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EFFECT OF TRANSLATION ON mRNA STABILITY

By

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ABSTRACT

EFFECT OF TRANSLATION ON mRNA STABILITY

By

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Regulation of any step in gene expression, including regulation at the level of mRNA stability, can affect the level of the functional gene product. mRNA degradation rates can vary over several orders of magnitude, and can be regulated in response to both endogenous and exogenous stimuli. In addition mRNA decay is tightly linked to translation. A well-characterized example of this is the destabilizing effect of premature nonsense codons on eukaryotic mRNA stability. This has been named nonsense-mediated mRNA decay.

Experiments described in this thesis show that plants have a nonsense-mediated mRNA decay pathway. Wild type *PHA* mRNA is degraded with a half-life of about two hours in tobacco cells. In contrast *PHA* mRNA containing a premature nonsense codon is degraded with a half-life of about 30 minutes. Analyses of several different *PHA* alleles showed that introduction of a nonsense codon in the first 60% of the normal coding region destabilizes the mRNA, while a nonsense codon at 80% of the coding region has no effect. In addition, nonsense codon-containing *PHA* mRNA was shown to accumulate to reduced levels in tobacco leaves and *Arabidopsis* seedlings, indicating that nonsense-mediated mRNA degradation is not restricted to tobacco cell lines

Nonsense-mediated mRNA decay has been reported to occur in a several eukaryotic systems. Although some aspects of nonsense-mediated mRNA decay are conserved among eukaryotes, some differences have been reported. Nonsense-mediated

mRNA decay appears to be a cytoplasmic event in yeast , but a nuclear event for some mammalian genes. Subcellular fractionation of tobacco cells expressing *PHA* mRNA subject to nonsense-mediated mRNA decay showed that a large fraction of this mRNA was bound to polyribosomes. This indicates that in tobacco cells nonsense-mediated mRNA decay of *PHA* occurs after the mRNA has been assembled into polyribosomes, and thus occurs in the cytoplasm.

A second link between translation and mRNA degradation that has been proposed in several reports is a destabilizing effect of rare codons on mRNA. Direct evidence for this effect has not been reported, and my experiments indicate that rare codons are not sufficient to destabilize a reporter transcript in tobacco.

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INTRODUCTION

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INTRODUCTION

Over the last several years, emphasis on the study of mRNA stability has increased significantly. This is especially true for mRNA stability in plants. The first part of this chapter will give an overview of what we have learned about mRNA degradation in plants, whereas the second part will concentrate on what we know about the influence of translation on mRNA stability.

CONTROL OF mRNA DECAY IN PLANTS

Plants have many unique features that make studies of mRNA turnover attractive from both a conceptual and a technical perspective. In cases where mechanisms are common to plants and other higher eukaryotes, plants can offer new approaches with the potential for novel findings. This chapter will not attempt to comprehensively review recent work, but instead to highlight those findings that are the most definitive and that reveal the most and the fewest similarities to work carried out in other systems. I will also attempt to identify relevant experimental attributes of plant systems that may not be familiar to those outside the field. For a broader presentation of work on plant mRNA decay, readers are referred to several recent reviews (Gallie, 1993; Sullivan and Green, 1993; Abler and Green, 1996).

SEQUENCES CONTROLLING INHERENT mRNA STABILITY

Plant mRNA decay rates appear to be similar to those in other higher eukaryotes, with the average mRNA having a half-life on the order of several hours (Siflow and Key, 1979; Sullivan and Green, 1993; Taylor and Green, 1995). Recent studies have focused primarily on unstable mRNAs with half-lives of an hour or less because, as in mammalian cells, such short-lived mRNAs often encode regulatory proteins or other proteins of interest that must be induced transiently. The rapid control of gene expression facilitated by unstable transcripts may be particularly important in plants because, as sessile organisms, they must respond rapidly to environmental conditions from which they cannot escape.

Relatively unstable transcripts with half-lives of about an hour or less include the *PHYA* mRNA encoding a major form of the plant photoreceptor phytochrome (Seeley et al., 1992), the Small-Auxin-Up RNAs (*SAUR*s) (McClure and Guilfoyle, 1989) that are rapidly regulated by the plant hormone auxin, transcripts of the *TCH3* and *TCH4* (Braam and Davis, 1990) genes induced transiently by touch, and nonsense mutants of the phytohemagglutinin gene (*PHA*) (van Hoof and Green, 1996), and other mRNAs (Taylor and Green, 1995). It appears that, as in other systems, rapid mRNA decay is an active process triggered by specific sequences. Recently, several sequences have been shown to function as mRNA instability determinants in tobacco. Thus far, the most detailed studies relate to sequences present in the unstable *SAUR* transcripts mentioned above (Newman et al., 1993; Gil and Green, 1996; Sullivan and Green, 1996). These transcripts are unique to plants, and instability determinants derived from them appear to be novel.

Plants also recognize AUUUA repeats as signals for rapid mRNA decay, as do mammalian cells (Chen and Shyu, 1995), and premature nonsense codons, as do yeast, *Caenorhabditis elegans*, and mammalian cells (see Maquat, 1995; and Weng et al., 1997).

Sequences derived from *SAUR* transcripts

The *SAUR* transcripts are among the most unstable plant mRNAs reported, with half-lives of 10-50 minutes depending on the method of measurement (McClure and Guilfoyle, 1989; Franco et al., 1990). Although the exact function of the SAUR proteins is not known, their expression patterns indicate that they may be involved in auxin-induced cell elongation (McClure and Guilfoyle, 1989). All unstable *SAUR* mRNAs reported to date contain a highly-conserved sequence about 40 nucleotides in length referred to as DST, for downstream element (McClure et al., 1989). Although this element does not resemble prominent mRNA instability determinants in other systems, it is found in the 3' untranslated region (UTR), a location similar to that of many sequences causing rapid decay in mammalian and yeast transcripts. A synthetic dimer of the soybean *SAUR 15A* DST element is sufficient to markedly destabilize β-*glucuronidase* (*GUS*) and β-*globin* reporter transcripts when mRNA half-lives are measured following actinomycin D (ActD) treatment of stably transformed tobacco (BY-2) suspension cultures (Newman et al., 1993). It is likely that DST sequences also target transcripts for rapid decay in intact plants because the presence of DST sequences causes coordinate decreases in mRNA abundance in transgenic tobacco plants (Newman et al., 1993;

Sullivan and Green, 1996).

The DST element consists of three highly conserved regions separated by two variable regions, as shown in figure 1-1. The ATAGAT and T--GTA of the second and third conserved regions, respectively, are invariant in all *SAUR* DST elements. These elements were the focus of a recent mutational analysis of the DST element carried out in stably transformed BY-2 cells and in transgenic tobacco plants (Sullivan and Green, 1996). The mutant DST elements were evaluated within the 3' UTR of a β-*globin* reporter transcript. Five- or six-base substitution mutations in the ATAGAT or the T--GTA regions were sufficient to inactivate the element, stabilizing the transcript to the level of spacer or no-insert controls. These mutations also inactivated the element in transgenic plants, based on mRNA accumulation levels. Smaller two-base mutations in these regions had varying effects, ranging from little or no effect to significant increases in reporter mRNA half-life and accumulation. Interestingly, a two-base substitution changing the invariant GTA to CCA inactivated the element in plants but not in BY-2 cells. Taken together, these results indicate that both the ATAGAT and GTA regions are required for DST to function as an instability determinant in leaves of transgenic plants or in BY-2 cells, and that the sequence requirements for DST to function in the former are more stringent (Sullivan and Green, 1996).

In addition to work on isolated DST elements, studies on one natural *SAUR* gene, *SAUR-AC1* (Gil et al., 1994), have been carried out to identify regions of the transcript that are responsible for its instability (Gil and Green, 1996). Similar to other *SAUR* promoters (Li et al., 1991, 1994), the *SAUR-AC1* promoter region mediates auxin induction of the gene (Gil and Green, 1997). In contrast, sequences downstream of the

Plant	Gene	DST Sequence
Soybean	15A	GGAG - 5 - CATAGATTG - 7 - CATTTTGTAT
Soybean	X15	GGAG - 5 - CATAGATTA - 7 - CATTTGGTAC
Soybean	6B	GGAG - 4 - CATAGATTA - 7 - CATTTTGTAC
Soybean	X10A	GGAT - 5 - GATAGATTA - 8 - AAATTTGTAC
Soybean	10A5	GGAG - 5 - GATAGATTA - 8 - AAATTTGTAC
Mungbean	ARG7	GGTT - 2 - CATAGATTA - 8 - ATTTTTGTAA
Arabidopsis	AC1	GGAA - 9 - CATAGATCG - 8 - CAATGCGTAT
Consensus		GGA g -$^{Avg.}_{5}$- c ATAGATT a -$^{7}/_{8}$- $^{C}/_{A}$ A $^{T}/_{A}$ T T t GTAc

Figure 1-1. Sequence of DST elements identified in seven *SAUR* genes. The element consists of three conserved regions (shown in gray) separated by two variable regions. Numbers indicate the number of bases between the conserved regions. DST sequences in five soybean *SAUR* genes (McClure et al., 1989), one mung bean gene (Yamamoto et al., 1992), and one *Arabidopsis thaliana SAUR* gene (Gil et al., 1994) are aligned. For the consensus, residues are shown as follows: underlined residues are invariant among all seven genes; capitalized residues are conserved in at least six genes; capitalized residues separated by a slash are conserved among all seven genes, with each residue present in three or four genes; lowercase residues are conserved in four or five of the genes.

SAUR-AC1 promoter limit mRNA accumulation in a manner that is independent of auxin (Gil and Green, 1996). Effects on mRNA stability were assayed in BY-2 cells using a tetracycline-repressible promoter system (Gossen and Bujard, 1992; Weinman et al., 1994) to shut off transcription. The results showed that the *SAUR-AC1* 3' UTR, but not the coding region, functions as a potent mRNA instability determinant in tobacco (Gil and Green, 1996). The *SAUR-AC1* 3' UTR has a DST element located 10 bases from the poly(A) addition site (Gil et al., 1994) which certainly could contribute to this instability. However, the 3' UTR also contains several other sequences resembling DST subdomains (i.e., the ATAGAT and T--GTA regions) which may also be involved. Based on work done with the *SAUR-AC1* gene (Gil and Green, 1996) and with the DST dimer (Newman et al., 1993), it appears that the *SAUR* mRNAs are unstable independent of auxin treatment, presumably to facilitate rapid changes in mRNA levels following auxin-mediated transcriptional changes (Franco et al., 1990).

AUUUA sequences

AU-rich elements (AREs) are perhaps the most actively studied mRNA instability sequences in mammalian cells (Chen and Shyu, 1995). These elements are found in the 3' UTRs of many unstable mammalian transcripts and often contain multiple AUUUA motifs (Caput et al., 1986; Shaw and Kamen, 1986). Studies arguing that AUUUA sequences are important mRNA instability determinants in mammalian cells include the demonstration that synthetic AUUUA sequences destabilize reporter transcripts (Vakalopoulou et al., 1991) and that mutation of AUUUA sequences in natural

AREs (e.g., in c-*fos*) can inactivate instability function (Shyu et al., 1991). AU-rich elements may contribute to mRNA instability in yeast, but AUUUA sequences do not appear to be critical determinants (Muhlrad and Parker, 1992).

To evaluate whether AUUUA sequences can function in plant cells, an AUUUA repeat consisting of 11 copies of the element, was inserted into the 3' UTR of *GUS* or β-*globin* reporter transcripts and mRNA half-lives were measured in stably transformed BY-2 cells following ActD treatment. Reporter transcripts containing the AUUUA repeat degraded much more rapidly than did control transcripts containing no insert or a spacer with interspersed G's and C's. Because a similar element containing an AUUAA repeat did not cause rapid decay, it appears that plants specifically recognize the AUUUA sequence, not simply sequences rich in As and Us. As in mammalian cells, not all AUUUA sequences act as instability determinants (Greenberg and Belasco, 1993; Walker et al., 1995), in part because the minimal domain appears to be longer in some cases (Lagnado et al., 1994; Zubiaga et al., 1995).

The effect of the AUUUA repeat on mRNA accumulation in BY-2 cells and transgenic plants was even greater than its effect on mRNA stability, as measured in the latter system (Ohme-Tagaki et al., 1993; Sullivan and Green, personal communication). A similar discrepancy between mRNA accumulation and mRNA stability was observed for an ARE from granulocyte-monocyte colony-stimulating factor (GM-CSF) in mammalian cells (Savante-Bhonsale and Cleveland, 1992). In the case of GM-CSF, it was suggested that mRNA half-lives measured in the presence of ActD may be overestimates if recognition of the ARE is dependent on transcription. A similar model could explain the data obtained with the AUUUA repeat in plants. Alternatively the

element may be recognized by more than one mechanism.

The effect of AUUUA sequences on mRNA stability and abundance in plants may have important relevance to biotechnology because some AU-rich foreign transcripts fail to accumulate in transgenic plants. The best example of this problem comes from attempts to express insecticidal proteins called *B.t.* toxins in plants using genes derived from the bacterium *Bacillus thuringiensis* (Diehn et al., 1996). Even when *B.t.* toxin genes are introduced into plants under the control of strong plant promoters, very little *B.t.* toxin mRNA can be detected. In a practical sense, this problem has been overcome by creating synthetic *B.t.* toxin genes that more closely resemble plant genes and are highly expressed (Perlak et al., 1990, 1991). The major modifications have involved removal of several AUUUA sequences and other potential RNA-processing signals, increasing the GC content, and improving the codon usage. It has been suggested that these changes stabilize the mRNA. In one case this hypothesis was examined directly by comparing the stability of wild-type and synthetic *B.t.* toxin transcripts. The wild-type *B.t.* toxin transcript (with a GC content of 34%) was much less stable than the synthetic version (with a GC content of 64%), but the contribution of AUUUA elements to this effect remains to be evaluated (EJ De Rocher and PJ Green, personal communication; Diehn et al., 1996).

NOVEL STIMULI AFFECTING mRNA STABILITY IN PLANTS

Fungal elicitors

Some of the most striking examples of differentially-regulated mRNA stability in plants are related to a plant's need to adapt rapidly to an ever-changing environment. One case that illustrates this point occurs during the response of common bean to pathogen attack. Plants respond to pathogen attack by changing the expression level of many genes (Alexander et al., 1994). Often these changes can be re-created by treating cell cultures with compounds of plant or pathogen origin, referred to as elicitors, that the cell recognizes as signals of pathogen attack. In common bean, the *PvPRP1* gene, which encodes a cell wall protein, is among those regulated in response to a fungal elicitor (extracellular polysaccharides from fungi) (Sheng et al., 1991). Presumably this gene is down-regulated by elicitor treatment to alter the composition of the cell wall, which may contribute to plant protection.

Several lines of evidence indicate that the decay rate of the *PvPRP1* transcript is regulated by elicitor treatment (Zhang et al., 1993). Most important, decay in the presence of elicitor was faster than in its absence when mRNA decay rates were measured following treatment of cultured bean cells with ActD. Further supporting evidence comes from nuclear run-on transcription experiments which showed a constant transcription rate, regardless of elicitor treatment. The magnitude of the elicitor effect on mRNA stability cannot be calculated directly because the decay of the *PvPRP1* transcript appears to be dampened by ActD. In the presence of ActD, the half-life of the *PvPRP1* transcript

is 18 h and 60 h with and without elicitor respectively, but the mRNA disappears with a half-life of only 45 min if elicitor is added in the absence of ActD. Decay of the *PvPRP1* transcript is also sensitive to the protein synthesis inhibitors emetine and anisomycin, leading to the proposal that the mechanism requires ongoing transcription and translation (Zhang et al., 1993).

Ultraviolet (UV) crosslinking experiments carried out with protein extracts from unelicited cells have identified a 50 kD polypeptide, called PRP-BP, that can be crosslinked to the 3' half of the *PvPRP1* transcript (Zhang and Mehdy, 1994). No proteins have been found to cross-link to the 5' half. Using a series of 3' and 5' deleted transcripts, the binding site for PRP-BP was mapped to a 27 nucleotide U-rich site that contains one copy of the AUUUA motif. Crosslinking of PRP-BP could be competed specifically by poly U and by poly AU but not by several other competitors (Zhang and Mehdy 1994). It remains to be shown that this binding site is necessary for regulated instability of the transcript, but interestingly, the binding activity responds to elicitor treatment. The binding activity increases about fivefold one hour after elicitor treatment, concurrent with *PvPRP1* destabilization. This increase in binding activity is not caused by its *de novo* synthesis, but seems to be regulated post-translationally. Binding activity in extracts from unelicited cells could be increased by treatment with dithiotreitol (DTT) or β-mercaptoethanol, and could be reversibly eliminated by treatment with N-ethylmaleimide (NEM) or diamide (Zhang and Mehdy 1994). One explanation of these results is that binding of PRP-BP triggers rapid decay of the *PvPRP1* transcript in a manner that is redox regulated (Zhang and Mehdy 1994).

Another legume transcript for a putative cell wall protein (*MsPRP2*) also appears

to be regulated post-transcriptionally. In this case, the regulatory signal is osmotic stress. This transcript was found to be induced in salt-treated cells without a change in transcription rate (Deutch and Winicov, 1995). Interestingly the 3' UTR of *MsPRP2* contains a sequence that is similar to the putative *cis*-acting element in *PvPRP1*. It will be interesting to see whether the two genes, which share only 26% amino acid identity, are using similar mechanisms to regulate mRNA stability in response to different signals.

Light

Light is perhaps the most important external signal to which plants must respond. Therefore, the effects of light on gene expression have been studied extensively, and post-transcriptional events have been found or suggested in a number of cases (Thompson and White, 1991). The best illustration of a gene that is post-transcriptionally regulated in response to light is the pea *fed-1* gene, which encodes the photosynthetic electron carrier ferredoxin I (Elliott et al., 1989). As with many other photosynthetic genes, *fed-1* expression is induced by light, with mRNA accumulating to about fivefold higher levels in the light than in the dark. Although no direct measurements of mRNA half-lives have been made due to technical limitations, strong circumstantial evidence argues that light regulation of *fed-1* occurs at the level of mRNA stability. When run-on transcription experiments were performed using nuclei from light-grown and dark-adapted plants, no differences in transcription rates were observed (unpublished data cited in Dickey et al., 1992). Moreover, the *cis*-acting sequences responsible for light-induction were localized to the transcribed region of the gene (Elliott et al., 1989). When different regions of *fed-1*

were fused to a reporter gene, the internal light regulatory element (iLRE) was found to include sequences from both the 5' UTR and the 5' end of the coding region. In particular, a fusion of the 5' UTR and the first 20 codons of *fed-1* to the chloramphenicol acetyl transferase coding region rendered the chimeric transcript light-responsive (Dickey et al., 1992, 1994). It has also been shown that an open reading frame is required for differential accumulation of *fed-1* in response to light (Dickey et al., 1994). Light-regulation of the *fed-1* gene is diminished or abolished by a missense mutation in the start codon and by nonsense, but not missense, mutations in the 5' portion of the coding region. This is the strongest evidence that light acts at the level of RNA stability because nonsense codons would not be expected to influence light-regulated transcription. It is possible that these data can be explained by nonsense-mediated decay overriding the normal stability of *fed-1* mRNA in the light. However, perhaps it is more likely that for the iLRE to function correctly, it must interact with a translating ribosome.

Other stimuli

A number of plant genes are regulated by the availability of nutrients such as carbohydrates. Recently, it has been shown that sucrose availability can influence α-amylase gene expression at the level of mRNA stability in rice suspension cultures. The addition of sucrose decreases the abundance of α-amylase transcripts (Yu et al., 1991) due to both transcriptional and post-transcriptional regulation (Sheu et al., 1994). When mRNA decay rates were measured following ActD treatment, the half-life of the pool of α-amylase mRNA was about 12 h in sucrose-starved cells but decreased to less than an

hour when sucrose was provided (Sheu et al., 1994). α-Amylase in rice is encoded by at least eight different genes that produce very little mRNA in the presence of sucrose. However, under sucrose starvation conditions, two transcripts give rise to 90% of the α-amylase pool. Although the individual half-lives of the two highly expressed transcripts and of a poorly expressed transcript differ, each is about fourfold more stable in the absence of sucrose (S.-M.Yu, personal communication).

A large number of other genes in plants have been proposed to be differentially regulated at the level of mRNA stability, but in most cases the work is at a relatively early stage. For example, it has been proposed that histone mRNAs can be post-transcriptionally regulated, possibly in response to the cell cycle in the case of histone H3 (Kapros et al., 1995). If this is the case, then the sequences and mechanisms responsible could be novel since plant histone H3 transcripts end with a poly(A) tail (Chaboute et al., 1988; Ohtsubo and Iwabuchi, 1994) rather than with the regulatory 3' inverted repeat well-characterized in mammalian cells (Marzluff and Hanson, 1993). In any event, it is clear from half-life measurements in nonsynchronized cells that the putative cell cycle-dependent forms of histone H3 mRNA degrade with half-lives of 1 h in tobacco (Taylor and Green, 1995) or 2 h in alfalfa cells (Kapros et al., 1995), while the mRNA for a putative replacement-type histone had a half-life of 6 h in alfalfa cells (Kapros et al., 1995). Aside from these examples, there are many other cases for which differential regulation of mRNA stability has been proposed (reviewed in Gallie et al., 1993; Abler and Green, 1996), the majority of which are based on observed discrepancies between nuclear run-on transcription rates and RNA accumulation levels.

CONTRIBUTION OF mRNA DECAY TO GENE SILENCING AND ANTISENSE MECHANISMS

The nature of gene silencing in plants

Perhaps the most intriguing phenomenon associated with the control of mRNA stability in plants is a form of gene silencing also known as cosuppression or sense suppression. This type of gene silencing was discovered during reverse genetic studies aimed at elucidating the function of cloned plant genes via overexpression approaches. Most often, these experiments involved introduction of the cloned plant gene into plants under the control of a strong (viral) promoter and the subsequent regeneration of transgenic plants. One would expect the expression of the transgene to vary among these plants due to chromosome position effects, so that high levels of overexpression would be achieved in some plants and lower to no overexpression in others. Indeed this occurs, but the surprising observation is that in some transgenic plants, accumulation of the mRNA from both the transgene and the endogenous gene is markedly suppressed. That is, in these cases, the transgene reduces its own expression and that of the endogenous gene (reviewed in Chasan, 1994; Flavell, 1994; Matzke and Matzke, 1995). The process is homology dependent, but the transgene and the endogenous gene do not need to be identical. Either one gene or several closely related genes can be silenced.

