U buffer (200 mM Tris-Cl, pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 100 μg/mL heparin, 2% polyoxyethylene 10 tridecyl ether, and 1% deoxycholic acid, as described in Davies and Abe [1995]). Extracts were spun at 15,000*g* for 15 min at 4°C. Seven hundred and fifty microliters of each supernatant was layered onto a sucrose gradient and spun in a Sorvall AH629 swinging bucket rotor at 26,000 rpm (88,300*g*) for 3.5 hr at 4°C. Gradients

were fractionated with a density gradient fractionator (model 185; ISCO, Lincoln, NE), and absorbance at 254 nm was monitored each time to ensure the reproducibility of the gradient fractionation.

Fractions (1 mL) were collected by dripping directly into an equal volume of phenol–chloroform–isoamyl alcohol supplemented with 25 μL of 10% SDS, 20 μL of 0.5 M EDTA, and 5 μL of 100 mM aurin tricarboxylic acid. The resulting mixtures were immediately vortexed and placed on ice before centrifugation for 10 min at 15,000*g* at 4°C. Five hundred microliters of the supernatant was precipitated with 2 volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate at −20°C overnight. Precipitated RNAs were resuspended in 22 μL of 100 μM aurin tricarboxylic acid and glyoxylated (Sambrook et al., 1989). Half of each sample was loaded in each lane of a 1% agarose gel and processed as described above for RNA gel blot analysis.

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REFERENCES

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

Adang, M.J., Brody, M.S., Cardineau, G., Eagan, N., Roush, R.T., Shewmaker, C.K., Jones, A., Oakes, J.V., and McBride, K.E. (1993). The reconstruction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. Plant Mol. Biol. **21,** 1131–1145.

Bevin, M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12,** 8711–8721.

Caponigro, G., and Parker, R. (1996). mRNA turnover in yeast promoted by the MATα1 instability element. Nucleic Acids Res. **24,** 4304–4312.

Caponigro, G., Muhlrad, D., and Parker, R. (1993). A small segment of the MATα1 transcript promotes mRNA decay in *Saccharomyces cerevisiae*: A stimulatory role for rare codons. Mol. Cell. Biol. **13,** 5141–5148.

Caspar, T., and Quail, P.H. (1993). Promoter and leader regions involved in the expression of the *Arabidopsis* ferredoxin A gene. Plant J. **3,** 161–174.

Davies, E., and Abe, S. (1995). Methods for isolation and analysis of polyribosomes. In Methods in Cell Biology, D.W. Galbraith, D.P. Bourque, and H.J. Bohnert, eds (San Diego, CA: Academic Press), pp. 209–222.

Dickey, L.F., Gallo-Meagher, M., and Thompson, W.F. (1992a). Ferredoxin gene expression and its regulation by light. In Control of Gene Expression, D.P.S. Verma, ed (Boca Raton, FL: CRC Press), pp. 221–232.

Dickey, L.F., Gallo-Meagher, M., and Thompson, W.F. (1992b). Light regulatory sequences are located within the 5′ portion of the *Fed-1* message sequence. EMBO J. **11,** 2311–2317.

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

Diehn, S.H., De Rocher, E.J., and Green, P.J. (1996). Problems that can limit the expression of foreign genes in plants: Lessons to be learned from B.t. toxin genes. In Genetic Engineering: Principles and Methods, Vol. 18, J.K. Setlow, ed (New York: Plenum Press), pp. 83–99.

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

Elliott, R.C., Pedersen, T.J., Fristensky, B.A., White, M.J., Dickey, L.F., and Thompson, W.F. (1989b). Characterization of a single copy gene encoding ferredoxin I from pea. Plant Cell **1,** 681–690.

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

Gallie, D.R., and Walbot, V. (1992). Identification of the motifs within the tobacco mosaic virus 5′-leader responsible for enhancing translation. Nucleic Acids Res. **20,** 4631–4638.

Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C., and Wilson, T.M.A. (1987). The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. Nucleic Acids Res. **15,** 8693–8711.

Gallie, D.R., Lucas, W.J., and Walbot, V. (1989). Visualizing mRNA expression in plant protoplasts: Factors influencing efficient mRNA uptake and translation. Plant Cell **1,** 301–311.

