The Plant Cell, Vol. 4, 47–58, January 1992

Degradation Products of the mRNA Encoding the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase in Soybean and Transgenic Petunia

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The degradation of a soybean ribulose-1,5-bisphosphate carboxylase small subunit RNA, *SRS4*, was investigated in soybean seedlings and in petunia plants transformed with an *SRS4* gene construct. Polyacrylamide RNA gel blot, primer extension, and S1 nuclease analyses were used to identify and map fragments of the *SRS4* mRNA generated in vivo. We showed that *SRS4* mRNA is degraded to a characteristic set of fragments in soybean and transgenic petunia and that degradation is not dependent on position of insertion of the gene construct within the genome, on the expression level of the *SRS4* mRNA, or on the *rbcS* promoter. Degradation products lacked poly(A) tails and fractionated with poly(A)-depleted RNA on oligo(dT)-sepharose columns. These products pelleted with polysomes and were released from polysomes prepared with EDTA. Sequences at the 5′ end of the *SRS4* mRNA were more stable than those at the 3′ end of the mRNA. Three models for *SRS4* mRNA degradation involving endonucleolytic and exonucleolytic degradation were presented to explain the origin of the 5′ proximal fragments.

INTRODUCTION

The interplay between transcriptional and post-transcriptional processes mediates levels of specific mRNAs in a cell. Post-transcriptional events regulate RNA levels for a variety of plant genes including those for alcohol dehydrogenase (Rowland and Strommer, 1986), heat shock (Belanger et al., 1986), α-amylase (Nolan et al., 1987), seed proteins (Walling et al., 1986), ferredoxin (Elliott et al., 1989), and phytochrome (Quail et al., 1986). RNA turnover in particular is highly regulated and is an important factor in maintaining RNA levels in cells (Shapiro et al., 1987; Belasco and Higgins, 1988). Recently, it has been shown that the light regulation of soybean *rbcS* RNA levels is controlled in part by RNA degradation in seedlings (Shirley and Meagher, 1990) and mature plants (Thompson and Meagher, 1990).

RNA degradation intermediates have been identified in several prokaryotic systems. The action of endonucleases and 3′ to 5′ exonucleases leads to degradation in the net 3′ to 5′ direction of the *Escherichia coli ompA* mRNA (Melefors and von Gabain, 1988) and thioredoxin mRNA (C. Arraiano and S. Kushner, personal communication). Alternatively, the concerted action of endonucleases and 3′ to 5′ exonucleases can also lead to more rapid degradation of sequences near the 5′ end of an mRNA, causing degradation to proceed in the "pseudo" 5′ to 3′ direction as occurs with the *lacZ* (Cannistraro et al., 1986) and pilin gene (Båga et al., 1988) mRNAs of *E. coli*. In general, mRNA degradation in bacteria is thought to proceed by a combination of endonucleolytic and 3′ to 5′ exonucleolytic action (Belasco and Higgins, 1988; Chen et al., 1988; Ebbole and Zalkin, 1988). There is no evidence for the existence of 5′ to 3′ exoribonucleases in *E. coli* (Deutscher, 1985).

Little information is available on mRNA degradation intermediates in eukaryotes, although degradation often appears to initiate near the mRNA 3′ end. The first step in histone H4 mRNA degradation disrupts a 3′ stem-loop structure that is likely to be inhibitory to 3′ to 5′ exonucleases, allowing rapid exonucleolytic degradation of the mRNA (Ross and Kobs, 1986; Ross et al., 1986). The first step in the degradation of c-*myc* mRNA is poly(A) tail shortening followed by the appearance of short-lived intermediates whose 3′ ends are located in an AU-rich sequence in the 3′ untranslated region (Brewer and Ross, 1988). Degradation of apolipoprotein II (Binder et al., 1989) and β-globin (Albrecht et al., 1984) mRNAs also initiates near the 3′ end, but rather than occurring at only two unique sites, as with the histone mRNA, cleavage occurs at many sites that share similar AU-rich sequences. The effect of these cleavages is to produce a set of RNAs that lack a poly(A) tail. In these examples, degradation seems to proceed first by removal of a 3′ sequence inhibitory to exonuclease action, followed rapidly by degradation that proceeds from the 3′ end of the molecule.

Even less is known about the mechanisms of plant RNA degradation. To understand better the process of *rbcS* RNA turnover in soybean plants, we have identified the major degradation

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products of the soybean *rbcS* RNA, *SRS4*. Our data support the hypothesis that sequences at the 5′ end of the *SRS4* mRNA are more stable than sequences at the 3′ end of the mRNA. Degradation generates a nested set of *SRS4* products, which are concentrated in the polysomal fraction.