There are at least two distinct mechanisms of gene silencing in plants, transcriptional and post-transcriptional. Transcriptional gene silencing may be similar to other epigenetic phenomena in plants, animals, and fungi (Matzke and Matzke, 1995).

Post-transcriptional gene silencing has been studied in the most detail in plants, but recently has also been described in *Neurospora crassa* (Cogoni et al. 1996). However, as discussed below, this type of gene silencing is also associated with some forms of plant viral resistance and may account for some of the position effects on transgene expression previously thought to affect only transcription. With respect to the latter, it has been observed that some transgenic plants that exhibit low transgene mRNA levels actually have high rates of nuclear run-on transcription and must therefore be silenced post-transcriptionally (e.g., Lindbo et al., 1993). There is a general assumption that post-transcriptional gene silencing involves accelerated decay of the mRNAs that are diminished, although the supporting data are somewhat indirect, as illustrated in the examples that follow. Within the confines of this chapter, it will only be possible to present a snapshot of post-transcriptional gene silencing and the major models proposed to explain it. For a more thorough discussion, readers are referred to several reviews on this topic (Chasan, 1994; Flavell 1994; de Carvalho Niebel et al., 1995b; Matzke and Matzke, 1995; Baulcombe and English 1996).

Examples of post-transcriptional gene silencing

One gene whose post-transcriptional gene silencing has been studied in detail is a gene for the basic isoform of β1,3-glucanase of *Nicotiana plumbaginifolia.* When this gene was introduced into tobacco plants (*Nicotiana tabacum*), the plant with the highest transgene mRNA level was selected for further study and self-fertilized (de Carvalho et al., 1992). Progeny of the original transformant that were homozygous for the transgene

did not express the transgene, while heterozygous progeny did. The endogenous basic β1,3-glucanase isoform, but not the acidic isoform, was also silenced in the homozygotes (de Carvalho Niebel et al., 1995a). The effect was observed at the RNA level, but nuclear run-on transcription experiments showed that there was no difference in transcription rate between heterozygotic unsilenced plants and homozygotic silenced plants (de Carvalho et al., 1992; de Carvalho-Niebel et al., 1995a). This was confirmed by analyzing the level of RNA copurifying with nuclei using a reverse transcriptase polymerase chain reaction (RT-PCR) assay. Both spliced and unspliced, polyadenylated RNA were more abundant in homozygous silenced nuclei than in heterozygous unsilenced nuclei (de Carvalho Niebel et al., 1995a). These data provide strong evidence that gene silencing occurs post-transcriptionally and presumably involves degradation of the mature form of the mRNA, either just before export from the nucleus or in the cytoplasm.

Other prominent examples of post-transcriptional gene silencing relate to the chalcone synthase (*CHS*) and dihydroflavonol-4-reductase (*DFR*) genes in petunia. Again, a percentage of the plants transformed with a *CHS* or *DFR* transgene showed gene silencing. Both genes are involved in flower pigmentation, so that in these cases, the silenced state (white flowers) can easily be distinguished from the normal state (purple flowers). The white flowers have low *CHS* or *DFR* RNA levels (Napoli et al., 1990; van der Krol et al., 1990), and, at least in the case of *CHS*, transcription rates and nuclear RNA levels are normal compared to those of purple flowers (van Blokland et al., 1994).

There are also instances of engineered virus resistance that are representative of post-transcriptional gene silencing and provide further insight into the process. Transgenic plants transformed with a virus-derived gene sometimes show resistance to

viral infection. In one particularly interesting example, some of the plants transgenic for the Tobacco Etch Virus (TEV) coat protein transgene were initially successfully infected by TEV (a cytoplasmically replicating RNA virus), but after some time they "recovered" (Lindbo et al., 1993). This recovery resulted in the production of new leaves that had no detectable virus. Protoplasts isolated from recovered leaves did not support virus replication, but protoplasts from untransformed and transformed uninfected plants did (Lindbo et al., 1993). The most interesting finding was that the transgene was silenced in the recovered tissue. mRNA accumulated to reduced levels in these recovered leaves even though the transcription rates of the transgene were the same as those in uninfected plants (Lindbo et al., 1993). Apparently, the viral infection had triggered the silencing of the transgene and viral resistance, most likely by causing rapid decay of transgene and viral transcripts (Lindbo et al., 1993).

English et al. (1996) recently provided additional evidence that transgene-mediated virus resistance can be due to post-transcriptional gene silencing. An important prediction of this hypothesis was that a silenced transgene should be able to confer virus resistance to viruses that have sequence similarity, even if this gene was not originally part of the virus. The cytoplasmically replicating Potato virus X (PVX) tolerates insertion of a *GUS* gene that is not normally part of the viral genome. This PVX.GUS virus was used to infect plants that contained either a post-transcriptionally silenced *GUS* gene, an expressed *GUS* gene, or no *GUS* gene. As expected, infection of nontransformed plants or plants that had not silenced the *GUS* gene showed numerous GUS-positive lesions, indicating a successful infection by PVX.GUS. These plants also accumulated PVX.GUS RNA to high levels. In contrast, plants carrying the silenced *GUS* gene showed very few

GUS-positive lesions after inoculation with PVX.GUS and accumulated little if any PVX.GUS RNA. Additional experiments showed that this outcome was critically dependent on sequence identity between the silenced transgene and the infecting virus. A silenced version of the PG or *nptII* gene conferred resistance to PVX viruses carrying the same gene, while none of these silenced genes affected infection by a PVX virus carrying the gene for green fluorescent protein (English et al., 1996). In this study and in previous analyses of these same transgenic lines (Hobbs et al., 1990), a correlation was observed between the post-transcriptional gene silencing phenomenon, the number of *GUS* genes inserted in the genome, and methylation levels of specific restriction sites in the *GUS* gene.

Similar observations have been reported for post-transcriptional gene silencing of an *nptII* gene. In this case, one particular transformant containing a silenced *nptII* gene was self-fertilized and the progeny were analyzed. All progeny transcribed their *nptII* genes at approximately the same rate, but some plants accumulated 20- to 40-fold more RNA than other progeny and the original transformant. The progeny that had silenced genes (and the original transformant) had two independently segregating loci of nptII and methylation of several restriction sites in the transgenes. In contrast, progeny that expressed *nptII* at high levels had only one locus (although multiple copies at that locus) of nptII and less or no methylation on these same restriction sites (Ingelbrecht et al., 1994). It is not clear whether methylation is a cause and/or an effect of gene silencing in these cases.

Models for post-transcriptional gene silencing

Two favored models that have been proposed to explain how post-transcriptional gene silencing is triggered are the threshold model and the ectopic pairing model. Neither of these models has been proven valid, and arguments for and against each can be made. Here we will briefly present both models, without attempting to comprehensively discuss all the supporting data. A proposed explanation for how these models lead to increased mRNA decay is also included. For a more detailed discussion the reader is referred to several recent reviews (Flavell 1994; de Carvalho Niebel et al., 1995b; Baulcombe and English, 1996)

In the threshold model (Lindbo et al., 1993), gene silencing is triggered when homologous mRNA accumulates above a certain threshold (Figure 1-2B). This excess mRNA is sensed by one or more *trans*-acting factors, which in turn trigger increased rates of mRNA decay. In the absence of a gene-silencing mechanism, this excess RNA would be expected to accumulate to a high level, as in Figure 1-2A. The *trans*-acting factors can be RNA-binding proteins, aberrant RNAs, or antisense RNAs (see below). An observation supporting this model is that those transgenes with the highest transcriptional activity are generally (but not always) the ones silenced. A possible weakness is that it is difficult to accommodate the observation that a promoterless transgene can cause silencing (van Blokland et al., 1994) without proposing technical problems with the experiments.

In the ectopic pairing model (reviewed by Baulcombe and English 1996), gene silencing is triggered by direct interaction of two DNA molecules, resulting in an altered

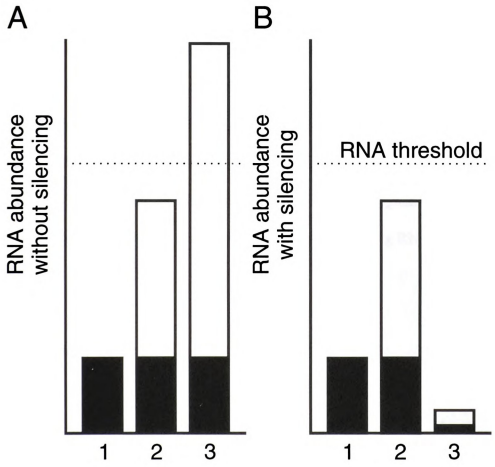

Figure 1-2. The histograms represent the mRNA abundance from a given gene (black bar) in the absence (1) or presence of an additional copy of that gene (open bar) transcribed at relatively low levels (2) or at high levels (3). **A** depicts the expected situation in the absence of a gene-silencing mechanism, and **B** depicts what has been found in several cases of gene silencing. The RNA threshold model proposes that gene silencing is triggered when the total amount of homologous RNA is higher than some threshold level. This trigger leads to a stable state in which RNA is rapidly degraded and therefore accumulates to very low levels.

state of the DNA (or chromatin). The exact nature of this altered state is unknown, but recent results showing a correlation between post-transcriptional gene silencing and methylation may indicate that methylation is involved. This altered state of the DNA leads to the production of a small amount of aberrant RNA in addition to the normal RNA. This aberrant RNA is then transported to the cytoplasm, where it triggers degradation of the mRNA. This model explains why a promoterless transgene can induce silencing (van Blokland et al., 1994), and it is easy to imagine how methylation could correlate with a post-transcriptional event that involves DNA-DNA interactions. However, a rather complicated hypothesis (Baulcombe and English 1996) is required to accommodate the observation that a cytoplasmically replicating RNA virus can induce silencing, as in the TEV example.

Role of antisense RNA

In both models, triggering gene silencing leads to an increased degradation rate of the homologous mRNA. It has been proposed that an RNA-dependent RNA polymerase (RdRP) may be involved in this process. RdRP activity has been characterized in several plant species and has been purified from tomato (Schiebel et al., 1993a; 1993b). The substrate for RdRP in the threshold model could be the excess RNA, while in the ectopic pairing model it could be the aberrant RNA. The antisense RNA produced in this manner is envisioned to form a hybrid with the sense RNA and thus be targeted for degradation by a double-strand specific RNase.

Antisense RNA has been widely used to reduce expression of homologous genes

in a variety of plants (reviewed in Bourque, 1995). In many cases, antisense effects in plants are associated with diminished levels of the sense RNA. The exact mechanism by which this occurs has not been elucidated, but there is precedent for the involvement of RNA degradation. This was directly demonstrated in transgenic tobacco expressing an antisense *RBCS* gene by measuring decay rates of the sense mRNA after addition of cordycepin (Jiang et al., 1994). In untransformed control plants *RBCS* mRNA decayed with a half-life of about 5 hours, versus about 1 hour in plants expressing antisense *RBCS* RNA.

Clearly, more detailed study of the mechanism(s) of gene silencing and antisense RNA effects in plants is required. To date, the power of genetic analysis has not been fully utilized, although one report has shown that at least two loci of *Arabidopsis* are required for silencing of the *rolB* gene (Dehio and Schell, 1994). Identification of additional loci and cloning of some of the genes involved should greatly advance our understanding of this intriguing phenomenon. A more detailed study of the involvement of methylation also seems warranted.

ANALYSIS OF mRNA DECAY PATHWAYS

The analysis of mRNA degradation intermediates is one of the most effective strategies to uncover steps in particular mRNA decay pathways. As in mammalian cells and yeast, prominent mRNA decay intermediates have not been detected for most transcripts in plants. This indicates that once degradation has been initiated, subsequent steps are very rapid. However, two plant transcripts that appear to be exceptions to this

rule are the oat *PHYA* mRNA and the soybean *SRS4* mRNA. Accordingly, detailed analysis of these transcripts has provided most of our insight into possible mRNA decay pathways in plants.

When the *SRS4* gene is expressed in soybean and transgenic petunia, a series of short *SRS4* transcripts can be observed in addition to the full-length mRNA on RNA gel blots (Thompson et al., 1992). These short transcripts have been proposed to be *bona fide* decay intermediates of *SRS4* mRNA for several reasons: (1) they accumulate when the gene is expressed from the Cauliflower Mosaic Virus 35S promoter, as well as from the *SRS4* promoter (Thompson et al., 1992), (2) the fragments are polysome associated (Thompson et al., 1992), (3) radioactive tracers added to homogenized samples are not degraded *in vitro* during subsequent RNA purification steps (Thompson et al., 1992); and, most importantly, (4) the same short transcripts can be produced by adding *in vitro*-synthesized *SRS4* RNA to a cell-free mRNA decay system containing polysomes (Tanzer and Meagher, 1994) or to an S150 extract (Tanzer and Meagher, 1995). Mapping the ends of the short transcripts showed that most proximal *SRS4* RNAs with intact 5' ends could be matched with a distal *SRS4* RNA with an intact 3' end, indicating that each pair of products probably arose from endonuclease cleavage of the intact *SRS4* mRNA, perhaps directed by local secondary structure (Tanzer and Meagher, 1995). This endonuclease cleavage appeared to be independent of decapping or of deadenylation of the transcript. On the basis of these and other data, the model of *SRS4* RNA decay shown in figure 1-3A was proposed (Tanzer and Meagher, 1995). According to this model, cleavage by a stochastic endonuclease initiates decay, followed by 3' to 5' or 5' to 3' exonuclease digestion of the proximal and distal products.

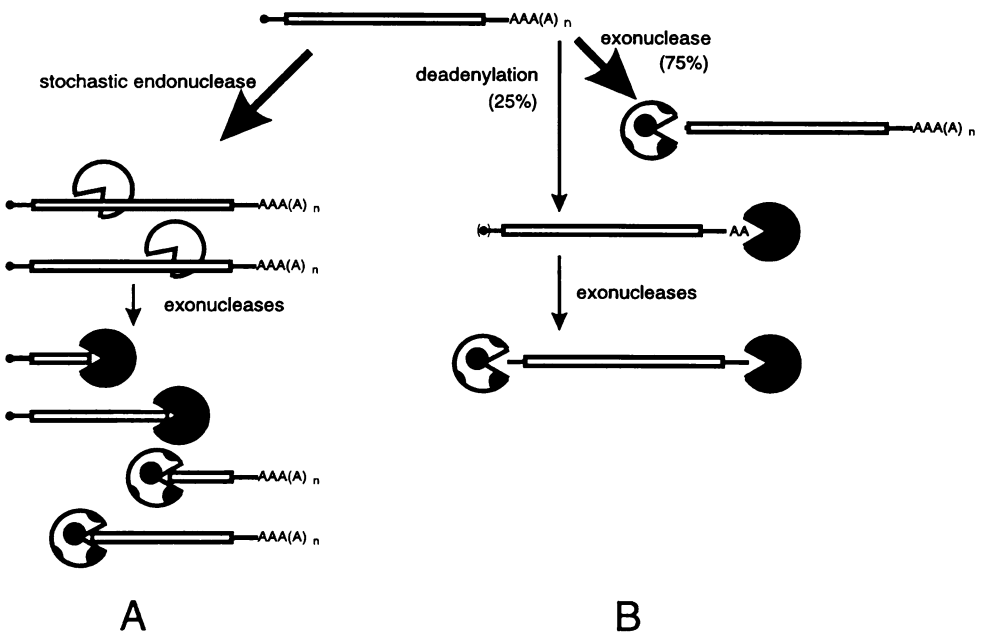

Figure 1-3. Proposed mechanisms of *SRS4* and *PHYA* mRNA decay in plants. **A** depicts stochastic endonucleolytic decay without prior deadenylation, as proposed for *SRS4* mRNA (Tanzer and Meagher, 1995). **B** depicts exonucleolytic decay pathways, as proposed for *PHYA* mRNA (Higgs and Colbert, 1994). The major degradation pathways for each transcript are indicated by heavy arrows. The 5' cap, the 3' and 5' untranslated regions, and the coding region of the transcripts are indicated by solid dots, thin lines, and open bars, respectively. $AAA(A)_n$ represents the poly(A) tail of the mRNA. White enzymes are stochastic endonucleases, gray and spotted enzymes are exonucleases. Parentheses around the 5' cap indicate that the presence or absence of the cap has not been determined.

RNA preparations from plants expressing *PHYA* genes also yield short RNA fragments in addition to the full-length transcript. Similar to the case of *SRS4*, three observations (Higgs and Colbert, 1994) argue that the short *PHYA* mRNAs are true decay intermediates: (1) the *PHYA* RNA fragments are present in RNA preparations isolated according to different procedures while other endogenous or added RNAs remain intact, (2) RNA isolated from a polysome-based *in vitro* system also contains the *PHYA* fragments, and (3) these fragments are associated with polysomes *in vivo* as well. Interestingly, the *PHYA* fragments are continuously distributed over a size range of about 200 nt to 4.2 kb (Higgs and Colbert, 1994), a pattern differing from that of the discrete sized fragments of the *SRS4* transcript discussed above. The structure of the PHYA fragments was analyzed using different probes. Based on the results, two pathways that primarily involve exonuclease activities were proposed (Higgs and Colbert, 1994) as illustrated in figure 1-3B. In this model, the majority (about 75%) of the *PHYA* mRNA is degraded by a 5' to 3' exoribonuclease before removal of the poly(A) tail. The remainder of the mRNA is deadenylated prior to degradation by 5' to 3' and 3' to 5' exoribonucleases (Higgs and Colbert., 1994). An alternative model involving a stochastic endoribonuclease cannot be ruled out; however, considering the continuous distribution of fragments, this seems less likely (Higgs and Colbert, 1994).

Only the deadenylated fraction of the *PHYA* mRNA (25%) appears to be a good candidate for decay by the major deadenylation-dependent decapping pathway elucidated in yeast by Parker and coworkers (reviewed by Tharun and Parker, 1997). The alternative deadenylation-independent decapping pathway also described in yeast (Muhlrad and Parker, 1994) could explain the decay of the remainder of *PHYA* mRNA, but at present it

is unknown whether or not decapping is required for degradation of the *PHYA* transcript. In contrast, a novel mechanism appears to be responsible for *SRS4* mRNA decay. It will be interesting to see if other plant mRNAs that do not give rise to visible mRNA decay intermediates degrade by the same pathways.

LINKAGE OF mRNA STABILITY AND TRANSLATION

There are numerous indications that translation and mRNA degradation are intimately linked processes *in vivo*. This is true for both prokaryotes and eukaryotes, but prokaryotic and eukaryotic mRNA structure and translation are sufficiently different from each other to suggest that one can not expect to be able to extrapolate findings from one to the other. In this section I will highlight some of the more interesting examples of links between the two processes in eukaryotes. Most of these links are not yet fully understood, and clearly more work is needed.

The poly(A) tail

Both ends of eukaryotic mRNAs play rate-determining and regulated roles in mRNA degradation and translation. A major pathway of mRNA decay in yeast is initiated by shortening of the poly(A) tail, followed by removal of the cap structure from the 5' end of the transcript (the deadenylation-dependent decapping pathway) (Decker and Parker, 1994). Although little is known about the pathways of mRNA degradation in higher eukaryotes, given the conservative nature of evolution (as exemplified in the

similarities in transcription, splicing, and polyadenylation mechanisms among all eukaryotes), it seems plausible that some of the basic mechanisms of mRNA decay are similar in higher eukaryotes. The limited amount of available data indicate that shortening of the poly(A) tail is also a critical step in the degradation of some higher eukaryotic mRNAs. However, it remains to be shown that this is indeed the case for the bulk of the mRNA. Removal of the poly(A) tail is the first step in the degradation of the unstable c-fos (Wilson and Treisman, 1988; Shyu et al., 1991) and c-myc (Swartwout and Kinniburgh, 1989; Laird-Ofringa et al., 1990) mRNAs in mammalian cells. Furthermore, determinants of poly(A) tail-shortening rates are identical to, or at least overlap with, the determinants of mRNA instability. Specifically, β-globin reporter transcripts that normally are stable and slowly deadenylated can be made to deadenylate and degrade quickly by insertion of the c-fos 3' UTR, a fragment of the c-fos coding region, or the sequence UUAUUUAUU (Shyu et al., 1991; Zubiaga et al., 1995), suggesting that removal of the poly(A) tail is a required initial step in the degradation of the c-fos message and possibly of other mRNAs. As discussed above removal of the poly(A) tail does not appear to be a critical step in the degradation of *SRS4* and *PHYA* transcripts in plants.

The poly(A) tail not only is important for regulating mRNA decay but also has an important role in translation. The presence of a poly(A) tail increases the translation rate of an mRNA both in rabbit reticulocyte lysates (Doel and Carey, 1976) and *in vivo*. For example, RNA synthesized *in vitro* and introduced into protoplasts by electroporation was more efficiently translated if a poly(A) tail was added to the 3' end (Gallie et al., 1989; Gallie, 1991).

The exact mechanism by which the poly(A) tail affects translation and mRNA degradation is not known, but the available evidence suggests that the poly(A) binding protein (PAB) is important (Caponigro and Parker, 1995; Jacobson, 1996). These data suggest that the first step in the degradation of an mRNA may inhibit further translation of the mRNA. It seems likely that more links exist, probably acting through PAB. Plants express what appear to be multiple PABs in a tissue-specific manner (Belostolsky and Maegher, 1993). It remains to be determined whether all these proteins function in regulating both translation and mRNA turnover. It is tempting to speculate that there may be differences in some of their functions, and that the repertoire of expressed PABs regulate translation or turnover in a cell type-specific manner.