Gallo-Meagher, M., Sowinski, D.A., Elliott, R.C., and Thompson, W.F. (1992). Both internal and external regulatory elements control expression of the pea *Fed-1* gene in transgenic tobacco seedlings. Plant Cell **4,** 389–395.

Helliwell, C.A., and Gray, J.C. (1995). The sequence surrounding the translation initiation codon of the pea plastocyanin gene increases translational efficiency of a reporter gene. Plant Mol. Biol. **29,** 621–626.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77,** 51–59.

Horsch, R.B., Fry, F.E., Hoffman, N.L., Eicholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

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

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

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

Kozak, M. (1988). Leader length and secondary structure modulate mRNA function under conditions of stress. Mol. Cell. Biol. **8,** 2737–2744.

Kozak, M. (1994). Features in the 5′ non-coding sequences of rabbit α- and β-globin messenger RNAs that affect translational efficiency. J. Mol. Biol. **235,** 95–110.

Laso, M.R.V., Zhu, D., Sagliocco, F., Brown, A.J.P., Tuite, M.F., and McCarthy, J.E.G. (1993). Inhibition of translational initiation in the yeast *Saccharomyces cerevisiae* as a function of the stability and position of hairpin structures in the mRNA leader. J. Biol. Chem. **268,** 6453–6462.

Linz, B., Koloteva, N., Vasilescu, S., and McCarthy, E.G. (1997). Disruption of ribosomal scanning on the 5′-untranslated region, and not restriction of translational initiation per se, modulates the stability of nonaberrant mRNAs in the yeast *Saccharomyces cerevisiae.* J. Biol. Chem. **272,** 9131–9140.

Marcotte, W.R., Jr., Russell, S.H., and Quatrano, R.S. (1989). Abscisic acid–responsive sequences from the Em gene of wheat. Plant Cell **1,** 969–976.

Muhlrad, D., Decker, C.J., and Parker, R. (1995). Turnover mechanisms of the stable yeast PGK1 mRNA. Mol. Cell. Biol. **15,** 2145–2156.

Parker, R., and Jacobson, A. (1990). Translation and a 42-nucleotide segment within the coding region of the mRNA encoded by the MATα1 gene are involved in promoting rapid mRNA decay in yeast. Proc. Natl. Acad. Sci. USA **87,** 2780–2784.

Peltz, S.W., Brewer, G., Bernstein, P., Hart, P.A., and Ross, J. (1991). Regulation of mRNA turnover in eukaryotic cells. Crit. Rev. Eukaryotic Gene Expression **1,** 99–126.

Petracek, M.E., Dickey, L.F., Huber, S.C., and Thompson, W.F. (1997). Light-regulated changes in abundance and polysome association of ferredoxin mRNA are dependent on photosynthesis. Plant Cell **9,** 2291–2300.

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

Rouwendal, G.J.A., Mendes, O., Wolbert, E.J.H., and de Boer, A.D. (1997). Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. Plant Mol. Biol. **33,** 989–999.

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

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

Savant-Bhonsale, S., and Cleveland, D.W. (1992). Evidence for instability of mRNAs containing AUUUA motifs mediated through translation-dependent assembly of a >20S degradation complex. Genes Dev. **6,** 1927–1939.

Stewart, C.N., Adang, M.J., All, J.N., Boerma, H.R., Cardineau, G., Tucker, D., and Parrott, W.A. (1996). Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis cryIAc* gene. Plant Physiol. **112,** 121–129.

Sullivan, M.L., and Green, P.J. (1993). Post-transcriptional regulation of nuclear-encoded genes in higher plants—The roles of messenger RNA stability and translation. Plant Mol. Biol. **23,** 1091–1104.

Thompson, W.F., Everett, M., Polans, N.O., Jorgensen, R.A., and Palmer, J.D. (1983). Phytochrome control of RNA levels in developing pea and mung bean leaves. Planta **158,** 487–500.

Van Hoof, A., and Green, P.J. (1997). Rare codons are not sufficient to destabilize a reporter gene transcript in tobacco. Plant Mol. Biol. **35,** 383–387.

Wada, K., Aota, S., Tsuchiya, R., Ishibashi, F., Gojobori, T., and Ikemura, T. (1990). Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. **18,** 2367–2368.