The cap structure

The 5' end of eukaryotic mRNAs consists of a modified G-nucleotide linked to the rest of the transcript by a unique linkage. Recently it has become evident that the second step of the major mRNA decay pathway in yeast is the removal of this cap structure (Decker and Parker, 1994). However, the role of decapping in the decay of mRNA in higher organisms is completely unknown. The cap structure also has a well-documented role in translation initiation (Sonenberg, 1996). The first step in translation is the binding of the cap by eIF4F. This is followed by assembly of a large complex, including the 40S ribosomal subunit, onto the 5' end of the mRNA (Merrick and Hershey, 1996). Assembly of such a large complex may influence the rate of decapping of the transcript by sterically hindering access to the cap by the decapping enzyme. Conversely, decapping would be

predicted to influence translation by preventing assembly of the preinitiation complex. Clearly more work needs to be done to investigate the importance of decapping in mRNA decay in plants and how this process interacts with translation.

Inhibition of translation stabilizes many unstable mRNAs

Inhibition of translation can have large effects on the degradation rates of specific mRNAs. Usually this is studied by inhibiting global translation with chemical inhibitors, but more refined analysis has been performed in certain cases (see below). The drug most widely used to inhibit translation is cycloheximide (chx). A large number of unstable transcripts are stabilized by addition of chx (Jacobson and Peltz, 1996), although there are also cases where chx has no effect on mRNA stability. This has also been found to be the case in plants. Taylor and Green (1995) identified unstable mRNAs by differential hybridization and studied the effect of chx on the disappearance of these transcripts during actinomycin D treatment. For all eight mRNAs analyzed the disappearance of the transcripts during actinomycin D treatment was inhibited in the presence of chx. However it is clear that induction by chx can also be mediated by promoter sequences (e.g. Gil and Green, 1996), thus it is important to measure mRNA half lives with and without chx, before concluding that chx influences the stability of a particular transcript.

Besides chx treatment, there are additional ways to inhibit global translation. There are other drugs that inhibit translation. Some act at the same step as chx, while others act at different steps in translation. Inhibition of global translation without the use of drugs can be achieved by using a (conditional) mutation in a factor involved in

translation. One example of this is a temperature sensitive mutation in the CCA1 gene of yeast. The CCA1 gene encodes tRNA-nucleotidyltransferase, the enzyme that adds the trinucleotide CCA to the 3' end of tRNAs. The 3' terminal CCA sequence is required for tRNA function, but it is not encoded by eukaryotic tRNA genes (Deutscher, 1990). In strains that have a defect in CCA1, the cell is depleted of functional tRNAs and translation is inhibited. Unstable mRNAs are stabilized in this strain at non-permissive temperature, presumably because of the defect in translation (Peltz et al., 1992).

It is unclear by what mechanism inhibition of global translation affects mRNA stability, and it seems likely that there is more than one way in which this may occur. The two simplest explanations are that either translation of the mRNA in *cis* is required for its normal degradation, or that inhibition of translation of some other mRNA (in *trans*) depletes the cell of some unstable protein involved in mRNA degradation. In the *trans* mechanism, The required protein could be an RNase or other factor important for degrading RNA. This model would predict that all drugs that inhibit translation would have the same effect, regardless of what step in translation they affect. Alternatively, under the *cis* model, association of the mRNA of interest with ribosomes is important for its normal degradation. One clear, but possibly unique, example of this mechanism is the degradation of β-tubulin mRNA in mammalian cells (Cleveland, 1988; Theodorakis and Cleveland, 1996). The degradation rate of β-tubulin mRNA is increased when free tubulin monomers accumulate. Degradation of the β-tubulin mRNA is initiated when the nascent peptide emerges from the ribosome. The N-terminus of the protein is recognized specifically, triggering the degradation of the mRNA. If translation of the tubulin mRNA is inhibited, it is stabilized because it is the mRNA-ribosome complex that is required for

recognition of β-tubulin mRNA.

In most other cases it is not clear which of these two models best explains translation-dependent degradation of a particular transcript. Addressing this question requires inhibition of translation of only the mRNA of interest. This can be achieved in several different ways. Translation of a particular mRNA can be inhibited by introduction of a stable secondary structure in the 5' UTR, by mutation of the start codon, or by introduction of a nonsense codon. As discussed below, each of these possibilities has been used in different studies. However introduction of a nonsense codon often has a destabilizing effect independent of the normal decay pathway for the specific mRNA (see the section on nonsense-mediated mRNA decay), so inhibiting translation by insertion of a nonsense codon may not be informative of the normal pathway of mRNA degradation. One disadvantage of all of three possible strategies is that they require mutations in the gene of interest, and thus the gene of interest has to be introduced as a transgene.

A stable secondary structure in the 5' UTR inhibits translation because during the normal translation initiation process, initiation factors and the 40S ribosomal subunit bind at the cap structure at the 5' end of the mRNA and subsequently scan the mRNA to find the start codon. Introduction of a sufficiently stable secondary structure in the 5' UTR inhibits the scanning of the ribosome and thus prevents it from reaching the start codon (Kozak, 1989). This strategy has been used in several cases. For example Aharon and Schneider (1993) introduced a hairpin structure into the 5' UTR of a reporter gene that also contained an AU-rich destabilizing sequence. This mRNA was inefficiently translated and was not targeted for rapid degradation. In an elegant control experiment translation of the mRNA was restored by introducing an internal ribosome entry site of

viral origin (IRES) downstream of the hairpin. This restores translation and instability of the mRNA, which shows that the hairpin in the 5' UTR functioned by inhibiting translation and not by some other means, for example inhibiting an exonuclease. This is an important consideration, because secondary structures are known to inhibit a wide variety of exonucleases in yeast (Vreken and Raue, 1992), Hela cell extracts (Ford et al., 1997), chloroplasts (Stern and Gruissem, 1987), and *E. coli* (Mott et al., 1985)

A variation on this scheme is the introduction of an iron responsive element (IRE) in the 5' UTR, similar to what is naturally present in the human ferritin message. The IRE is a stem loop structure capable of binding a *trans*-acting factor, the iron responsive protein (IRP). The IRE structure by itself is not sufficiently stable to inhibit ribosomal scanning, but when the IRP is bound translation of the mRNA is inhibited (Raoult et al., 1996). In mammalian cells, the binding activity of IRP is regulated by the availability of iron. Thus translation of specific mRNAs can be regulated by iron, and its effect on mRNA stability can be examined. The IRE/IRP system has been used to investigate the link between translation and stability of reporter genes destabilized by insertion of AU rich sequences from c-fos (Koeller et al., 1991; Winstall et al., 1995) and GM-CSF (Winstall et al., 1995) or to study translation-dependent stability of a modified transferrin mRNA (Koeller et al., 1991). Koeller et al. (1991) found that IRP binding inhibited translation of the reporter transcripts as effectively as chx, but that the stability of the reporter mRNAs was not affected. This suggested that a labile *trans*-acting factor is necessary for the rapid degradation of these mRNAs. Interestingly, Winstall et al. (1995) using the same experimental design, but slightly different reporter genes and a different mammalian cell line, reached the opposite conclusion. In this case IRP binding led to

decreased translation of the reporter transcripts and decreased RNA turnover. This effect was critically dependent on a functional IRE in the reporter genes; control transcripts that had a defective IRE because of a one-base deletion were not affected. It is not clear why these two experiments resulted in opposing conclusions.

Yeast and plants do not have an IRE-binding activity (Rothenberger et al., 1990; Oliveira et al., 1993). Instead plants appear mainly to use regulation of mRNA abundance as the major control of ferritin expression (van der Mark, 1983; Lescure et al., 1991; Gaymard et al., 1996), while yeast does not appear to use ferritin as the major iron storage form (Raguzzi et al., 1988). However, all the essential components of the IRE/IRP system have been cloned from mammals and thus can be introduced into yeast or plants. This was successfully accomplished in yeast by expressing the IRP from a strong, but tightly regulated promoter and introducing an IRE in a luciferase reporter gene (Oliveira et al., 1993). This resulted in regulated expression of luciferase, but the system has not been widely used to address questions related to mRNA stability. A similar strategy was followed to introduce this system into plants, but luciferase was expressed approximately equally in the presence or absence of an IRE and/or IRP (van Hoof and Green, unpublished). It is not clear whether this reflects a difference between the two organisms, or whether technical problems, for example a failure to produce sufficient levels of IRP, are responsible for this.

Translation can also be inhibited by mutation of the start codon. To inhibit translation completely one has to mutate all AUG triplets in all three reading frames, which for most transcripts means introducing multiple mutations along the transcript. According to the universally accepted scanning model of translation initiation, this would

lead to scanning of the 40S ribosome until it reaches the 3' end of the transcript, which is an unnatural situation. This strategy is not often used because it requires a lot of effort to generate an unnatural situation. More often only the normal start codon is mutated, while downstream AUGs are left unchanged. For example, mutating the normal start codon of the pea *fed1* gene led to a reduction in the magnitude of regulated stability, indicating that translation of the internal light regulatory element is important for its function (Dickey et al., 1994).

Coding region instability determinants

Further indications that translation and mRNA degradation are linked are that some determinants of mRNA half-life have been found in the coding region, and that their effects are dependent on their translation. One well-characterized example of a coding region determinant that links translation and mRNA degradation can be found in the yeast matα1 gene. A 65 bp region of the matα1 gene can destabilize a reporter gene when it is inserted into the coding region (Parker and Jacobson, 1990; Caponigro et al., 1993). This region consists of two parts. The first part is rich in rare codons, while the second part is AU-rich (Parker and Jacobson, 1990). That translation is important for the function of this determinant was indicated by the fact that the first part can be replaced by other rare codons (Caponigro et al., 1993). This part of the coding region by itself does not have destabilizing activity but can stimulate the destabilizing activity of the AU rich region, probably by pausing the translating ribosomes so that they are in the correct position to interact with the second part of the coding region (Caponigro et al., 1993;

Hennigan and Jacobson, 1996).

Other coding region determinants of mRNA stability have been found in the mammalian c-myc and c-fos transcripts and in the yeast STE3 and SPO13 transcripts (Shyu et al., 1991; Wisdom and Lee, 1991; Heaton et al., 1992; Surosky and Esposito, 1992) but are not as well-defined. Whether any of these coding region determinants interact directly with ribosomes is unknown, but at least in some cases their translation is required. Normally stable reporter genes can be destabilized by insertion of *c-myc* or *c-fos* coding region determinants (of 250- 320 bp), but this effect is overcome if translation of the mRNA is prevented by inserting a stable secondary structure in the 5' UTR (Schiavi et al., 1994) or by mutating the normal start codon (Wisdom and Lee, 1991). However, recognition of the *c-fos* coding region determinant is dependent on the RNA sequence itself (Wellington et al., 1993), not on the amino acid sequence or the codon usage (as described above for *β-tubulin* and *matα1* respectively).

In contrast to the coding region determinants that require translation for their activity, some determinants normally found in the 3' UTR are inactive when inserted into the coding region. This was demonstrated for the AU-rich region of the 3' UTR of GM-CSF. This element can destabilize a β-globin reporter transcript when inserted into the 3' UTR of the reporter gene but does not confer instability when it is inserted in the coding region of the same reporter gene (Savant-Bhonsale and Cleveland, 1992).

One explanation for the different effects of inserting instability determinants in the coding region versus the 3' UTR is that to function correctly these elements need to bind specific proteins, and ribosomes may interfere with the correct assembly of this RNA-protein complex. Alternatively, passing ribosomes may alter the secondary structure of

the mRNA, which may affect the function of instability determinants.

The *fed1* internal light regulatory element mentioned above (which includes the 5' UTR and part of the coding region) is the only characterized coding region determinant from a plant gene. The *SAUR-AC1* coding region also contains sequences that result in decreased mRNA abundance. However, transcripts containing the *SAUR-AC1* coding region were found to be stable when mRNA half-lives were measured directly (Gil and Green, 1996). It is not clear what step in gene expression is affected by the *SAUR-AC1* coding region.

Nonsense-mediated mRNA decay

In most if not all eukaryotes some mRNAs that contain nonsense mutations are rapidly degraded. This process is the best-characterized example of a link between translation and mRNA degradation and has been named nonsense-mediated mRNA decay or mRNA surveillance. Nonsense-mediated mRNA decay has been studied in yeast (Peltz et al., 1994), mammals (Maquat, 1995), *Xenopus* (Whitfield et al., 1994), *C. elegans* (Pulak and Anderson, 1993) and plants (see chapters two and three of this thesis). Similar effects described in *E. coli* (e.g. Nilson et al., 1987) may be functionally alike to nonsense-mediated decay, but may operate by a different mechanism.

Conserved characteristics of Nonsense-mediated mRNA decay

A number of the main characteristics of nonsense-mediated mRNA decay are conserved among all eukaryotes. In yeast (Losson and Lacroute, 1979; Peltz et al., 1993), mammals (Cheng et al., 1990), and plants (see chapter two), nonsense codons in the 5' part of an mRNA are effective in triggering the decay of the transcript, while nonsense codons in the 3' part of the same transcript have no effect. In both yeast and mammals this is dependent at least in part on certain sequences downstream of the premature nonsense codon, although the sequence requirements are very different in these two systems (see below).

The available evidence indicates that recognition of nonsense codon-containing transcripts occurs by the translational machinery. In both yeast and mammals nonsense codon-containing mRNAs are stabilized by expression of suppressor tRNAs capable of translating the appropriate nonsense codon, but not by other tRNAs (Losson and Lacroute, 1979; Takeshita et al., 1984; Gozalbo and Hohmann, 1990; Belgrader et al., 1993). In addition inhibition of translation by insertion of a strong secondary structure in the 5' UTR stabilizes mammalian mRNAs containing premature nonsense codons (Belgrader et al, 1993).

In both yeast (Leeds et al., 1991; 1992) and nematodes (Pulak and Anderson, 1993), there are specific *trans*-acting factors that are required for nonsense-mediated mRNA decay, and some of these factors are homologous to each other. For example, the best-characterized factor involved in nonsense-mediated mRNA decay is UPF1p from yeast. The smg2 gene product from *C. elegans* is also involved in nonsense-mediated

mRNA decay, and has extensive sequence similarity to UPF1p. Recently cDNAs with sequence similarity to UPF1p were isolated from mouse and human, and a fusion gene encoding most of the human protein and N- and C-terminal parts of the yeast UPF1 protein was able to complement a defect in the yeast gene (Perlick et al., 1996), which may indicate that this protein functions in nonsense-mediated mRNA decay in mammals. However, the UPF1 gene product has two genetically separable functions. In addition to its role in nonsense-mediated mRNA decay UPF1p also functions in translation (Weng et al., 1996a, b). It remains to be shown whether the mammalian homologs also function in both roles.

As has been discussed above, poly(A) shortening is an important first step in the degradation of most mRNAs in yeast and of at least some mRNAs in mammalian cells. The decay of nonsense codon-containing transcripts in these same systems does not require shortening of the poly(A) tail. In yeast, nonsense-mediated mRNA decay is achieved by decapping the fully polyadenylated transcript, followed by a 5' to 3' exonucleolytic decay of the body of the transcript (Muhlrad and Parker; 1994). The requirement of poly(A) shortening for nonsense-mediated mRNA decay in mammals was tested by fusing a nonsense codon containing *β-globin* coding region to the *c-fos* promotor. Transient induction of the *c-fos* promotor by serum addition enabled the measurement of both mRNA degradation and poly(A) shortening rates. While this mRNA was very unstable, the poly(A) tail was shortened slowly (Shyu et al., 1991). In addition, expression of *β-globin* genes with inserted nonsense codons in transgenic mice leads to accumulation of shorter-than-expected transcripts (as well as full length mRNA). These shorter transcripts appear to be decay intermediates, and are missing varying

amounts of the 5' end of the transcript, yet copurify with poly(A)$^+$ RNA (Lim et al., 1989; 1992). This suggests that nonsense-mediated mRNA decay mechanisms may be conserved between yeast and mammals, and that it involves 5' to 3' exonucleolytic decay without previous shortening of the poly(A) tail.

Differences is nonsense-mediated mRNA decay in eukaryotes

In contrast to the conserved features of nonsense-mediated mRNA decay mentioned above, there are certain variations among the process in different eukaryotes. Two distinctions have been described in some detail. The first difference concerns the mechanism by which cells discriminate between normal nonsense codons and premature nonsense codons. To distinguish between the two types of nonsense codons, additional sequence elements are needed. One of the elements from yeast (TGYYGATGYYYYY) is fairly well-characterized (Zhang et al., 1995) and appears to function by destabilizing transcripts if present downstream of the nonsense codon, while having no effect when located upstream of the nonsense codon. It is clear that yeast has additional *cis*-acting sequences, but these have not been well-characterized (Peltz et al., 1993). Mammalian cells discriminate between premature and wild-type nonsense codons by their position relative to introns: Nonsense codons upstream of the last intron of the coding region of TPI, aprt and MUP genes are recognized as being premature nonsense codons and the transcripts are targeted for rapid degradation (Urlaub et al., 1989; Cheng et al., 1990; 1994; Kessler and Chasin, 1996; Belgrader and Maquat, 1994). In contrast nonsense codons in the last exon of the coding region are not recognized as being premature, and

hence the mRNA is stable. In neither yeast nor mammalian cells is it clear exactly how these additional sequences function.

The other well-characterized difference in nonsense-mediated decay is the subcellular location of the pathway. In yeast nonsense-mediated mRNA decay is a cytoplasmic process, while in mammalian cells at least some nonsense codon-containing transcripts are degraded while they are still associated with the nucleus. The evidence supporting these conclusions is discussed in detail in the introduction of chapter three of this thesis.

SCOPE OF THIS THESIS

From the data described above it seems clear that translation and mRNA degradation are intimately linked processes. A wide variety of links have been described in mammalian cells and yeast, but comparatively little is known about these links in plants. The work described in this thesis is aimed at providing us with a better understanding of the effects of translation on mRNA stability in plants.

Chapter 2 of this thesis describes the first convincing evidence that plants, like other eukaryotes, contain a nonsense-mediated mRNA decay pathway. This was achieved by directly measuring the stability of transcripts with premature nonsense codons and comparing that with the stability of the wild-type transcript. In addition chapter 2 describes data that show that nonsense codons in the 5' part of an mRNA are effective in triggering mRNA degradation, while nonsense codons further downstream have no such effect, and that this does not require the presence of introns. Chapter 3

describes my attempts to determine the subcellular location of the nonsense-mediated mRNA decay pathway in plants. The results indicate that this pathway appears to be cytoplasmic, making plants more similar to yeast than mammals in this respect.

In contrast to the well documented effects of premature nonsense codons on mRNA stability, there has been speculation but very little data on the effects of rare codons on mRNA stability. Chapter 4 describes an attempt to show directly that rare codons can destabilize PHA reporter transcripts, but the resulting data indicate that rare codons are not sufficient to destabilize this reporter mRNA.

An appendix to this thesis describes the characterization of an unusual set of transcripts from *Arabidopsis* and tobacco. The first report of this group of transcripts came from a screen for genes with unstable transcripts (GUTs) by Taylor and Green (1995), but subsequently, similar transcripts were found in *Arabidopsis*, cucumber, and potato. These transcripts are not typical protein-encoding mRNAs and are unlikely to be translated. This may be related to the extremely rapid degradation of the transcript in tobacco.

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CHAPTER 2

PREMATURE NONSENSE CODONS DECREASE THE STABILITY OF PHYTOHEMAGGLUTININ mRNA IN A POSITION-DEPENDENT MANNER

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ABSTRACT

Premature termination of translation has often been associated with decreased mRNA accumulation in plants, but the affected step in gene expression has not been identified. To investigate this problem, I have examined the expression of wild-type and mutant alleles of the bean phytohemagglutinin (*PHA*) gene in tobacco cells and transgenic plants. Measurement of mRNA decay rates in stably transformed cell lines demonstrated that premature nonsense codons markedly destabilized the mRNA. This decreased stability was also reflected by decreased accumulation of transcripts containing premature nonsense codons in transgenic plants. The positional dependence of the nonsense codon effect was evaluated by introducing premature nonsense codons at different distances from the *PHA* AUG start codon. Transcripts with nonsense codons about 20%, 40% or 60% of the way through the normal *PHA* coding region yielded highly unstable mRNAs, whereas a transcript with a nonsense codon at 80% was as stable as wild-type. The ability to recognize and rapidly degrade certain transcripts with early nonsense codons could provide plant cells with a means to minimize the production of wasteful and possibly deleterious truncated proteins.

INTRODUCTION

Eukaryotic gene expression is a highly regulated process that involves many steps. A majority of the studies on gene expression have focused on transcription and the regulation thereof. More recently it has become clear that control of gene expression can also result from regulation exerted at later steps, such as splicing (Smith et al., 1989; Luehrsen et al., 1994), mRNA degradation (Sullivan and Green, 1993; Surdej et al., 1994), or translation (Altmann and Trachsel, 1993; Hershey, 1991). Considerable evidence suggests that these steps are not independent, but are influenced by each other (e.g. Urlaub et al., 1989; Dickey et al., 1994). In particular, it appears that translation and mRNA degradation can be tightly coupled (Peltz et al., 1991; Sullivan and Green, 1993). For some transcripts, translation is necessary for normal rates of mRNA degradation (Cleveland, 1988; Edwards and Mahadevan, 1992; Aharon and Schneider, 1993), whereas for others interruption of translation leads to increased degradation rates (Peltz et al., 1992; Losson and Lacroute, 1979).

In plants, most of the data indicating a linkage between translation and mRNA stability derive from the effects of mutations creating premature nonsense codons. Several studies indicate that these mutations lead to decreased transcript accumulation. In one case it was found that a natural allele of a Kunitz trypsin inhibitor gene (*Kti3*) of soybean caused low accumulation of mRNA. This allele contained a point mutation about 70% into the coding region that introduced a premature nonsense codon (Jofuku et al., 1989). Transcription rates of the wild-type and mutant genes, as measured by nuclear run-on analysis, were not significantly different. This suggested that the effect was post-

transcriptional, possibly at the level of mRNA stability (Jofuku et al., 1989). In an other case it was found that a natural allele of the phytohemagglutinin (*PHA*) gene from common bean (*Phaseolus vulgaris*) led to greatly reduced levels of *PHA* mRNA (Voelker et al., 1986). Sequencing showed that this allele contained a frame-shift mutation that led to a premature nonsense codon in the new reading frame about 20% of the way through the PHA coding region. Replacement of the wild-type coding region with that from the frame-shift allele decreased expression in transgenic plants, and correction of the mutation restored wild-type expression levels (Voelker et al., 1990). From these studies it can be concluded that the frame-shift mutation decreased mRNA levels, but the affected step in gene expression was not identified. It appears that a premature nonsense codon may also decrease mRNA accumulation for a chimeric patatin gene in transgenic tobacco (Vancanneyt et al., 1990). An additional example of premature nonsense codons interfering with normal transcript accumulation has been found for the pea ferredoxin gene (*FED1*) which is post-transcriptionally regulated by light (Dickey et al. *1994*). The *cis*-acting sequences regulating the response to light partially overlap with the coding region. When nonsense codons were introduced into the *FED1* coding region, the accumulation of the mRNA failed to respond to light (Dickey et al., 1994). If light stabilizes the *FED1* mRNA as has been proposed (Dickey et al. 1992), then the introduction of nonsense codons could interfere with this effect. Alternatively, nonsense codons may destabilize or otherwise affect the abundance of the *FED1* message by a pathway that is independent of light. Measurements of mRNA decay rates will be required to resolve among these possibilities. Finally, recent advances in map-based cloning have led to the molecular characterization of a number of mutant alleles from

Arabidopsis thaliana and other plants. In several cases, a correlation between the presence of nonsense or frame-shift mutations and reduced mRNA levels can be seen (e.g. Dehesh et al., 1993; Reed et al., 1993). This suggests that premature nonsense codons may decrease mRNA accumulation when present in a wide variety of plant genes.

Premature termination of translation also has a pronounced effect on post-transcriptional mRNA metabolism in *Saccharomyces cerevisiae* (yeast). The effect was shown to occur at the level of mRNA stability by measuring mRNA decay rates after transcriptional inhibition, or by estimating mRNA decay rates by approach to steady state labeling (for a discussion of this method, see Ross, 1995). Prominent examples include transcripts of the yeast genes URA3 (Losson and Lacroute, 1979), URA1 (Pelsy and Lacroute, 1984), LEU2 and HIS4 (Leeds et al., 1991), and PGK1 (Peltz et al., 1993), all of which were shown to be destabilized by premature nonsense codons. Molecular genetic studies in yeast and *Caenorhabditis elegans* indicate that several gene products participate in nonsense-mediated mRNA decay (Leeds et al., 1991; Pulak and Anderson, 1993).

Examples of reduced mRNA abundance caused by premature termination of translation in mammalian systems have also been reported. Similar to the situation in yeast, premature nonsense codons decreased the stability of the human (Maquat et al., 1981) and rabbit β-globin mRNAs (Shyu et al., 1991), and the Rous sarcoma virus gag pre-mRNA (Barker and Beeman, 1991), when mRNA half-lives were monitored in cells following transcriptional inhibition. However, premature nonsense codons that decrease mRNA levels are not always associated with decreased mRNA stability in similar experiments. Messenger RNA decay measurements made following transcriptional

inhibition, were not indicative of accelerated mRNA decay for hamster dihydrofolate reductase (Urlaub et al., 1989), human triosephosphate isomerase (Cheng et al., 1990), v-src of avian sarcoma virus (Simpson and Stoltzfus, 1994), or minute virus of mice NS2 (Naeger et al., 1992) mRNAs that contained premature nonsense codons. The exact mechanism(s) by which nonsense codons decrease mRNA abundance in these cases, (some of which depend on the presence of introns), is not known (Maquat, 1995).

Our knowledge of the effects of premature nonsense codons in plants is even more limited because, prior to this report, rates of mRNA degradation were never measured for any of the plant examples. The goal of this study was to use the *PHA* gene as a model to better understand the decreased mRNA accumulation caused by the presence of premature nonsense codons in certain plant transcripts. To this end, I investigated the expression of a set of natural and *in vitro* generated *PHA* alleles containing premature nonsense codons in cultured tobacco cells and transgenic plants. These studies demonstrate that premature nonsense codons decrease *PHA* mRNA accumulation at the level of mRNA stability, and therefore indicate that a nonsense-mediated mRNA decay pathway exists in higher plants. I also examined how far into the PHA coding region a nonsense codon can be positioned before it no longer causes rapid mRNA decay.

RESULTS

Premature nonsense codons lead to decreased mRNA accumulation in protoplasts.

The *PHA* gene system from common bean has several features that make it attractive for the study of premature nonsense codons. First, the wild-type (WT) allele and a natural frame-shift (FS) allele of *PHA* have been well characterized. As illustrated in figure 2-1, a frame-shift mutation in codon 11, which creates a nonsense codon at codon 53, is the only difference between the FS and WT alleles (Voelker et al., 1990). Second, the decreased mRNA accumulation attributable to the FS allele in bean can be recapitulated in tobacco (Voelker et al., 1990), which is easy to transform and regenerate. Finally The *PHA* structure is relatively simple. It contains a 5'UTR of 10-15 bp (Hoffman and Donaldson, 1985), a coding region of 825 bp that has a typical codon usage and is not interrupted by introns (Hoffman and Donaldson, 1985 and data not shown) and a 3'UTR of 126-132 bp (as determined by RT-PCR of the 3'UTR, data not shown).

It seemed likely that the decreased abundance of the FS *PHA* mRNA was caused by the premature nonsense codon, but alternative effects of the FS mutation could not be excluded. To resolve this issue, I constructed a third allele, designated STOP, by introducing a nonsense codon via site-directed mutagenesis. The STOP mutation was derived from WT by making a two base substitution in codon 53 to create a nonsense codon (Figure 2-1). Thus, the only common characteristic differentiating FS and STOP from WT is a premature nonsense codon at codon 53. Previous studies comparing *PHA* alleles in tobacco were carried out in seeds where the *PHA* promoter is preferentially

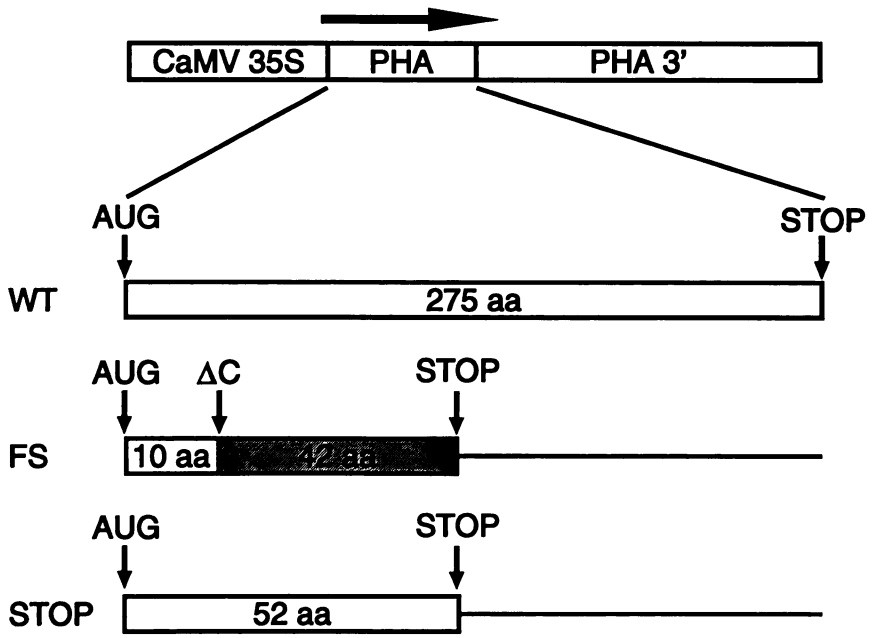

FIGURE 2-1. *PHA* alleles introduced into tobacco cells and transgenic plants. Initially three different *PHA* alleles (WT, FS and STOP) were constructed. Each allele contained the CaMV 35S promoter (CaMV 35S), a PHA coding region (PHA) and 1.4 kb of 3' flanking sequence (PHA3'). The transcribed region is indicated by an arrow. A 1-bp deletion in the frame-shift allele (FS) is indicated by ΔC. This deletion leads to a frame-shifted reading frame, indicated by the shaded box and a premature nonsense codon in the 53rd codon. A control allele (STOP) was constructed that contains a premature nonsense codon in the same position as the FS, without the frame-shift mutation.

expressed (Voelker et al., 1990). To facilitate analyses using tobacco leaf tissue and cultured tobacco cells, the *PHA* promoter was replaced by the 35S promoter in alleles used for this study.

Previous work has shown that the established tobacco cell line BY-2 (also called NT1) provides a useful system for mRNA stability measurements (Newman et al., 1993; Ohme-Takagi et al., 1993). Therefore, in preparation for such analyses each of the *PHA* alleles was initially examined for expression levels following electroporation into BY-2 protoplasts. Total RNA was isolated 14 hr after electroporation and analyzed by RNA gel blotting. Figure 2-2a shows that the mRNA from WT *PHA* accumulated to a substantial level, while the mRNA from FS or STOP *PHA* was barely detectable. Quantitation of the transcript levels for the different *PHA* alleles relative to that of a *GUS* gene, co-electroporated as an internal standard is represented in Figure 2-2b. These data show that the abundance of the *PHA* FS and STOP transcripts was reduced approximately fivefold as compared to WT *PHA* mRNA. The faint extra band that can be seen above the *PHA* mRNA in Figure 2-2a was observed whenever *PHA* was expressed, but is more prominent after the long exposures needed to visualize destabilized versions of the *PHA* mRNA (e.g. Figure 2-3b). The band was enriched in poly A^+ fractions, was DNase I resistant, hybridized to a strand-specific probe, was not observed with other 35S promoter constructs, but was observed when another promoter (from the wheat cab1 gene) was used to control *PHA* (data not shown). Therefore, this band most likely represents polyadenylation at a site far downstream of the major polyadenylation site. Because the FS and STOP alleles cause decreased mRNA accumulation in the absence of the *PHA* promoter, it is likely that the effect is post-transcriptional. The decreased transcript

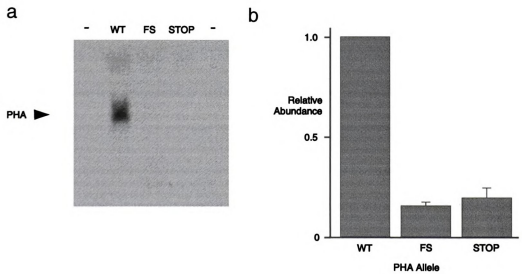

FIGURE 2-2. Decreased expression of the FS and STOP transcripts in tobacco protoplasts. **(a).** *PHA* mRNA accumulation. Tobacco BY-2 protoplasts were electroporated with Bluescript derivatives containing the WT, FS, and STOP *PHA* alleles described in Figure 2-1a. A Bluescript derivative containing a *35S-GUS* gene was co-electroporated with each *PHA* allele to serve as an internal standard. RNA was isolated 14 hr after electroporation and 20µg was analyzed on RNA gel blots. (-) represents control electroporations without *PHA* DNA.**(b).** Relative abundance of *PHA* transcripts. RNA levels of three independent experiments were quantified using a PhosphorImager. The *PHA* transcript level for the different alleles was standardized to that of the *GUS* transcript and the relative transcript accumulation for WT was set to 1. Shown is the mean relative abundance of *PHA*. Error bars indicate the standard error (SE).

accumulation associated with the FS mutation in BY-2 cells is analogous to the effects of the FS mutation originally observed in seeds of bean and tobacco (Voelker et al., 1986; 1990). This indicates that the BY-2 system will be a valid model for further study of premature nonsense codons.

Premature nonsense codons cause rapid degradation of mRNA

To test whether the premature nonsense codons exert their effect at the level of mRNA stability, the WT, FS and STOP alleles were stably introduced into BY-2 cells using *Agrobacterium*-mediated transformation. Messenger RNA decay rates were measured in individual transformed cell lines that were grown in liquid culture. Following treatment with actinomycin D to stop transcription, total RNA was isolated from cells harvested at regular time intervals and analyzed by RNA gel blotting. Representative experiments measuring the decay of the WT and FS mRNAs are shown in figure 2-3. WT *PHA* mRNA was quite stable (Figure 2-3a and 2-3c), whereas the FS mRNA disappeared rapidly (Figure 2-3b and 2-3c; note the different time scale between Figures 2-3a and 2-3b). These mRNA half-life measurements were repeated at least four times on at least two independent transformants for each of the three *PHA* alleles. The mean half-lives of the *PHA* transcripts as well as the standard error are represented in Figure 2-4. The half-lives of both the FS and STOP transcripts were reduced about 3-fold relative to the WT (36±6 and 43±4 min respectively as compared to 129±14 min), showing that premature nonsense codons can cause rapid degradation of the *PHA* mRNA in tobacco.

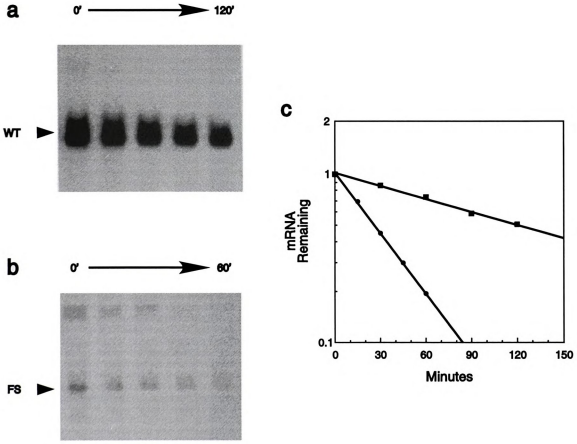

FIGURE 2-3. Destabilization of the *PHA* mRNA by the FS mutation in stably transformed tobacco cells. **(a).** RNA gel blot monitoring the decay of WT *PHA* mRNA. Tobacco BY-2 cells were stably transformed with a pMON505 derivative containing the WT *PHA* allele. A cell line that expressed the *PHA* gene was treated with actinomycin D (ActD) to stop transcription and RNA was isolated at 30 min intervals for 2 hr thereafter. The gel blot contained 20µg total RNA per lane and was probed for the *PHA* transcript. **(b).** RNA gel blot monitoring the decay of the FS *PHA* mRNA. As in a, except that a cell line expressing the FS allele of *PHA* was used and RNA was isolated from samples collected at 15 min intervals after ActD treatment for a period of 1 hr. **(c).** Graphic representation of mRNA decay rates. Signals from the blots shown in a and b were quantified using a PhosphorImager, normalized to the zero time point, and subjected to linear regression analysis to calculate mRNA half-lives. Boxes represent the WT transcript from a. Circles represent the FS transcript from b.

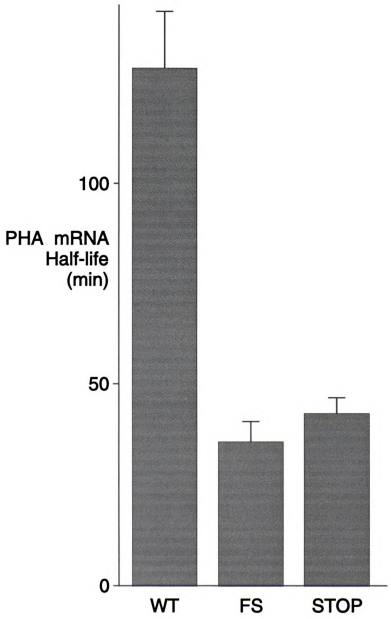

FIGURE 2-4. Effects of the FS and STOP mutations on *PHA* mRNA stability in tobacco. Messenger RNA half-life measurements were made as shown in Figure 2-3 from at least four independent experiments carried out using at least two independent tobacco BY-2 cell lines per construct. Mean *PHA* mRNA half-lives for the individual constructs were calculated. Error bars represent the SE.

Premature nonsense codons lead to decreased mRNA accumulation in transgenic plants

To study the effect of premature nonsense codons in transgenic plants, the WT, FS, and STOP alleles were introduced into tobacco. Nine to ten transgenic tobacco plants expressing *PHA* were regenerated for each construct. RNA was isolated from an individual leaf from each plant and analyzed by RNA gel blotting. *PHA* mRNA levels were quantitated and are shown in figure 2-5a. Although there is considerable variation in expression levels between individual transformants (presumably due to position effects), statistical analysis shows that premature nonsense codons significantly decrease the accumulation of *PHA* mRNA. Compared to WT, the average mRNA level of FS and STOP was reduced 5 to 10-fold and this difference is highly significant ($P>0.975$), according to the test of Wilcoxon-Mann-Whitney (Nap et al., 1993). As expected there was no significant difference between mRNA levels for the FS and STOP alleles. Decreased mRNA accumulation was also clearly evident when equal amounts of RNA from FS and STOP plants were pooled and analyzed in the RNA gel blot in figure 2-5c. As a further confirmation the WT and STOP constructs were introduced into *Arabidopsis* and mRNA levels were again compared in individual transgenic plants and pooled RNA samples. The results were similar to those obtained with tobacco as shown in Figure 2-5b and d. The average mRNA accumulation for the FS construct was reduced 5-fold, and this difference was statistically significant ($P>0.999$). Based on these results, it is likely that premature termination of *PHA* mRNA translation leads to increased mRNA degradation rates in transgenic tobacco and *Arabidopsis* plants, as it does in tobacco cells.

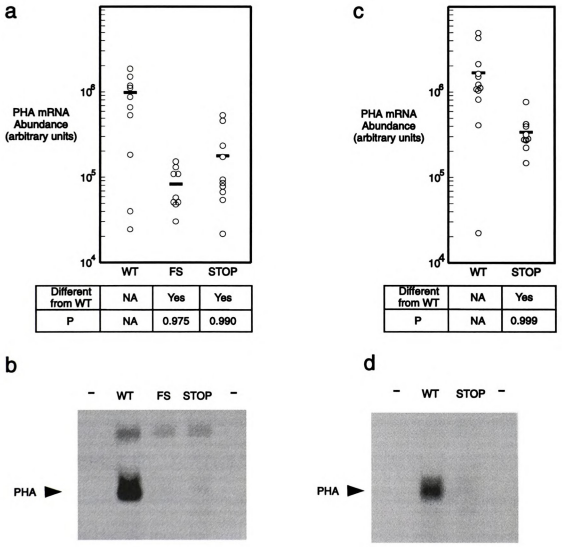

FIGURE 2-5. Reduced accumulation of the FS and STOP transcripts in transgenic plants. **(a).** Relative abundance of different *PHA* transcripts in transgenic plants. RNA levels of at least nine independent transgenic plants per construct were quantified using a PhosphorImager. Points represent the data from individual plants. The average for each construct is indicated by a black bar. Statistical analyses using the Wilcoxon-Mann-Whitney test are presented in the table below the plot. NA indicates not applicable and P indicates the probability that the expression level is different from that of WT. **(b).** Same as a except that RNA from at least 11 independent *Arabidopsis* transformants was used. **(c)**. Equal amounts of the RNA from each plant were pooled and 10µg was used for RNA gel blot analysis. Hybridization was as in Figure 2-3. (-) represents control RNA from an untransformed tobacco plant. The band above the *PHA* transcripts is the same as that in Figures 2-2a and 2-3b and probably represents polyadenylation at a site downstream of the normal site, as explained in the text. **(d)**. Same as c except that *Arabidopsis* RNA was used.

The effect of premature nonsense codons is position-dependent

In all of the aforementioned experiments, the premature nonsense codon was located at the same position, 20% of the way through the *PHA* coding region. However it is unclear whether premature termination of PHA translation further downstream could also decrease mRNA stability. To address this question three additional alleles were made, each containing a premature nonsense codon about 40%, 60%, or 80% of the way through the normal *PHA* coding region as shown in Figure 2-6a. Each of these *PHA* alleles was stably transformed into BY-2 cells and mRNA decay was monitored as described above for the FS, STOP (20%), and WT constructs. Multiple half-life measurements were made on two to four independent cell lines for the 60 and 80% constructs and one cell line for the 40% construct. The half-lives of transcripts with a nonsense codon at 40% (35±4 min) or 60% (38±3 min) of the way through the coding region were indistinguishable from those of the unstable FS and STOP transcripts (Figure 2-6b). In contrast, the transcript with a nonsense codon at 80% was at least as stable (161±18 min) as WT *PHA* mRNA. These data indicate that nonsense codons up to 60% of the way through the coding region can trigger the nonsense-mediated mRNA decay of *PHA* mRNA in tobacco, but the machinery apparently fails to discriminate between the 80% nonsense codon and the normal nonsense codon.

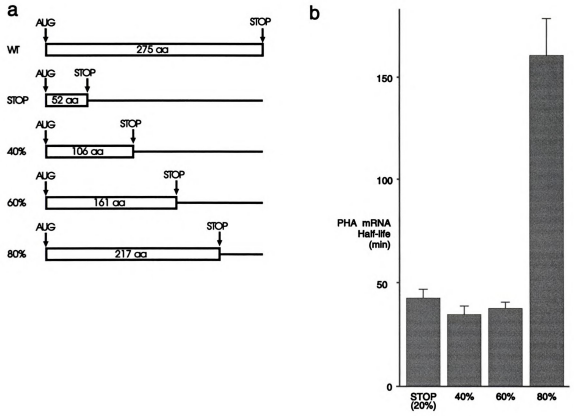

Figure 2-6. Premature nonsense codons in the first 60% of the *PHA* coding region cause mRNA instability. **a.** Introduction of nonsense codons at different positions within the *PHA* coding region. *PHA* alleles containing premature nonsense codons about 40%, 60%, and 80% of the way through the coding region are compared diagrammatically to the WT and STOP (20%) alleles. Open bars represent the open reading frame and indicate the number of amino acids (aa) before the nonsense codons that are indicated by stop arrows. Untranslated regions are represented by lines. All alleles were cloned into pMON505 derivatives for stable introduction into BY-2 cells. **b.** Effects of premature nonsense codons on *PHA* mRNA stability. Multiple mRNA half-life measurements were made for the cell lines stably expressing each *PHA* allele in a. Mean half-lives, calculated as in Figure 2-4, are represented in the histogram. Error bars represent the SE.

DISCUSSION

In this report I examined the effect of premature nonsense codons on *PHA* mRNA metabolism. The half-life of *PHA* transcripts containing a frame-shift (FS) or nonsense (STOP) mutation in tobacco BY-2 cells was significantly reduced as compared to the wild-type (WT) *PHA* transcript, showing that premature termination of translation (20% of the way through the coding region) can lead to increased rates of mRNA decay. These results were confirmed by demonstrating that *PHA* transcripts with nonsense codons 40% or 60% of the way through the coding region were also rapidly degraded. Further support for a destabilizing effect of premature nonsense codons was obtained by analyzing the effect of the FS and STOP mutations on accumulation of *PHA* mRNA in BY-2 protoplasts and transgenic tobacco and *Arabidopsis* plants. FS and STOP mRNA accumulated to barely detectable levels in all three systems.

It was reported previously that the FS mutation reduced the accumulation of *PHA* mRNA in bean and transgenic tobacco seeds (Voelker et al., 1986; 1990). Based on these reports, it was not clear whether this effect was caused by premature termination of translation, a change in the structure of the mRNA, or a change in codon usage in the alternative reading frame. Another consideration was that the FS mutation, located in codon 11, disrupted the PHA signal sequence, which could potentially change its site of translation from membrane-bound to soluble polyribosomes. To differentiate among these alternative explanations, in this study I created the STOP allele, which had the same early nonsense codon as the FS, but no alteration in the reading frame. The FS mutation (a one-base deletion) and the STOP mutation (a two-base substitution) were separated by more

than 100 nucleotides, which makes it very unlikely that they both disturb a stabilizing sequence in the transcript or have a similar effect on secondary structure. In addition, the codon usage of the STOP allele is identical to that of the WT *PHA*. Therefore, premature termination of translation is the only plausible cause of the rapid degradation of the mutant transcripts. Moreover, a premature nonsense codon is the common denominator among the four *PHA* transcripts that were found to be unstable (FS, STOP, 40%, and 60%) in this report. This work extends previous studies both by showing that it is premature termination of translation that causes low accumulation of *PHA* mRNA, and by demonstrating that the degradation of the transcripts is markedly accelerated.

The nonsense-mediated mRNA decay pathway that facilitates the rapid decay of certain *PHA* transcripts may extend to other transcripts in higher plants. As discussed in the introduction, a number of other transcripts with premature nonsense codons also fail to accumulate to wild-type levels in plant cells. In addition, E.J. De Rocher and P.J.Green (personal communication) have identified a derivative of a synthetic *Bacillus thuringiensis* toxin gene containing a premature nonsense codon. This mutant gene gives rise to much lower transcript levels than does the wild-type in maize and tobacco protoplasts and stably transformed BY-2 cells . The work in this report demonstrates that early nonsense codons in *PHA* can also be recognized in different plants, organs, and cells in which *PHA* is not normally expressed. On the basis of all of these observations it seems likely that other mRNAs in addition to *PHA* transcripts may be subject to nonsense-mediated mRNA decay in plants and that this mechanism operates in at least three different plant species.

The effect of premature nonsense codons on *PHA* mRNA can be greatly

influenced by their position in the transcript because termination of translation 80% of the way through the *PHA* coding region did not trigger rapid decay. The information that allows plants to recognize certain nonsense codons as being premature is not contained in the nonsense codon itself. Therefore, there must either be an additional *cis*-acting sequence contained in the transcript, or simply the length of the translated or untranslated region may render the mRNA unstable. At present, there is no evidence for a minimal length of a coding region or a maximal length of a 3' untranslated region for efficient gene expression. However, there is precedence for the presence of *cis*-acting sequences for nonsense-mediated mRNA decay in yeast (Peltz et al., 1993). This has been studied in the most detail for the *PGK*1 gene. Premature nonsense codons in the first two-thirds of the *PGK*1 coding region cause rapid degradation of the mRNA, whereas nonsense codons in the last quarter do not (Peltz et al., 1993). Nonsense-mediated mRNA decay of the *PGK*1 mRNA appears to be mediated by two types of *cis*-acting sequences, one of which has been delineated fairly precisely and must be located downstream of a nonsense codon to have an effect on mRNA abundance (Peltz et al., 1993; Zhang et al., 1995). The second more loosely defined sequence, located between 67 and 92% of the *PGK*1 coding region, has been hypothesized to prevent downstream nonsense codons from causing rapid degradation when it is translated (Peltz et al., 1993).

These results point to the sequences located between 60 and 80% of the way through the *PHA* coding region as probable candidates for *cis*-acting elements controlling nonsense-mediated decay. An mRNA instability sequence that acts when it is downstream of a premature stop codon could be present in this region. This scenario would explain why the *PHA* transcripts with nonsense codons located in the first 60% of the *PHA*

coding region are unstable, whereas those which terminate translation in the last 20% are not. If such an element exists, it is likely to differ from the *cis* element that must be located downstream of a premature stop codon in *PGK*1, discussed above. The *PGK*1 sequence is not found in *PHA* and the closest match is found upstream of the 60% position. Another possibility is that the region between 60% and 80% contains a sequence that stabilizes the transcript if it is translated, similar to that of the second element hypothesized to act in *PGK*1. In any event, the requirement for additional sequences might indicate that not all messages can be destabilized by premature nonsense codons. In addition, the exact location of these *cis*-elements in different transcripts could easily differ and thus determine the positions in the transcript where nonsense codons would have an effect. Clearly, more experiments will be required to examine the nature of putative *cis*-elements in the *PHA* transcript and to explain how these elements mediate recognition of premature nonsense codons in a position-dependent manner.

The location in the cell where nonsense-mediated mRNA decay takes place is somewhat controversial. There is considerable evidence for recognition and degradation of these transcripts during export from mammalian nuclei and for a likely cytoplasmic recognition and degradation event in yeast (Maquat, 1995). It is not clear whether these are fundamentally different mechanisms or variations on the same theme. With the tools and knowledge resulting from the present study, it should be possible to address the question of where *PHA* transcripts with premature nonsense codons are degraded in plant cells and to investigate other aspects of the mechanism.

Plants, yeast (Leeds et al., 1991), mammals (Maquat, 1995) and *C. elegans* (Pulak and Anderson, 1993) all appear to have evolved mechanisms to accelerate the decay of

certain transcripts containing early nonsense codons. A major advantage associated with having a nonsense-mediated mRNA decay pathway would be that it allows organisms to minimize the production of truncated proteins from defective mRNAs. RNA processing reactions are not completely accurate, giving rise to abnormal messages by incorporating an incorrect nucleotide during transcription or by aberrant or incomplete splicing. Premature nonsense codons could also arise by somatic or germline mutation. Not only is the production of truncated proteins wasteful, but it could be detrimental to the cell. In *C. elegans*, some myosin nonsense mutations that are recessive in a wild-type background become dominant in mutants that lack the nonsense-mediated mRNA decay pathway (Pulak and Anderson, 1993). Presumably, the overproduction of truncated myosin polypeptides interferes with the assembly and/or function of normal myosin. Truncation of many other proteins could also cause ill effects. Thus, the ability to degrade even a subset of transcripts with premature nonsense codons may increase evolutionary fitness.

MATERIALS AND METHODS

Plasmid construction

Standard procedures were used for plasmid manipulation (Sambrook et al., 1989). Each chimeric *PHA* gene in Figure 2-1 contained the 35S promotor of CaMV, including 28 bp of the 5'UTR, followed by 12 bp of linker sequence (CAAGCTCAGATCTG). This was fused to *PHA* sequences consisting of 3 bp of the 5'UTR, all of the coding region and 1.4 kb of 3' flanking sequence (ending at the *Acc* I site in the genomic clone Voelker et

al., 1990). *PHA* genes were introduced between the *Sac* I and *Cla* I sites of a pMON 505 derivative that also contained a *35S-GUS* gene (as in Newman et al., 1993). Additional details of the gene construction are available on request. For electroporation experiments, the *PHA* genes were introduced between the *Sac* I and *Sal* I site of Bluescript II SK+ (Stratagene, La Jolla, CA).

Nonsense codons were introduced using the Muta-gene kit (Biorad, Hercules, CA) and the (antisense) oligonucleotides 5'GATTGGTTTATCGTAACTG3' for the STOP (20%) allele, 5'CAAAGGCAAGGCCCTAGGCGGGTCC3' for the 40% allele, 5'AGTTCACGTCGATTTAAATATGACG3' for the 60% allele, and 5'GGAAGAACGCTCTACTAGTCCACTGT3' for the 80% allele. The introduced nonsense codons are underlined. The mutagenic oligonucleotides for the 40%, 60% and 80% alleles also introduced a restriction endonuclease site that was used to identify putative mutants. All mutant alleles were confirmed by dideoxy sequencing. The resulting *PHA* alleles were cloned into pMON 505, as described above. After this work was completed it was found that the binary vector containing the FS allele contains a small (0.8kb) insertion outside the *35S-PHA* gene. This is unlikely to have an effect on its expression because the behavior of the STOP gene which lacks this insertion is identical to that of the FS gene.

BY-2 cell manipulations

Cell culture and transformation of Bright Yellow 2 (BY-2; Nagata et al., 1992; also known as NT1) tobacco cells was performed as described by Newman et al. (1993)

with the following modifications. All pMON505 derivatives were introduced into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation. Putative BY-2 transformants were screened for expression of the *GUS* gene by a histochemical assay using the substrate X-Gluc (Clontech, Palo Alto, CA; Jefferson et al., 1987) and for *PHA* expression by RNA gel blot analysis.

Protoplasts for electroporation were prepared by incubating BY-2 cells, three days after subculture, for 3-5 hr, at 28° C, in 2% cellulysin (Calbiochem, La Jolla, CA), 1% cytolase (Genencor International, Rolling Meadows, IL) and 0.2% pectolyase (Karlan, Santa Rosa, CA) in NT wash solution [3.4 g/l MS salts (Gibco BRL, Gaithersburg, MD), 30 g/l sucrose, 3 μM thiamine, 0.56 mM myoinositol, 1.3 mM KH_2PO_4, 54.1 g/l glycine betaine and 10^{-6} M 2,4-D pH 5.7]. The protoplasts were washed 3 to 4 times in approximately 20 volumes NT wash solution, and resuspended in electroporation buffer (10 mM HEPES pH 7.2, 150 mM NaCl, 4 mM $CaCl_2$, 400 mM mannitol) to a concentration of $4x10^6$ protoplasts per ml. One half ml of electroporation buffer containing 100 μg/ml of PHA encoding plasmid, 100 μg/ml of GUS encoding plasmid (under control of the 35S promoter) and 100 μg/ml sheared single-stranded salmon sperm DNA was added to 0.5 ml of protoplasts. Protoplasts were electroporated in a 24-well plate on ice at 350 V, 500 μF, and a 6 mm distance between electrodes. The protoplasts were harvested 14 hr after electroporation, and total RNA was isolated as described below. A 20 μg aliquot of total RNA was treated with 1 unit DNase I (RQ1; Promega, Madison, WI) for 15 min at 37° C to digest residual plasmid DNA.

Messenger RNA decay measurements following treatment with 100 μg/ml actinomycin D (Act D) were as described by Newman et al. (1993), except that for

analysis of FS and STOP constructs time points were 15 min apart instead of 30 min apart. Newman et al. (1993) showed that this concentration of actinomycin D inhibits transcription by 94%.

Transgenic tobacco

Transformation and regeneration of transgenic tobacco plants were performed as described by Newman et al. (1993). Plants were analyzed for GUS activity using 20 μg of protein extract of leaves in enzymatic assays using 4-methylumbelliferyl-β-D-glucuronide (United States Biochemical, Cleveland, OH; Jefferson et al., 1987) as substrate. Transgenic plants with detectable GUS activity were grown to the 10-15 leaf stage and RNA was isolated from a leaf near full expansion as described below. Statistical analyses were carried out using the test of Wilcoxon-Mann-Whitney (Nap et al. 1993).

Transgenic *Arabidopsis*

Binary vectors were introduced into *Agrobacterium* strain C58C1(pMP90) by electroporation. Transformation of *Arabidopsis* was done by vacuum infiltration, based on the method of Bechtold et al. (1993) with the following modifications. Plants of the ecotype Columbia were grown under a regime of 16h light, 8h dark at 20° C until the primary bolt was 5-15 cm long. *Agrobacterium* from a 500 ml overnight culture (of YEP medium [10 g/l Yeast extract, 10 g/l bacto peptone, 5 g/l NaCl, pH 7.0] supplemented with 50 mg/ml rifampicin, 25 mg/ml gentamycin and 100 mg/ml spectinomycin) was

pelleted and resuspended in 1 l of infiltration medium (0.5XMS salts [Gibco BRL, Gaithersburg, MD], 100 µg/ml myo-inositol, 10 µg/ml thiamine-HCl, 1 µg/ml nicotinic acid, 1 µg/ml pyridoxine-HCl, 5% sucrose, 2.5 mM MES pH 5.7, 44 nM Benzylaminopurine, and 200 ppm Silwet L-77 [OSI Specialties, Danbury, CT]). *Arabidopsis* plants were infiltrated with this suspension under 450 mm Hg vacuum for five min and returned to the growth chamber. Seeds of each plant were harvested separately. Approximately 2000 seeds from each plant were plated on media containing 30 µg/ml kanamycin and 500 µg/ml vancomycin. Using this method >95% of the infiltrated plants were transformed and 1-5% of the seed from an individual plant was transgenic. To ensure that each analyzed transformant was from an independent event, only one kanamycin resistant seedling from each infiltrated plant was used. This transformant was transferred to soil and the seed was harvested. In the next generation approximately 500 seeds from each independent transformant were plated on AGM plates (Taylor et al., 1993) supplemented with kanamycin (30 µg/ml) and vancomycin (500 µg/ml). Seedlings were harvested 7 days after germination and immediately frozen in liquid nitrogen.

RNA isolation and analysis

RNA was isolated by the method of Puissant and Houdebine (1990) with modifications as described by Newman et al. (1993), with the following exceptions. When RNA was isolated from protoplasts, the step involving grinding in liquid nitrogen was replaced by vortexing in GTC buffer (Newman et al., 1993) for 1 min. When RNA

was isolated from *Arabidopsis* seedlings, the step involving grinding in liquid nitrogen was replaced by lyophilizing the tissue and grinding it by vortexing in the presence of about 20 three mm glass beads for 30 seconds before addition of GTC buffer. RNA was separated on 1% agarose/formaldehyde gels, blotted, and hybridized as described by Newman et al. (1993). For the electroporation experiments, where a *35S-GUS* construct was co-electroporated with the *PHA* constructs, blots were probed with *PHA*, analyzed, stripped, and then reprobed with *GUS* and analyzed again. The *PHA* probe consisted of 8bp of the 5' UTR and all of the coding region. The *GUS* probe was as described by Newman et al. (1993). Both probes were labeled with ^{32}P by the random priming method (Feinberg and Vogelstein, 1983). RNA levels were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and half-lives determined by linear regression analysis using Sigma Plot software.

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CHAPTER 3

POLYRIBOSOMAL *PHA* mRNA CAN BE DEGRADED BY THE NONSENSE-MEDIATED mRNA DECAY PATHWAY

ABSTRACT.

In every eukaryote examined, it has been found that mRNA containing premature nonsense codons is preferentially degraded. Although general features of the nonsense-mediated decay pathway are conserved among eukaryotes some characteristics vary. Premature nonsense codon-containing transcripts from yeast and some mammalian genes appear to be degraded in the cytoplasm, while other mammalian nonsense-containing transcripts appear to be degraded while they are associated with the nucleus. Here I describe experiments to localize the nonsense-mediated decay of *PHA* mRNA in tobacco cells. A majority of nonsense-containing *PHA* mRNA was found to be associated with polyribosomes, indicating that it is located in the cytoplasm. This indicates that *PHA* mRNA containing premature nonsense codons is exported to the cytoplasm and assembled into polyribosomes before it is degraded.

INTRODUCTION.

Although nonsense-mediated decay appears to be universal in eukaryotes, important mechanistic differences among eukaryotes may exist. One of the most important differences in nonsense-mediated decay relates to the subcellular location of the pathway. In yeast and *Xenopus* nonsense-mediated decay appears to be cytoplasmic, while conflicting evidence exists for the subcellular location of this decay pathway in mammals. Some data support a nuclear nonsense-mediated mRNA decay process, while other data suggests a cytoplasmic mechanism.

The best evidence for a cytoplasmic nonsense-mediated mRNA decay pathway comes from microinjection experiments of *Xenopus* oocytes and embryos. Whitfield et al. (1995) injected mRNAs that contained premature nonsense codons and several control transcripts into the cytoplasm of *Xenopus* oocytes and embryos, and followed the decay of these transcripts over time. In these experiments the nonsense codon-containing mRNAs were degraded more rapidly than several control transcripts. This indicates that cytoplasmic mRNA can be degraded in a nonsense codon-dependent manner.

A large body of evidence strongly suggests that nonsense-mediated mRNA decay in yeast is also a cytoplasmic event, but alternative explanations for at least some of the results are possible. First, UPF1p, a *trans*-acting factor involved in nonsense-mediated decay has been localized in two independent studies (Peltz et al., 1993; 1994; Atkin et al., 1995). In both cases the majority of UPF1p was localized to the cytoplasm and was associated with polyribosomes. However, subsequently it was shown that UPF1p has two genetically separable functions (Weng et al. 1996 a, b) One function of UPF1p is related

to translation, and thus it is not surprising that UPF1p localizes to polyribosomes. A small amount of UPF1p has been localized to the nucleus (Peltz et al., 1994) and it seems possible that the role of UPF1p in nonsense-mediated mRNA decay is carried out by this subset. Second, translation, a cytoplasmic process, appears to be important for recognition of nonsense codon-containing transcripts by the nonsense-mediated decay pathway in yeast. Nonsense codon containing-mRNAs are stabilized both by expression of suppressor tRNAs capable of translating the appropriate nonsense codon, but not by other tRNAs (Losson and Lacroute, 1979; Gozalbo and Hohmann, 1990;) and by treatment with cycloheximide (chx; Zhang et al. 1997). Similar observations have been made for nonsense-mediated mRNA decay in mammalian cells (Takeshita et al., 1984; Belgrader et al., 1993), yet other evidence (that is discussed below) suggests that in these cases degradation occurs during export from the nucleus. Third, polysome-associated nonsense-containing mRNA that accumulates during chx treatment is sensitive to nonsense-mediated mRNA decay upon removal of the chx (Zhang et al., 1997). Other data have also been used to argue that nonsense-mediated mRNA decay in yeast is a cytoplasmic process (see Zhang et al., 1997), but the three lines of evidence mentioned above are the strongest.

Nonsense codons in a wide variety of mammalian genes reduce the steady state level of the mRNA by a post-transcriptional mechanism. While in some cases this can be explained by increased turnover of cytoplasmic mRNA (e.g. Shyu et al., 1991; Kessler and Chasin, 1996), in other cases there appears to be a nuclear mechanism to decrease the abundance of nonsense-containing mRNAs. There are three main lines of evidence to support this hypothesis. First, measurements of (cytoplasmic) mRNA decay rates of

some mRNAs with premature nonsense codons do not reflect the decrease in mRNA abundance. Second, the level of mRNA that copurifies with nuclei is reduced for some nonsense-containing mRNAs. Finally, in some cases an intron is a required *cis*-acting sequence for nonsense-mediated reduction of RNA levels.

Cytoplasmic mRNA decay rates in mammalian cells can be measured by inhibiting transcription and purifying cytoplasmic RNA at regular time intervals. Measurements of half-lives of total mRNA using actinomycin D (Act D; Maquat et al 1981) or cytoplasmic mRNA using a regulated promotor (Shyu et al 1991), show an increased degradation rate of nonsense-containing *β-globin* mRNAs. For other genes the half-lives are the same for nonsense-containing and wild-type transcripts (Urlaub et al., 1989; Cheng et al., 1990; Baserga and Benz 1992; Carter et al., 1995). One possible explanation is that nonsense codons affect the nuclear fate of the mRNA, while a small fraction of the mRNA escapes to the cytoplasm and is stable. Alternatively, it is well established that inhibition of global transcription can inhibit the decay of some mRNAs, thus masking a difference in decay rates (Peltz et al., 1991; Ross, 1995; Abler and Green, 1996). This is very well demonstrated by equal stability of nonsense-containing and wild-type aprt transcripts in the presence of actD, but a 4-fold difference in half-lives of the same mRNAs when measured using a regulated promoter (Kessler and Chasin, 1996). In contrast, Cheng and Maquat (1993) reported that nonsense codon-containing and wild-type TPI mRNA were equally stable when measured using actD or a regulated promoter, suggesting that the actD results are not an artifact.

A further indication that the degradation of nonsense-containing transcripts may be a nuclear event comes from cell-fractionation studies. In a large number of studies

RNA was isolated from purified nuclei and purified cytoplasm and the amount of nonsense-containing mRNA that copurified with nuclei was reduced relative to the wild type control mRNA (Takeshita et al., 1984; Baserga and Benz, 1992; Cheng and Maquat, 1993; Belgrader and Maquat, 1994; Carter et al., 1996; Kessler and Chasin 1996). Several controls indicated that this reduced copurification reflects an *in vivo* reduction in nuclear RNA (Belgrader et al., 1994; Kessler and Chasin, 1996)

In mammalian cells the position of the nonsense codon relative to introns seems to be important for nonsense-mediated decay. This has been used as supporting evidence for a nuclear site of degradation because introns are removed from the RNA in the nucleus, before export of the mRNA. In TPI, aprt, MUP and presumably other transcripts, nonsense codons upstream of the most 3' intron of the coding region are effective in triggering decreased mRNA accumulation, while nonsense codons in the final exon of the coding region are ineffective (Urlaub et al., 1989; Cheng et al., 1990; 1994; Kessler and Chasin, 1996; Belgrader and Maquat, 1994). Even though the accumulation level of the mRNA depends on the presence of introns, it is the mature mRNA (or partially spliced RNA), and not the pre-mRNA that is recognized and degraded (Cheng and Maquat, 1993; Cheng et al., 1994; Carter et al., 1996; Zhang and Maquat, 1996). The requirement of a downstream intron has been used as an argument for a nuclear nonsense codon-dependent degradation mechanism, but the available data can also be explained by hypothesizing that some factor associates with the mRNA in an intron-dependent manner and remains associated with the mRNA after splicing. It may be this hypothetical protein (and not the intron itself) that is required for nonsense-mediated mRNA degradation in mammalian cells, which then may occur in any subcellular compartment.

The current model (Maquat, 1995; Weng et al., 1997) to explain how nonsense codons can determine the nuclear fate of an mRNA is that (in mammalian cells) the 5' end of the mRNA is exported first, and translation starts when the mRNA is still associated with the nucleus. When a premature nonsense codon is encountered, most of the mRNA is degraded before it completely dissociates from the nucleus. It is not clear whether this reflects a fundamental difference in nonsense-mediated mRNA decay between yeast and mammalian cells, whether the data can all be explained by minor variations on one mechanism, or whether a nuclear and a cytoplasmic pathway operate in parallel in all eukaryotes.

It has been shown that plants also contain a nonsense-mediated mRNA decay pathway (van Hoof and Green, 1996), but little is known about the mechanism and subcellular location of this pathway in plants. The existence of a nonsense-mediated mRNA decay pathway in plants was proven by measurements of mRNA half-life made following actD treatment of stably transformed tobacco cells. Although actD timecourses are often assumed to measure cytoplasmic decay rates, this is not necessarily the case. Such timecourses measure the decay of pre-existing mRNA and thus, if most of the mRNA of interest is located in the cytoplasm at the beginning of the timecourse, the cytoplasmic decay rate is measured. However if most of the mRNA of interest at the start of the timecourse is in the nucleus the measured half-life reflects either the decay rate of the mRNA in the nucleus, or the rate of export from the nucleus and subsequent decay in the cytoplasm. To distinguish between these alternatives it is necessary to determine the localization of the bulk of the mRNA at steady state (i.e. before the addition of actD). In the experiments described here I have compared the subcellular location of *PHA* mRNA

destabilized by insertion of a nonsense codon at 60% of the normal coding region with that of a stable control *PHA* mRNA with a nonsense codon at 80% of the normal coding region. I have determined that the majority of the 60% mRNA copurifies with polyribosomes, similar to the 80% mRNA and thus the 60% mRNA is likely to be cytoplasmic. This indicates that nonsense-mediated decay of *PHA* mRNA is likely to be a cytoplasmic event.

RESULTS.

It has been shown that insertion of a nonsense codon in the first 60% of the normal coding region of *PHA* causes a reduction in the accumulation of the mRNA (van Hoof and Green; 1996). Furthermore, this reduction is caused by an increase in the rate of degradation of this mRNA. This was determined by measuring the decay rate of the mRNA by treating stably transformed BY-2 cells with actD. *PHA* mRNA with a nonsense codon at 60% (or 20% or 40%) of the coding region was 3-4 fold less stable than *PHA* mRNA with a nonsense codon at 80% of the normal coding region or wild type *PHA* mRNA. In addition, the difference in mRNA accumulation (about 4 fold) is fully explained by the difference in half-lives. This is true when comparing accumulation of *PHA* mRNA with a nonsense codon at 20% of the normal coding region with wild type (van Hoof and Green; 1996) and when comparing the 60% and 80% constructs (data not shown). Thus analysis of the subcellular distribution of the 60% mRNA at steady state should allow us to localize the nonsense-mediated decay pathway in plant cells.

One way to address whether nonsense-mediated decay is a nuclear event is to

isolate RNA from purified nuclei and compare the accumulation of wild-type and premature nonsense codon-containing mRNA levels. Experiments comparing nuclear RNA levels are one of the main lines of evidence supporting a nuclear location of the nonsense-mediated mRNA decay pathway in mammalian cells. Despite exhaustive attempts I have been unable to purify nuclei of sufficient purity and containing intact mRNA from BY-2 cells for analysis of nuclear RNA. In various attempts I either obtained intact RNA from nuclei that were contaminated with large amounts of cytoplasmic mRNA, or degraded RNA. This is probably caused by release of RNases from the large central vacuole (which contains the majority of RNase activity; Boller and Kende, 1979) when the cells are lysed. This also prevents isolation of cytoplasmic RNA simply by gently lysing the cells, as is routinely done with mammalian cells.

An alternative approach to measure the subcellular distribution of *PHA* mRNA is to isolate polyribosomes and determine to what extent *PHA* mRNA copurifies with polyribosomes. For these experiments polyribosomes were isolated from stably transformed BY-2 cell lines expressing *PHA* mRNA with a nonsense codon at 60% of the normal coding region. This RNA was chosen because it is susceptible to nonsense-mediated decay, but still contains a fairly large coding region, and thus should be associated with reasonably large polyribosomes. As a control cells expressing *PHA* mRNA with a nonsense codon at 80% of the normal coding region were also analyzed. Most published polyribosome isolation procedures include addition of chx to the cells or lysate to stabilize the association of mRNA with ribosomes. However, I omitted chx because it also inhibits the degradation of premature nonsense codon-containing *PHA* mRNA (data not shown) and thus might artifactually increase the association of *PHA*

mRNA with polyribosomes. Typically about 50 to 70% of the total RNA copurified with polyribosomes during centrifugation trough a 1.8 M (62% w/v) sucrose cushion. The RNA from the polyribosomal fraction was further purified and analyzed by RNA gel blotting. Figure 3-1 shows a typical RNA gel blot of total and polyribosomal RNA. *PHA* mRNA was slightly enriched in the polyribosomal RNA fraction relative to total RNA and thus since a majority of total RNA is polyribosomal, a majority a the 60% *PHA* mRNA appears to be polyribosomal.

To confirm that *PHA* mRNA is indeed associated with polyribosomes the isolated polyribosomes were further separated on 15-60% w/v sucrose gradients. After centrifugation the gradients were analyzed for the distribution of UV absorbing material (i.e. RNA) and fractionated in 24 fractions (Figure 3-2). RNA was purified from each fraction and analyzed by RNA gel blotting. Analysis of the UV absorption profiles shown in figure 3-2B and ethidium bromide stained gels showed little RNA in fraction 1, 40S ribosomal subunits typically in fractions 2 and/or 3, 60S subunits in fractions 3 and/or 4, 80S monosomes in fractions 4 and/or 5, and polyribosomes in denser fractions. Figures 3-2 A and C show typical UV absorption profiles for preparative sucrose gradients. These preparative gradients were loaded with a larger amount of material than the gradient in figure 3-2B and as a result the peaks of 40S, 60S and 80S are not well resolved, but monosomes were easily identified as a shoulder on the large peak representing subunits. Disomes and larger polyribosomes were always well separated from monosomes and free subunits. The large peak containing subunits and monosomes typically contained about 40% of the UV absorbing material and the polyribosome fractions contained the remaining 60% of UV absorbing material.

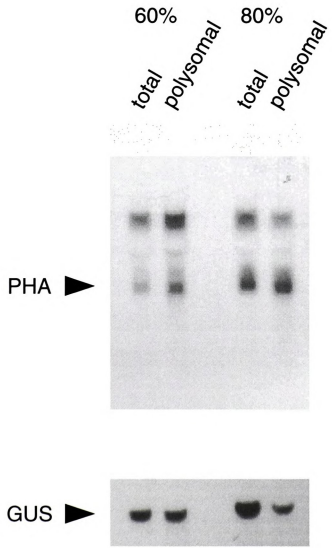

Figure 3-1. *PHA* transcripts with premature nonsense codons copurify with polyribosomes. Protoplasts were prepared from BY-2 cells expressing *PHA* alleles with a nonsense codon at 60% or 80% of the normal coding region and polyribosomes were isolated. Shown is a northern blot containing 20 µg of total or polyribosomal RNA probed with a *PHA* probe (upper panel), stripped and reprobed with a *GUS* probe (lower panel.

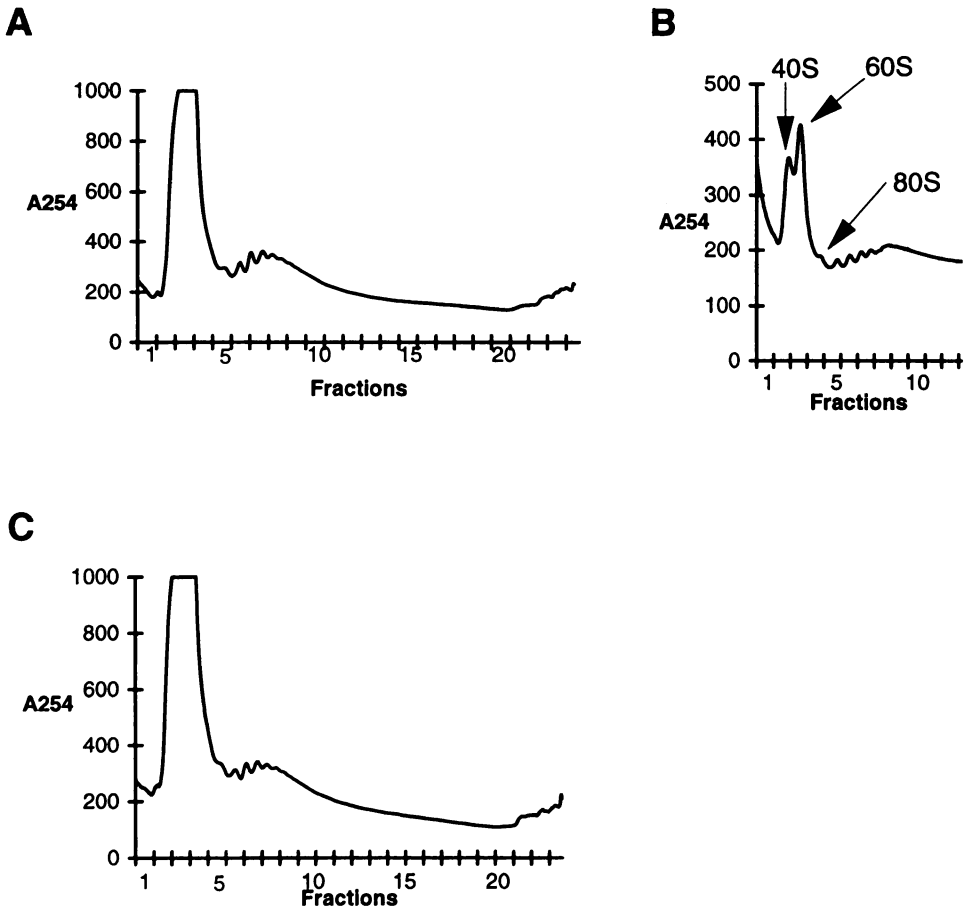

Figure 3-2: Absorption profiles of sucrose gradients. Polyribosomes corresponding to 500 µg (A and C) or 100 µg (B) RNA were separated on 15-60% w/v linear sucrose gradient and fractionated. A and B show typical profiles for a cell line expressing *PHA* with a nonsense codon at 60% of the normal coding region. C shows a typical profile from a cell line expressing *PHA* mRNA with a nonsense codon at 80% of the normal coding region. The peaks corresponding to 40S and 60S ribosomal subunits and 80S monosomes are indicated in B

RNA gel blot analysis (Figure 3-3 and 3-4) of the fractions from these same sucrose gradients showed that *PHA* mRNA was generally absent in the first three or four fraction (i.e. those containing free RNA and free subunits) but was present in fractions 4 or 5 to 24 (i.e. those containing monosomes and polyribosomes). This was true for *PHA* with a nonsense codon at 60% of the normal coding region, as well as for *PHA* with a nonsense codon at 80% of the normal coding region. In addition upon reprobing the RNA gel blots it was evident that *GUS* mRNA was present in the same fractions (data not shown). Based on the spacing of ribosomes along mRNA, one might not expect to find polyribosomes or *PHA* mRNA in the bottom part of the sucrose gradient (i.e. in fractions denser than fraction 15). However, *GUS* mRNA and rRNA were also present in these same fractions (data not shown) indicating that the material in these fractions is indeed polyribosomal. All these data confirm that *PHA* mRNA was indeed associated with polyribosomes. One possible explanation of finding polyribosomes in denser fractions than predicted is that the polyribosome pellet was very tightly packed at the bottom of the sucrose pad after an overnight centrifugation step. This pellet of polyribosomes could be resuspended only partially, and the sample loaded onto the sucrose gradient may have contained some nonspecific aggregates.

As a final confirmation that *PHA* mRNA is indeed polyribosome associated (and not associated with some other very dense material) the sucrose gradients were repeated under conditions that disrupt polyribosomes. Polyribosomes can be specifically disrupted into ribosomal subunits and free mRNA by the omission of $MgCl_2$ from the resuspension buffer and gradient, and inclusion of chelating agents (Cox and Goldberg 1988). Polyribosomes were solubilized as before and loaded onto duplicate sucrose pads. After

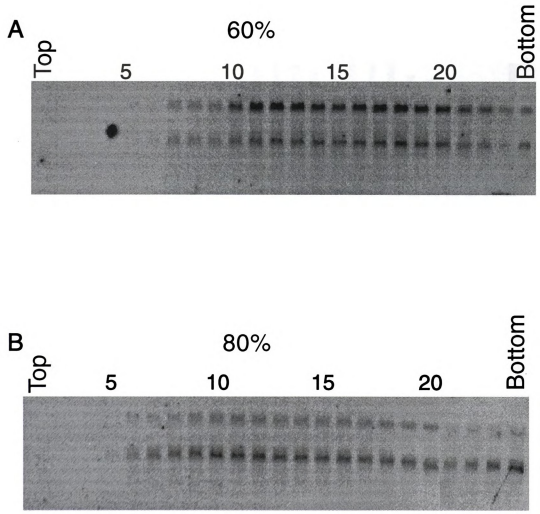

Figure 3-3: RNA gel blot analysis of fractionated sucrose gradients. Polyribosomes purified from BY-2 cell lines expressing *PHA* mRNA with a nonsense codon inserted either at 60% (A) or at 80% (B) of the normal coding region were separated by sucrose gradient centrifugation. 24 fractions were collected from the top of the gradient, RNA was purified from each and analyzed by RNA gel blotting. Shown are typical RNA gel blots probed with a *PHA* probe.

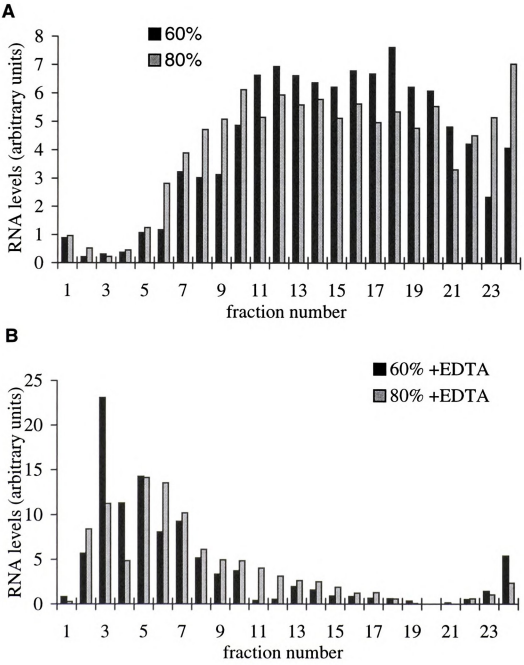

Figure 3-4: Distribution of *PHA* mRNA on sucrose gradients. Signals from RNA gel blots of fractionated sucrose gradients containing polyribosomes (A) or disrupted polyribosomes (B) were quantitated using a PhosphorImager.

centrifugation the pellet under one sucrose pad was resuspended as before, while the other pellet was resuspended under conditions that disrupt polyribosomes, and both were loaded onto sucrose gradients. Figure 3-5 shows the superimposed UV absorption profiles of both sucrose gradients. The polysome gradient shows the same distribution as described above, while in the other gradient only one large peak can be seen. Whereas polyribosome gradients never resulted in a large amount of RNA in fraction one (and to a lesser extent fraction 2), the gradients with disrupted polyribosomes did contain a large amount of RNA in these fractions. Presumably this is caused by disruption of the polyribosomes, which yield free mRNA and free 40S and 60S subunits. RNA gel blot analysis of fractions of these gradients revealed that *PHA* (and *GUS*) mRNA was shifted to the top of the gradient: Most of the *PHA* mRNA was now present in fractions one to five, while it was barely detectable in fractions 10 and higher. These same fractions now contained rRNA as indicated by the ethidium bromide stained gel (data not shown), and *GUS* mRNA (data not shown), confirming that *PHA* mRNA was indeed associated with polyribosomes.

CONCLUSIONS

The subcellular location of the nonsense-mediated mRNA decay pathway in plants is of considerable interest because there appears to be variation in this aspect among eukaryotes. Nonsense-containing mRNAs in yeast and *Xenopus* appear to be degraded in the cytoplasm while mammalian cells may have both a cytoplasmic and a nuclear nonsense-mediated decay pathway. The location within the plant cell was

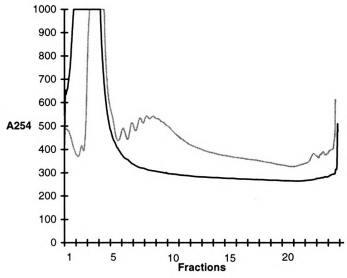

Figure 3-5. Absorption profiles of sucrose gradients. Polyribosomes (black line) or disrupted polyribosomes (grey line) corresponding to 500 µg RNA were separated on 15-60% w/v linear sucrose gradients and fractionated. Typical profiles are shown for a cell line expressing *PHA* with a nonsense codon at 60% of the normal coding region.

investigated by localizing *PHA* mRNA that was destabilized by insertion of a nonsense codon at 60% of the normal coding region. Aproximately 50% to 70% of the destabilized *PHA* mRNA copurified with polyribosomes through a sucrose pad. Additional fractionation of these isolated polysomes by sucrose gradient fractionation and analysis of a control stable *PHA* mRNA strongly indicate that this copurification reflects an *in vivo* association of *PHA* mRNA with polyribosomes. This indicates that a mayor fraction of the unstable *PHA* mRNA is cytoplasmic and thus that its nonsense-mediated decay is cytoplasmic.

Measurements of nonsense-mediated degradation rates of cytoplasmic or total mRNA from some mammalian genes show that the mRNA is stable. This has been suggested to indicate that a subset of the mRNA is completely exported from the nucleus and is stable (Maquat, 1995). Measurements of degradation rates of nonsense-containing *PHA* mRNA in tobacco cells and similar experiments in yeast do not show a significant population of stable transcripts, which is consistent with a cytoplasmic degradation pathway.

Reduced copurification of nonsense-containing mRNA with nuclei is one of the main arguments supporting a nuclear location of the nonsense-mediated mRNA decay pathway in mammals, and ideally one would want to compare similar experimental designs to localize the pathway in different eukaryotes. Attempts to purify nuclear RNA from BY-2 cells were unsuccessful because of technical limitations. Despite extensive efforts nuclear RNA that was intact, and not contaminated with cytoplasmic RNA, could not be obtained, probably because upon lysing the cells large amounts of RNase activity were released from the vacuole. Experiments assaying copurification of nonsense-

containing mRNA with yeast nuclei have not been reported either, possibly because of similar difficulties.

Recently Zhang et al. (1997) showed that polysome-associated mRNA is a substrate for nonsense-mediated mRNA decay in yeast. One complication is that to show association of nonsense-containing mRNA with polyribosomes the mRNA was stabilized by treatment with chx. Polyribosomal RNA that accumulates during chx treatment is subject to nonsense-mediated mRNA decay upon removal of the drug, indicating that nonsense mediated mRNA decay is a cytoplasmic event. Copurification of mammalian nonsense-containing messages with polyribosomes has to my knowledge not been reported. A direct comparison of results of similar experiments using yeast, plant and mammalian cells is therefore not possible.

MATERIALS AND METHODS.

Construction of *PHA* alleles with nonsense codons at 60% or 80% of the normal coding region, stable transformation of BY-2 cells, protoplast isolation, RNA isolation and RNA gel blotting methods are described in Chapter 2.

Polyribosome isolation.

Polyribosomes were isolated from protoplasts essentially as described by Cox and Goldberg (1988): A 50 ml culture of stably transformed BY-2 cells was used to make protoplasts. One tenth of the protoplasts were frozen in liquid nitrogen and used for

isolation of total RNA. The remaining protoplasts were lysed in 20 ml of polyribosome extraction buffer (0.2 M TRIS-HCl pH 9.0, 0.1 M KCl, 25 mM EGTA, 35 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 M Sucrose, 1 mM spermidine, 5 mM 2-mercaptoethanol) and nuclei were pelleted (10 min 1300g). Membranes (and membrane bound polyribosomes) were solubilized by addition of 1% each of Brij 35, Tween 40 and Nonidet P40 and stirring for 30 min on ice. Insoluble material was removed by pelleting (20 min 12000g). The supernatant was split in two and layered on 5 ml sucrose pads (1.8 M sucrose, 40 mM TRIS-HCl pH 9.0, 0.2 M KCl, 5 mM EGTA, 30 mM $MgCl_2$, 5 mM 2-mercaptoethanol) and polyribosomes were pelleted (16h 120000g in a Beckman 70TI rotor). The pellet was either resuspended in GTC buffer (van Hoof and Green 1996) for isolation of polyribosomal RNA, or gently resuspended on ice in 1 ml 40 mM TRIS-HCl pH 9.0, 0.2 M KCl, 5 mM EGTA, 30 mM $MgCl_2$, 5 mM, 2-mercapto-ethanol for 2 hours for further fractionation of polyribosomes.

Polyribosome fractionation.

Insoluble material was removed from resuspended polyribosomes (10 min 120g) and the O.D. of the supernatant was measured at 260 nm. 500 µg RNA (assuming all material absorbing at 260 nm was RNA) was loaded onto a 12 ml 15-60% w/v linear sucrose gradient (in 40 mM TRIS-HCl pH 8.5, 20 mM KCl, 10 mM $MgCl_2$) and centrifuged for 45 min at 300000g (in a Beckman SW40 rotor). For the gradient shown in figure 3-2B only 100 µg RNA was loaded. The gradient was fractionated on an ISCO 640 gradient fractionator, Absorbance at 254 nm was measured during fractionation in a

flow cell (path length 5 mm) and 24 0.5 ml fractions were collected. Polyribosomes in each fraction were precipitated by the addition of 10 μg glycogen (as a carrier), sodium acetate pH 5.0 to 0.2 M, and 2 volumes of ethanol and incubated at -20°C overnight. The pellet was resuspended in 0.5 ml urea lysis buffer (Brewer and Ross, 1990), extracted twice with an equal volume of phenol/chloroform and once with an equal volume of chloroform/isoamyl alcohol, and precipitated by the addition of sodium acetate pH 5.0 to 0.2 M and 1 volume isopropanol. Half of the resulting RNA pellet was analyzed by RNA gel blotting as described.

For EDTA release gradients isolated polyribosomes were resuspended in buffer lacking $MgCl_2$ and fractionated on gradients containing 1 mM EDTA instead of $MgCl_2$. and analyzed exactly as described above for polysome fractionation gradients.

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CHAPTER 4

RARE CODONS ARE NOT SUFFICIENT TO DESTABILIZE A REPORTER GENE TRANSCRIPT IN TOBACCO.

Parts of this chapter will be published in *Plant Molecular Biology*.

Reference: A van Hoof and PJ Green (1997). Rare Codons Are Not Sufficient to Destabilize a Reporter Gene Transcript in Tobacco. in Press.

ABSTRACT

In plants as in other eukaryotes, most synonymous codons of the genetic code are not used with equal frequency. Instead some codons are preferred, whereas others are rare. Circumstantial evidence led to the suggestion that rare codons have a negative influence on mRNA stability. To address this question experimentally, rare codons encoded by a *B.t.* toxin gene (cryIA(c)) or a synthetic sequence were introduced into a phytohemagglutinin *(PHA)* reporter gene. In neither case was the mRNA stability appreciably diminished in stably-transformed tobacco cell cultures nor was the accumulation of mRNA in transgenic plants affected. Thus rare codons do not appear to be sufficient to cause rapid degradation of the *PHA* mRNA and potentially other mRNAs in plants.

INTRODUCTION

It was shown more than 30 years ago that the genetic code is degenerate (Crick et al., 1961; Nirenberg et al., 1963), i.e. several codons are available to dictate the incorporation of a particular amino acid residue into a protein. The usage of synonymous codons does not appear to be random, since certain codons are used less frequently than others. The underlying reason for this biased codon usage is not clear, but it is thought to be related to the abundance of isoaccepting tRNAs. tRNAs corresponding to rare codons are less abundant in *Escherichia coli* and yeast than tRNAs corresponding to preferred codons (Ikemura , 1982; Ikemura and Ozeki, 1983). The choice of codons has been suggested to influence the rate of translation and mRNA degradation, although little evidence exists that the latter is indeed the case. One possible mechanism by which rare codons can affect the rate of translation is that a ribosome may pause when encountering a rare codon, because it may take longer for a rare isoaccepting tRNA to enter the A-site of the ribosome. A shortage of a specific charged tRNA in bacteria can cause the ribosome to pause at codons translated by that tRNA. As few as one (Hsu et al., 1985) or two (Yanofsky, 1981) codons for which there is little charged tRNA available can cause ribosomal pausing. More recently it was suggested that a stretch of rare codons causes the ribosome to stall on the yeast matα1 mRNA (Caponigro et al., 1993; Hennigan and Jacobson, 1996). The possible effect of codon usage on mRNA stability is not only intriguing from a purely scientific view, but also relevant to plant biotechnology. In several cases it has been problematic to express foreign genes in plants (Diehn et al., 1996). Often, these problems can be overcome by redesigning the genes to alter the

codon usage.

Evidence for a general role of rare codons in regulating mRNA stability in eukaryotes comes mainly from two types of experiments. First, in yeast there is a statistical correlation between the presence of rare codons and transcript instability (Herrick et al., 1990). This might be an indication that rare codons are sufficient to trigger the rapid decay of an mRNA, but this observation could equally well be explained by two separate evolutionary pressures on the genes for abundant proteins. That is, there may have been evolutionary selection for mRNAs that are both stable and efficiently translated (Herrick et al., 1990). Second, several studies have addressed the effect of rare codons on transcript stability in yeast (e.g. Hoekema et al., 1987; Kotula and Curtis, 1991) and plants (e.g. Perlak et al., 1991), by either altering codons in highly expressed mRNAs to change them to rare codons, or, more commonly, by altering codons to more typical codon usage in low abundance mRNA, and measuring the resulting change in mRNA abundance. In general, the results are consistent with the hypothesis that rare codons have an effect on mRNA stability, but alternative explanations can not be excluded. For example the rare codons introduced into the yeast *PGK1* gene, resulting in decreased mRNA accumulation (Hoekema et al., 1987), were in a region later shown to contain a transcriptional enhancer element (Caponigro et al., 1993; Mellor et al., 1987).

Additional examples of changes in RNA abundance as a result of large scale alterations in codon usage have been found for engineered *Bacillus thuringiensis* (*B.t.*) toxin genes expressed in plants (reviewed in Diehn et al., 1996). However, elimination of rare codons in *B.t.* toxin genes significantly increased the GC content, thereby eliminating AU-rich sequences that may be responsible for improper recognition of introns

(Filipowicz et al., 1995) and polyadenylation sites (Hunt, 1994), as well as removing instability determinants (Ohme-Takagi et al., 1993). Thus, the effect of changing the *B.t.* toxin gene may be to eliminate aberrant RNA processing. In only one case were the stabilities of the modified and nonmodified versions of *B.t.* toxin mRNA compared. In this case, the altered *B.t.* toxin transcript was more stable than the wild type control, but the difference in stability was not large enough to fully explain the effect on steady state mRNA levels (De Rocher and Green, personal communication). Moreover, these experiments do not address whether codon usage changes or RNA sequence changes cause the observed difference in *B.t.* toxin mRNA stability.

In only one case has it been clearly shown that rare codons can influence mRNA metabolism in eukaryotes. In yeast, rare codons enhanced by about 2-fold the effect of a downstream instability determinant from the matα1 gene (Caponigro et al., 1993). Several other sequences rich in rare codons could substitute for the matα1 rare codons to enhance mRNA decay, whereas control sequences did not. However, rare codons by themselves were not sufficient to cause mRNA instability. Additional matα1 sequences were also required to trigger rapid mRNA degradation. The role of the rare codons appears to be to stall the ribosome, to allow interaction with these additional sequences (Caponigro et al., 1993; Hennigan and Jacobson, 1996).

RESULTS

Insertion of rare codons does not affect *PHA* mRNA stability in BY-2 cells

To test whether rare codons are sufficient to destabilize a reporter transcript in plants, two sequences rich in rare codons were inserted into the phytohemagglutinin *(PHA)* gene from *Phaseolus vulgaris*. *PHA* is well-suited to serve as a reporter gene for this study. Unlike more frequently used reporter genes (such as *GUS* or *luc*), *PHA* is a plant gene that has a typical plant codon usage. In addition, stopping translation early in the *PHA* coding region leads to increased degradation of the mRNA (van Hoof and Green, 1996). As discussed above, rare codons have been suggested to act by stalling the ribosome. If this is indeed the case, an effect of rare codons may be more readily detectable in a reporter gene that can be destabilized by stopping its translation. The sequences that were inserted into the *PHA* gene were chosen not only because they were rich in rare codons, but also because they contained an alternative reading frame that was both open and showed a typical plant gene codon usage. Insertion of the sequence in one reading frame would lead to the incorporation of a stretch of rare codons in the reporter transcripts (Figure 4-1). If a given construct caused rapid degradation of the *PHA* mRNA, then insertion of the same sequence in the alternative frame could be used to distinguish between an effect caused by the presence of rare codons and an effect caused by insertion of the RNA sequence *per se*.

The first construct was designed to test whether a stretch of rare codons from a *B.t.* toxin gene could trigger rapid mRNA decay. It is firmly established that codon usage

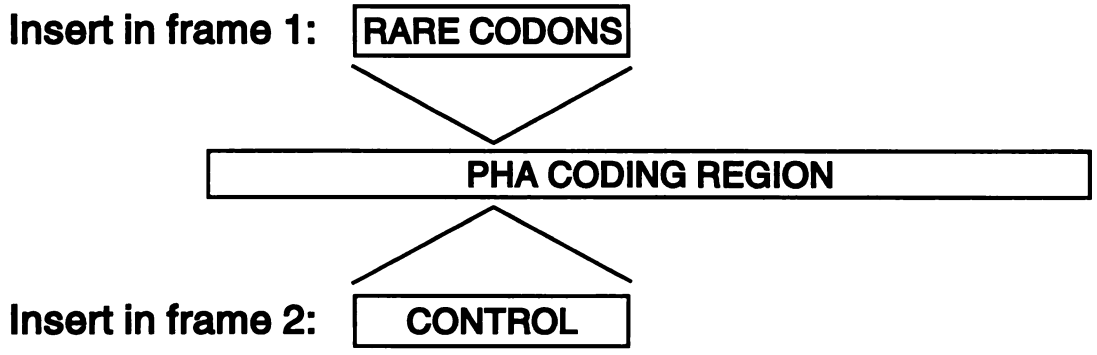

Figure 4-1. Experimental design used to test the effect of rare codons on mRNA stability. The same fragment of DNA can be inserted into the *PHA* coding region in two different reading frames. In the first reading frame it contains multiple rare codons, while in the second reading frame it contains more typical plant codons. DNA fragments containing rare codons were inserted into a *Sty* I restriction site 123 nt from the beginning of the 825 nt-long coding region.

in *B.t.* toxin genes poorly matches the preferred codons in plants, and it has been suggested that this difference may (in part) cause the low abundance of *B.t.* toxin transcripts in transgenic plants (reviewed in Diehn et al., 1996). Specifically, two copies of codons 317 to 339 from a *cryIA(c)* gene (Adang et al., 1985) were inserted into the *Sty* I site of the *PHA* gene under control of the CaMV 35S promoter. This chimeric gene (*BT-PHA*) was stably transformed into tobacco BY2 cells, and the stability of the mRNA was measured as previously described (van Hoof and Green, 1996). Insertion of the *B.t.* toxin segment, even in two copies, did not significantly alter the stability of the mRNA (Figure 4-2). Because insertion of the fragment from *B.t.*-toxin had no effect, analysis of the alternative reading frame control was not necessary. One possible explanation for the lack of effect of the *B.t.* toxin rare codons is that only very rare codons cause an effect. To test this possibility, six very rare codons were inserted into the *Sty* I site of *PHA*. If rare codons stall translation, six very rare codons should be sufficient because a limited number of very rare codons have been shown to stall translation in bacteria (Hsu et al., 1985; Yanofsky, 1981), and only four rare codons were needed to pause ribosomes in yeast (Caponigro et al., 1993; Hennigan and Jacobson, 1996). CGA is one of the rarest codons in plants: Only 7% of the 2915 arginine codons in 207 plant genes are CGA (Murray et al., 1985). However, inserting six copies of a CGA codon would also introduce a fairly stable stem-loop structure. Instead, I introduced the sequence $(CGA)_4(CUA)_2$. CUA is used only for 8% of 5285 leucine codons (Murray et al., 1985). The stability of the resulting mRNA was slightly reduced (1.5 fold, Figure 4-2), but this difference was much smaller than the effect of previously-characterized *bona fide* instability determinants. Instead, it was comparable to the effect of inserting certain

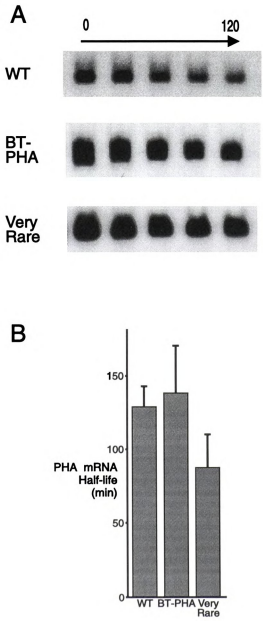

Figure 4-2. Insertion of rare codons does not affect *PHA* mRNA stability in BY-2 cells. Tobacco BY-2 cells were stably transformed with either the *WT-PHA* gene or a *PHA* gene containing rare codons (*BT-PHA* or *Very Rare*). At least two independent transformed cell lines were used for each construct. Messenger RNA half-lives were determined as described in chapter 2. (A) Shown are RNA gel blots from representative half-life measurements for wild type *PHA* and two chimeric genes containing rare codons. (B) The histogram represents the average half-life calculated from at least four experiments and the standard error.

random control sequences into a reporter gene (Newman et al., 1993).

Insertion of rare codons does not affect *PHA* mRNA levels in transgenic tobacco plants

To confirm that the results obtained in cultured tobacco cells were representative of what occurs in intact plants, 12 to 21 transgenic tobacco plants expressing either the wild type *PHA* gene or the two versions with rare codons inserted were generated. RNA was isolated from a leaf of each plant, and the abundance of *PHA* mRNA was determined by RNA gel blotting and quantitation with a PhosphorImager. As shown in figure 4-3A, the average level of the *BT-PHA* and *WT-PHA* RNA were virtually identical, while the average RNA level for the *Very Rare* gene was slightly reduced. Statistical analysis of these data by the Wilcoxon-Mann-Whitney test, showed that this difference was not statistically significant. An RNA gel blot showing the difference in average *PHA* mRNA levels is shown in figure 4-3B. To generate this blot, equal amounts of RNA from each transgenic plant were mixed and analyzed by RNA gel blotting. Thus neither the *BT-PHA* nor the *VERY RARE* gene showed a significant reduction in mRNA stability in BY2 cells or in mRNA abundance in tobacco plants. This is unlikely to be due to technical limitations because effects of nonsense codons on *PHA* mRNA stability and mRNA abundance were easily detected in both systems in previous studies (van Hoof and Green, 1996). In addition, immuno gel blot analysis indicated that both wild-type and rare codon-containing *PHA* transcripts are translated in BY2 cells (data not shown). Thus, the failure to find an effect of rare codons on mRNA stability cannot be explained by a failure of these mRNAs to be translated.

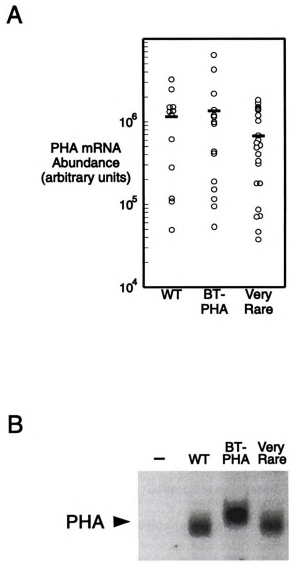

Figure 4-3. Insertion of rare codons does not affect *PHA* mRNA levels in transgenic tobacco plants. Tobacco leaf explants were stably transformed with either the *WT-PHA* gene or the *PHA* genes containing rare codons. For each construct at least twelve independent plants expressing *PHA* were regenerated and transferred to soil. Plants were grown to the 10-15 leaf stage and total RNA was isolated from one leaf near full expansion. *PHA* mRNA levels were quantitated by RNA gel blot using a PhosphorImager. (A) Shown is the mRNA level for each individual plant (circles) as well as the average for each construct (bar). Statistical analysis (van Hoof and Green, 1996) showed that there was no significant difference in RNA levels. (B) Shown is an RNA gel blot of pooled RNA from these same plants. Equal amounts of RNA from each individual plant were mixed and 10 µg of each pool was analyzed by RNA gel blot.

Rare codons may affect translation rates

In addition to affecting mRNA stability, rare codons may also reduce the rate of translation, resulting in reduced levels of protein. I addressed this hypothesis by measuring PHA synthesis rates in cell lines stably transformed with either *WT-PHA* or one of the two *PHA* genes with rare codons inserted. Because WT-PHA, BT-PHA and Very Rare-PHA likely decay at different rates, a difference in protein accumulation can not be assumed to be caused by a difference in the rate of synthesis. Instead, translation rates were measured directly by labeling newly-synthesized protein with tritiated leucine, extracting proteins, and immunoprecipitating PHA with either a polyclonal serum previously shown to be effective in immunoprecipitation (Kjemtrup et al., 1994; Bollini and Chrispeels, 1979) or commercially available immunopurified polyclonal antibodies (Pierce, Rockford IL). Neither of these antibodies immunoprecipitated detectable amounts of tritiated PHA even though the cells expressed *WT-PHA* as determined by RNA gel blot and immunoblot analysis (data not shown). It is not clear whether this was caused by insufficient labeling of PHA or by a failure to immunoprecipitate PHA.

CONCLUSIONS

The results described in this chapter indicate that rare codons are not sufficient to destabilize a transcript. It seems likely that if there is a general effect of rare codons on transcript stability, then the experiments described above should have detected it. However, rare codons can have an indirect effect in certain specific cases. For example

the yeast matα1 instability determinant is clearly stimulated by upstream rare codons (Caponigro et al., 1993; Hennigan and Jacobson, 1996), and similar situations may occur in some plant genes. It is possible that for rare codons to have a direct effect on mRNA stability, they need to be present at a specific location within the coding region (for example just downstream of the start codon), or at a high frequency throughout the transcript (as they are in the *B.t.* toxin gene). Testing these hypotheses would require the insertion of rare codons at many different positions within the coding region or construction of two full-length genes that are nearly identical in nucleotide sequence, but differ by the reading frame that is read.

Whether rare codons reduce the translation rate of a transcript remains an unresolved question. In general initiation of translation is the slowest step (about 6.5 seconds) in translation, while elongation is more than 10-fold faster (about 1/8 to 1/3 second; Mathews et al., 1996). Thus for rare codons to affect translation rates they would have to have a very large effect on the elongation step. In addition rare codons that are translated by rare isoacccepting tRNAs may not be translated much slower because they compete with fewer other codons for these tRNAs. In any case, an effect of rare codons on protein synthesis rates would be most easily addressed by constructing a set of genes coding for identical proteins and producing similar steady state levels of mRNA, but differing in their codon usage. In the process of analyzing the cause(s) of the low accumulation of *B.t.*-toxin mRNA, such constructs have fortuitously been made. De Rocher et al. (1997) constructed a synthetic *B.t.*-toxin gene with preferred codon usage, and several derivatives with segments of the nonmodified *B.t*-toxin gene replacing the corresponding synthetic segment. Genes containing certain segments of the nonmodified

B.t.-toxin gene accumulate as much mRNA as fully synthetic *B.t.*-toxin genes (De Rocher et al., 1997), and thus are ideally-suited to test the effect of rare codons on translation.

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CHAPTER 5

CONCLUSIONS AND FUTURE PROSPECTS

NONSENSE-MEDIATED mRNA DECAY

At the outset of the research described in this thesis, little was known about the regulation of mRNA stability in plants, and only slightly more was known for other eukaryotes. Since then, significant progress has been made in identifying *cis*-acting sequences that can confer rapid degradation on reporter transcripts. The characterization of mRNA degradation triggered by one of these elements (premature nonsense codons) is described in this thesis (chapters two and three). In addition, rare codons had been suggested to confer rapid degradation, and this was investigated directly in the experiments described in chapter four.

The data described in chapter two provide the first conclusive evidence that plants contain a nonsense-mediated mRNA decay pathway. Direct measurement of decay rates shows that *PHA* mRNA is destabilized by nonsense codons in the first 60% of the normal coding region. Some of the characteristics of nonsense-mediated mRNA decay are conserved in all eukaryotes, while others vary. One of the interesting differences is the subcellular location of this turnover pathway (Weng et al., 1997). My results on the subcellular location of the pathway in tobacco cells are described in chapter three. Cell fractionation demonstrates that a substantial amount of *PHA* mRNA destabilized by a nonsense codon at 60% of the normal coding region is associated with polyribosomes. This suggests that this mRNA is cytoplasmic, and thus, that its nonsense-mediated decay is likely also cytoplasmic.

A second difference in nonsense-mediated mRNA decay can be found in the signals that mediate discrimination between normal and premature nonsense codons.

Yeast recognizes a premature nonsense codon by its position relative to additional *cis*-acting sequences (Weng et al., 1997), while mammalian cells use the position of nonsense codons relative to introns (Maquat, 1995). Nonsense-mediated mRNA decay in plants does not require introns in contrast to the process in mammalian cells. However, it is not clear whether plants and yeast use similar *cis*-acting sequences; the region between 60% and 80% of the normal *PHA* coding region that appears to be involved in discriminating between those nonsense codons that cause mRNA instability and those that don't has no obvious resemblance to a functionally similar well-characterized element from yeast. Further characterization of the putative *PHA* element is required to prove that it indeed functions by destabilizing transcripts when it is inserted downstream of a nonsense codon. Additional explanations of the differential degradation of mRNAs from different *PHA* alleles, such as the difference in length of the coding region and/or 3' UTR, can not be ruled out based on the results presented here. Additional alleles of *PHA* are needed to resolve this.

Recognition (or degradation) of premature nonsense codons in yeast and *C. elegans* requires specific *trans*-acting factors not required for degradation of typical mRNAs. None of the cloned genes shown to code for nonsense-mediated mRNA decay factors (i.e. UPF1, UPF2, UPF3 and smg2) have strong sequence similarity to known A*rabidopsis* sequences. Recently, He and Jacobson (1995) used the two-hybrid screen to identify additional yeast factors that interact with UPF1p. One of these factors, named NMD3, has significant sequence similarity with ESTs from *Arabidopsis* (119F15T7) and maize (W21741 and P38861). The *Arabidopsis* EST represents a partial cDNA clone which contains 708 bp, including 513 bp of coding region (data not shown). The deduced

amino acid sequence is 38% identical and 49% similar to NMD3 (data not shown), but it has not been shown that NMD3 is involved in nonsense-mediated decay, and thus the role of the *Arabidopsis* gene is not clear. Sequencing of the complete *Arabidopsis* genome will probably identify *trans*-acting factors involved in nonsense-mediated decay.

Identifying novel *trans*-acting factors involved in nonsense-mediated decay in plants may be difficult. In principle one could use both biochemical and genetic methods. Biochemical methods to isolate *trans*-acting factors involved in mRNA degradation have not been very successful in most systems. Some *in vitro* systems for mRNA decay have been developed using mammalian cell extracts (Ross, 1995), but it is not clear to what extent mRNA degradation in these systems mimics *in vivo* mRNA turn-over. Additionally, these systems are generally not translationally competent, and thus one would not expect to be able to reconstitute nonsense-mediated mRNA decay. On the other hand wheat germ extract is able to translate added RNAs, but *PHA* mRNA is stable in wheat germ extract during an overnight incubation regardless of wether it contains a premature nonsense codon or not (data not shown).

Currently mutants defective in AUUUA- or DST-mediated mRNA decay are being isolated in Pam Greens laboratory. This can be achieved by destabilizing the transcript coding for a selectable marker, and selecting mutants that are able to grow on higher concentrations of the corresponding antibiotic. The results described in this thesis indicate that it may be possible to adapt this strategy to find mutants in nonsense-mediated mRNA decay. The most likely explanation of the results in chapter two is that the region between 60% and 80% of the normal *PHA* coding region destabilizes transcripts when it is present downstream of a nonsense codon. This would predict that a

transcript coding for a selectable marker could be destabilized by inserting this region of *PHA* into the 3' UTR and that the same transcript would be stable in any mutants in nonsense-mediated mRNA decay. The hypothesis that a selectable marker mRNA can be destabilized by this putative *cis*-acting sequence is certainly testable.

Perhaps one of the most interesting questions concerning nonsense-mediated mRNA decay in plants concerns the events following recognition for rapid decay. In both yeast and mammals, shortening of the poly(A) tail appears to be the first hydrolytic event in the degradation of a typical mRNA (Tharun and Parker, 1997). One prominent exception to this is the degradation of nonsense-containing transcripts (Tharun and Parker, 1997). In both systems, deadenylation rates of nonsense-containing mRNAs are slow, while their decay is fast. It will be interesting to see whether this difference holds true for mRNA decay in plants.

Nonsense codons trigger rapid mRNA decay in both prokaryotes and eukaryotes (reviewed by Weng et al., 1997), indicating that there is an evolutionary advantage to maintaining this pathway. In *C. elegans,* certain nonsense mutations that are recessive in a wild type background become dominant when combined with a mutation in nonsense-mediated mRNA decay (Pulak and Anderson, 1993). This indicates that one function of nonsense-mediated mRNA decay may be to prevent these mutations from having a deleterious effect on the survival of the individual (Pulak and Anderson, 1993). From an evolutionary standpoint, survival of an individual carrying a deleterious mutation may not be an advantage. Nonsense-mediated mRNA decay may be advantageous for the survival of the species because it allows for genotypic variation to be maintained in the heterozygote, while it suppresses a negative phenotypic effect. This genotypic variation

may be used for the generation of new advantageous genes or alleles by further mutation.

The machinery that degrades nonsense codon-containing transcripts in yeast does not appear to be required for degradation of typical mRNAs (Reviewed by Weng et al., 1997). However, higher eukaryotes may use the nonsense-mediated mRNA decay machinery to degrade certain specific wild-type transcripts as well as mutant transcripts. One possible example of a wild-type transcript that is degraded by the nonsense-mediated mRNA decay pathway is that of the mammalian UHG gene (Tycowski et al. 1996). The functional products of the UHG gene are snoRNAs that are processed from the excised introns. The spliced mRNA does not appear to code for a protein or other functional product and is rapidly degraded. The degradation of UHG mRNA was suggested to be mediated by the nonsense-mediated mRNA decay pathway because the mRNA contains many AUG codons followed closely by nonsense codons, is associated with polyribosomes and is increased in abundance in response to protein synthesis inhibitors (Tycowski et al. 1996). Similar natural targets for the nonsense-mediated mRNA decay pathway may be the *GUT15* transcripts from tobacco and *Arabidopsis,* described in the appendix of this thesis, and related transcripts. Degradation of *GUT15* transcripts by the nonsense-mediated mRNA decay pathway is consistent with the facts that they contain many nonsense codons in all three reading frames, are rapidly degraded, are polyadenylated, and are induced by cycloheximide.

Another category of transcripts that may be degraded by the nonsense-mediated mRNA decay pathway is antisense RNA introduced for research or biotechnological goals. As described in chapter one introduction of an antisense copy of a gene often leads to a reduction in expression of the corresponding gene. It seems reasonable to postulate

that an artificial antisense transcript is unstable because it contains nonsense codons. It may be possible to increase the abundance of the antisense RNA by removing these nonsense codons. This, in turn, may lead to an increase in the effectiveness of antisense technology, although given that the mechanism of antisense inhibition is not clear, an increase in antisense RNA abundance may not result in an increased inhibition of gene expression.

Insufficient data have been gathered to completely describe how the cell targets some mRNA for nonsense-mediated mRNA decay at the molecular level, but the following model does account for most of the essential features. mRNA does not occur as an isolated molecule in the cell, but is associated with various proteins. During nuclear processing certain proteins are associated with the mRNA, while other proteins are associated with the mRNA in the cytoplasm. The essential part of my model for nonsense-mediated mRNA decay is that the ribosome plays an essential cytoplasmic role in removing certain proteins from the coding region of the mRNA. These proteins would associate with the mRNA in the nucleus, and the mRNA would be exported as an mRNP particle. During translation by the first ribosome these proteins would be removed. Thus, if a transcript contains a premature nonsense codon, these nuclear proteins would not be removed from part of the normal coding region. The cell would recognize this as an abnormal mRNP and degrade the RNA. This model is appealing because it does not require very many new *trans*-acting factors. Nuclear and cytoplasmic mRNP proteins have been described. In addition it explains why very many different mutant transcripts are degraded by the nonsense mediated decay pathway. Under this model the required additional *cis*-acting downstream sequences are degenerate binding sites for specific

nuclear proteins. TGYYGATGYYYYY could be one such binding site for a protein in yeast, while in mammals specific proteins could remain associated with the junction of exons after splicing has been completed. In both systems (and in other systems such as plants) other *cis*-acting sequences that bind other nuclear proteins may still remain to be discovered.

mRNA DEGRADATION IN PLANTS

Obviously, a thorough understanding of mRNA degradation is required for an understanding of links between translation and mRNA degradation. The next challenge in the field of plant mRNA degradation will be the identification and characterization of the *trans*-acting factors that are important players in mRNA degradation and the mechanisms by which specific transcripts are recognized and degraded in higher eukaryotes. The only systems where significant numbers of *trans*-acting factors have been identified are *E. coli*, yeast, and chloroplasts (see Sugita and Sudiura 1996, Nicholson, 1997, and Tharun and Parker, 1997 for reviews). The main methods of identifying *trans*-acting factors have been classical biochemical methods of purifying RNases and RNA-binding proteins and/or identifying mutants defective in mRNA turnover. Both of these approaches should also be valuable tools to identify *trans*-acting factors involved in the degradation of cytosolic transcripts in plants. Currently both genetic and biochemical means are being used in Pam Green's lab to identify *trans*-acting factors involved in AUUUA- and DST-mediated decay. Specific RNA-binding proteins are being sought through gel-shift experiments and the yeast three hybrid system

(Sengupta et al., 1996), *Arabidopsis* mutants with increased levels of a normally unstable selectable marker transcript are being selected, and RNA degrading activities will be purified and characterized.

Both genetic and biochemical approaches for the characterization of nonsense-mediated mRNA decay are complicated by the dual effect of nonsense codons on translation and on mRNA stability. However, any factors and mechanisms identified to be important for general mRNA degradation can be used to check whether similar mechanisms and the same *trans*-acting factors are involved in nonsense-mediated mRNA decay. It seems likely that some *trans*-acting factors (for example those conferring specificity) are only required for recognizing (and degrading) mRNAs with one specific instability determinant. In contrast, some of the actual mRNA-degrading enzymes might be involved in the decay of a larger number of transcripts. Once mutants defective in AUUUA- and DST-mediated decay have been obtained, these ideas will be easily testable by introducing genes containing premature nonsense codons into a mutant background by crossing or by *Agrobacterium*-mediated transformation.

In addition to the methods mentioned above, large-scale *Arabidopsis* genome and cDNA sequencing projects will help to identify putative *trans*-acting factors. Several RNA-degrading activities (and the corresponding genes) from *E. coli* are well-characterized, and at least three homologous genes are present in the dbEST database. EST ATTS0840 is similar to RNase III (N.D. LeBrasseur, M.L. Abler, and P.J. Green, personal communication), which is an endoribonuclease specific for certain dsRNA secondary structures. RNase III participates in rRNA processing and some rate-limiting cleavages of mRNA in *E. coli* (Nicholson, 1997). Currently, research is in progress to see

whether the *Arabidopsis* EST has similar functions. The other *Arabidopsis* ESTs with interesting similarities to *E. coli* RNases are 143N9T7 and 116G11T7, which are similar to polyribonucleotide phosphorylase (PNPase) and RNase PH respectively. Both of these enzymes are 3' to 5' exoribonucleases from *E. coli*. EST 143N9T7 may be involved in chloroplastic mRNA degradation, since it is more closely related to PNPase from spinach chloroplasts (GenBank accession number U52048) than to any other PNPase (69% identity over 68 amino acid residues and 88% identity over an additional 9 amino acid residues; data not shown). Unfortunately, the EST clone does not contain the 5' end of the coding region, so it is unknown whether it contains a chloroplast targeting signal. RNase PH is one of the least well-characterized RNases of *E. coli*, and its exact role *in vivo* is not clear (Nicholson, 1997). EST 116G11T7 is more closely related to hypothetical proteins from *C. elegans, S. pombe* and yeast than to prokaryotic RNase PHs (data not shown), suggesting that it probably does not function in the chloroplast. However, this cDNA is also a partial clone, so it is unknown whether it contains an N-terminal targeting signal. This gene may be involved in any of a number of different RNA metabolism pathways. If it is involved in mRNA degradation, its possible roles include shortening of the poly(A) tail and/or degrading the body of the mRNA.

In addition to homologs of *E. coli* enzymes, dbEST also contains sequences similar to yeast mRNA decay factors. The most interesting of these are ESTs H4B8T7 and H4B9T7 which likely represent one *Arabidopsis* gene with strong sequence similarity to a group of 5' to 3' exoribonucleases (J.P. Kastenmayer, A. van Hoof and P.J. Green, unpublished data) of which Xrn1p from yeast is the best-characterized example. Xrn1p is the main enzyme responsible for the degradation of at least some mRNAs in yeast

(Muhlrad and Parker, 1994), and its known substrates include premature nonsense codon-containing mRNAs. In addition, the biochemical characteristics of XRN1p, and the less well-characterized 5' exonuclease 2 (Stevens and Poole, 1995; Poole and Stevens, 1995) are similar to the classically defined "exonuclease I" class of enzymes from plants (Bariola and Green 1997; J.P. Kastenmayer, A. van Hoof and P.J. Green, unpublished data). Clearly, experimental data are required to show whether any of these *Arabidopsis* genes play a role in (nonsense-mediated) mRNA degradation.

In addition to the ESTs mentioned above, dbEST also contains *Arabidopsis* sequences with low, but possibly significant, similarity to other genes thought to be involved in mRNA decay, such as yeast DCP1 (Beelman et al., 1996) and zebrafish narI (Gaiano et al., 1996), numerous ESTs with high sequence similarity to RNA-binding proteins and helicases, and ESTs with high sequence similarity to secretory RNases (data not shown). This latter class includes EST 62B4T7, which is similar to fungal bifunctional nucleases (i.e. capable of degrading both RNA and DNA) (M.L. Abler, D.M. Thompson, A. van Hoof, N.D. LeBrasseur and P.J. Green, manuscript in preparation) and likely represents the first cloned representative of the biochemically-defined "nuclease I" enzymes (reviewed in Bariola and Green, 1997).

It seems clear that it will be relatively easy to identify RNases and RNA-binding proteins (using a combination of biochemistry and sequence analysis). The real challenge will be to differentiate activities involved in mRNA degradation from those involved in other processes, and to identify the specific role of each *trans*-acting factor. Some of the cloned genes mentioned above are being mapped (L.R. Danhof and P.J. Green, personal communication) and thus may be found to be identical to genes identified by the genetic

selection described above. In addition, it is now theoretically possible to identify insertion mutants in any cloned gene and this approach is also being pursued (N.D. LeBrasseur and P.J. Green personal communication).

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APPENDIX

APPENDIX

GUT15 CDNAS FROM TOBACCO AND *ARABIDOPSIS* CORRESPOND TO TRANSCRIPTS WITH UNUSUAL METABOLISM AND A SHORT CONSERVED ORF.

Parts of this appendix have been previously published in Plant Physiology.

Reference: A van Hoof, JP Kastenmayer, CB Taylor and PJ Green (1997). *GUT15* cDNAs from tobacco (Accession No. U84972) and *Arabidopsis* (Accession No. U84973) correspond to transcripts with unusual metabolism and a short conserved ORF (PGR97-048). Plant Physiol. 113:1004

An important aspect of gene expression is regulation exerted at the level of mRNA stability. The majority of transcripts in eukaryotic cells are stable, with half lives on the order of hours. In contrast to these stable messages, a subset of transcripts is rapidly degraded, with half lives on the order of minutes (Sullivan and Green, 1993). Previously, tobacco Genes with Unstable Transcripts (GUTs) were isolated by differential hybridization. The most unstable transcripts found in this study were those encoded by the *GUT15* gene (Taylor and Green, 1995).

The complete sequence of the original tobacco *GUT15* cDNA was determined in an effort to elucidate the function of the *GUT15* gene (GenBank accession Number. U84972). The *GUT15* cDNA is unusual in that it does not contain a long open reading frame (ORF); the longest ORF in any of the three reading frames is only 76 codons. To determine whether the original *GUT15* cDNA represented the functional *GUT15* transcript, a transcript from a pseudogene, or was a cloning artifact, additional cDNA clones were isolated from the same library. Eleven clones were partially sequenced and could be divided into two classes. One class consisted of cDNAs that were identical in sequence to the original clone, although their 5' and 3' ends were different. The other class contained some small insertions and deletions and base substitutions (Taylor and Green, personal communication), indicating that there are at least two *GUT15* genes in tobacco (confirming the results of Southern blotting; Taylor and Green, 1995). All of this information combined suggested that the original cDNA was not a cloning artifact and was likely to represent the functional transcript. 11 ESTs in dbEST that represent a putative *GUT15* homolog in *Arabidopsis thaliana* were also identified. Although the overall degree of sequence similarity between the genes from *Arabidopsis* and tobacco is

not very high, they share many common characteristics. In addition, analysis of the EST sequences combined with Southern blotting indicated that *Arabidopsis* contains only one *GUT15* gene. Therefore further analysis was concentrated on *Arabidopsis*, although most of the results were confirmed in tobacco.

RNA gel blot analyses of *Arabidopsis* RNA using the *GUT15* cDNA as a probe resulted in two bands of approximately 1.2kb and 1.6kb of similar intensity (data not shown). A comparable pattern was observed in tobacco, in which transcripts of approximately 1.7kb and 1.9kb were detected (confirming the data of Taylor and Green, 1995). This pattern of transcripts was also seen when tobacco or *Arabidopsis* RNA enriched for poly(A)$^+$ RNA was examined (Figure 6-1 and data not shown), indicating that transcripts of both sizes are polyadenylated. Analysis of all the *Arabidopsis* and tobacco cDNA clones suggested that the larger of the two transcripts retained an intron relative to the smaller transcripts. These putative introns have the characteristic features of plant introns, such as a high AU content, and conserved splice sites. The intron is conserved in sequence as well as position between *Arabidopsis* and tobacco. Preliminary data from RNase H cleavage experiments (Kleene et al, 1984) using oligonucleotides complementary to the intron sequence also indicated that the putative intron was present in the 1.6kb species seen in RNA blot analysis in *Arabidopsis*, but absent from the 1.2kb species (Kastenmayer and Green, personal communication).

Taylor and Green (1995) reported that cycloheximide (chx) stabilized the smaller *GUT15* mRNA species in tobacco cells. chx also induces the smaller of the two transcripts in *Arabidopsis*. The differential effect of chx on the two transcripts may be indicative of a difference in their degradation pathways. Alternatively, the larger

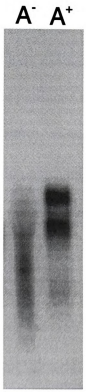

Figure 6-1. GUT15 probes hybridize to two polyadenylated transcripts. Shown is an RNA gel blot of 20 µg polyA$^-$ and 0.4 µg polyA$^+$ RNA from tobacco BY2 cells probed with a cDNA clone of the tobacco *GUT15* gene.

transcript may be a precursor of the smaller transcript that is slowly processed. Both explanations are consistent with the disappearance of the larger transcript during actinomycin D time courses as observed by Taylor and Green (1995).

Sequence analysis of the longest *Arabidopsis* cDNA clone (GenBank accession number U84973) showed that it also lacks a long ORF, but the longest ORF in tobacco is conserved in *Arabidopsis* (Figure 6-2A). The putative peptides do not show significant sequence similarity with known proteins. In all three genes this ORF is located 3' of multiple AUGs, which is unusual for an eukaryotic transcript. Therefore the encoded peptide would not be expected to be produced in large amounts by the normal scanning mechanism of translation (Kozak, 1989). However, it is conceivable that this ORF is translated by an alternative translation mechanism (Jackson and Kaminski, 1995). If the ORF is translated, the peptide may function as a signaling molecule that is needed in very small amounts (as has been described for the product of the *ENOD40* gene; van de Sande et al, 1996). To test this hypothesis, transgenic *Arabidopsis* plants were generated to overexpress the peptide. Specifically, the *GUT15* ORF was inserted between a double enhanced 35S promoter and NOS polyadenylation signals, and the sequence just upstream of the AUG was changed to the preferred context for plants. However, none of the 40 independent transgenic plants that were generated displayed an obvious phenotype. Thus the available data provides no support for the hypothesis that the conservation of the small ORF in the *GUT15* gene is biologically important. It seems possible that the *GUT15* transcript itself, or an intron within it (Tycowski et al, 1996), may be the functional gene product, and conservation of the ORF may reflect underlying conservation of important nucleotides.

A

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Tobacco          MMGALAWQV...TIPNKVSIFCFG.RGDGTGILPG..APLFVSS
                 | |||||||   .: .|  :::::  |:.|  ::||  .|| |||
Arabidopsis      MSGALAWQVKKLILNKKKILWVLERRSHGLLFFPGISSPLCVSS

Tobacco          RLLFSSL.....FPRYYTQDQYHQERHIRLL
                   |   ||     | .:.... . .. | . |
Arabidopsis      CLCSISLPALSHFISFLNAHIHSKTDHKQSL
```

B

Clone	Species	
GUT15	tobacco	CCGACCUUUGCCAUGA**U**GGGUGCGCUCGCAUGGCAGGUCA
GUT15	*Arabidopsis*	CCGACCUUUGCCAUGUCAGGUGCGCUUGCAUGGCAGGUCA
CR20	cucumber	CCGACCUUUGCCAUGACAGGUGCGCUUGCAUGGCAGGUCA
CR20	*Arabidopsis*	CCGACCUUUGCCAUGACAGGUGCGCUUGCAUGGCAGGUCA
sre1a	potato	CCGGCCUUUGCCAUGACGGGUGCGCUCGCAUGGCAGGUCA
sre1b	potato	CCGGCCUUUGCCAUGCCGGGUGCGCUCGCAUGGCAGGUCA
sre1c	potato	CCGGCCUUUG**U**CAUGCCGGGUGCGCUCGCAUGGCAGGUCA
Consensus		CCGRCCUUUGCC<u>AUG</u>NCRGGUGCGCUYGCAUGGCAGGUCA

Figure 6-2. Comparison of the conserved regions in *GUT15* genes. **A**. Comparison of the conserved open reading frame in tobacco and *Arabidopsis GUT15* genes. The deduced amino acid sequences were aligned using the bestfit program (genetics computer group, Madison, WI), using the default parameters. **B**. Alignment of a 40 nucleotide segment of GUT15 genes from tobacco and *Arabidopsis* and related sequences from CR20 genes of *Arabidopsis* (D79218) and cucumber (D79216) and from three cDNA clones that probably represent different alleles of one gene from potato (Wegener and Scheel, personal communication). The consensus shows nucleotides that are identical in at least six of the seven sequences. R indicates A or G, Y indicates U or C, N indicates a nonconserved nucleotide. The AUG underlined in the consensus corresponds to the initiation codon for the open reading frame show in A. The two nucleotides deviating from the consensus are indicated in bold.

As a further step toward functional analysis the *Arabidopsis GUT15* gene has been mapped to chromosome 2, 1cM distal of marker m216 (LR Danhof, A van Hoof and PJ Green, unpublished data), using the recombinant inbred lines developed by Lister and Dean (1993). Several mutants for which the corresponding genes have not been cloned map close to this location. Further research is needed to investigate the possibility that the phenotype of one of these mutants is caused by a mutation in the *GUT15* gene.

Recently Teramoto et al. (1996) reported the isolation of genomic and cDNA clones for the *CR20* gene from cucumber that was isolated by a differential screen for cytokinin repressed transcripts. Similar clones were also isolated from potato during a differential screen for transcripts induced during pathogen attack (Wegener and Scheel, personal communication), but preliminary results indicate that the *Arabidopsis GUT15* gene is not induced during pathogen attack (Kastenmayer, Lawton, van Hoof, Ryals and Green, unpublished data). The cucumber and potato clones share many of the unusual features of *GUT15* clones from tobacco and *Arabidopsis*. In particular, several transcripts were detected when these clones were used as probes on RNA gel blots (Teramoto et al., 1996; Wegener and Scheel, personal communication). Several cDNA clones from cucumber were isolated and appeared to differ by the presence or absence of one or more introns. Teramoto et al. also isolated a cDNA from *Arabidopsis* (*AtCR20-1*) that is distinct from, but similar to the *Arabidopsis GUT15* gene. *AtCR20-1* and *GUT15* share many of the same characteristics and very limited sequence similarity. The sequence similarity is not sufficient for probes for *GUT15* to cross-hybridize to *AtCR20-1* since only one gene was detected in Southern blotting. These data combined indicate that *GUT15* genes from *Arabidopsis* and tobacco are members of a large class of genes. The

ORF that is conserved in *Arabidopsis* and tobacco *GUT15* genes is not conserved very well in some of the cucumber and potato clones (although the AUG codon is conserved in all clones). A small segment of these RNAs is highly conserved (Figure 6-2B) and may be (an essential part of) the functional gene product. A similar sequence is not present in any other sequences in GenBank, indicating that this noncoding RNA may be unique to plants. Outside this 40nt area the clones do not share extensive sequence similarity. The presence of a small region of sequence conservation in the RNA sequence, combined with the absence of a highly conserved ORF suggests that these genes are transcribed into noncoding RNA. The function of this RNA remains unknown and additional experiments will be needed to resolve this.

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