RESULTS

Identification of *rbcS* RNA Degradation Products Generated in Vitro

We were interested in characterizing the degradation products of soybean *rbcS SRS4* mRNA in soybean plants and in petunia plants transformed with an *SRS4* construct. A construct was made that fused the soybean *rbcS* gene *SRS4* to the constitutively expressed promoter for the 35S transcript of cauliflower mosaic virus (CaMV; see Methods), separating a possible link between light-induced transcription and *SRS4* mRNA degradation. Petunia plants were transformed with this construct, diagrammed in Figure 1A, and transgenic plants were regenerated and analyzed. Primer extension analysis (data not shown) of five independent isolates showed that the 5′ end of the CaMV-promoted *SRS4* mRNA (Figure 1A) is at the same adenosine nucleotide in transgenic plants as *SRS4* mRNA in soybean seedlings (Berry-Lowe and Meagher, 1982; Grandbastien et al., 1986).

SRS4 RNAs were compared in soybean and transgenic petunia plants. Figure 2 shows the result of probing polyacrylamide RNA gel blots with the 5′ portion of the *SRS4* cDNA clone (probe A; Figure 1B). Soybean RNA contained not only the full-length *SRS4* mRNA but also several RNA fragments with lower molecular weights than the full-length RNA. These fragments ranged in size from ~630 nucleotides to 115 nucleotides, the approximate detection limit of the system. The low molecular weight RNAs detected with the *SRS4* cDNA probes were of similar size in soybean and transgenic petunia. RNA purified by very different methods showed the same pattern of low molecular weight RNAs (see Methods).

The spectrum of *SRS4* low molecular weight RNAs was independent of position of DNA insertion in the petunia genome and of expression levels because the four independent transgenic plants gave similar patterns. DNA gel blots confirmed that these plants were the result of independent transformation events, and quantitative dot blot analysis (Shirley and Meagher, 1990) showed that *SRS4* mRNA levels varied over 100-fold in these plants (data not shown). However, despite the variation in RNA levels, in the four transgenic petunia lines tested (T975-1, T1075-6, T1099-12, and T1099-13), the spectrum of low molecular weight RNA fragments was the same. The faint pattern of T1099-13 was due to the extremely low level of *SRS4* expression in this plant. Longer exposures suggested a nearly identical pattern (data not shown). The plant that expressed *SRS4* mRNA at the highest level (~50% of soybean *SRS4* RNA expression), T1099-12, was examined

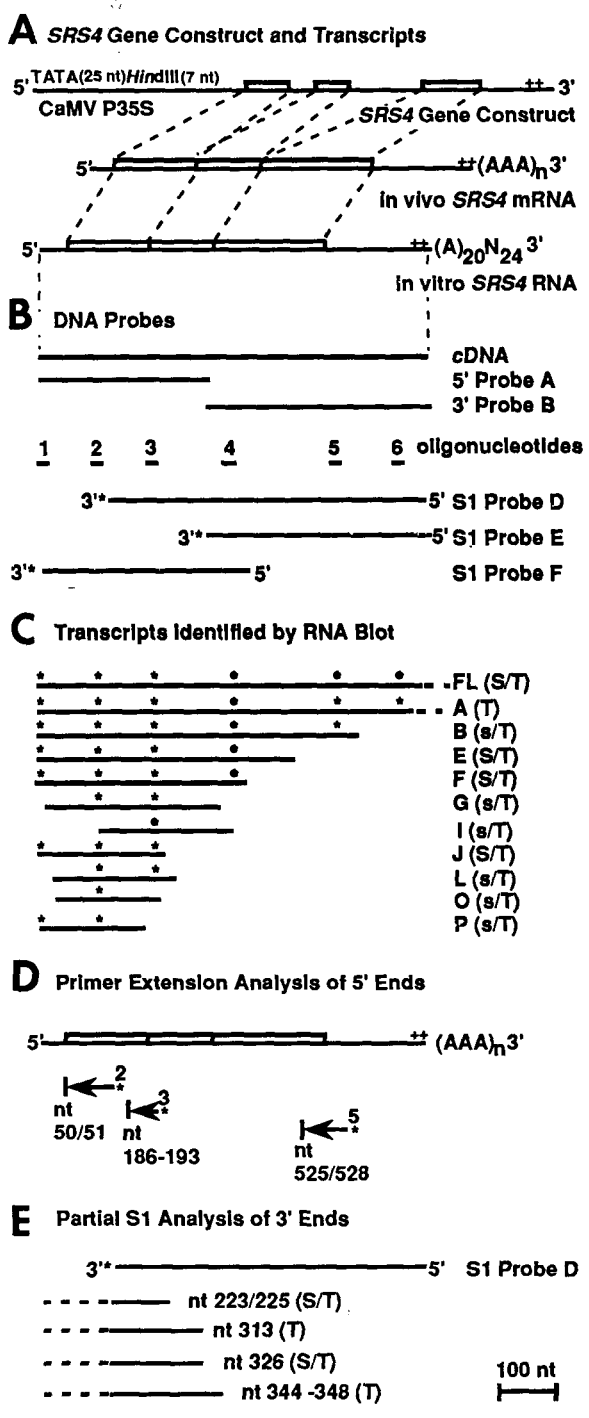

Figure 1. Diagram of the *SRS4* Gene Including Hybridization Probes and Results of RNA Gel Blot, Primer Extension, and S1 Nuclease Analyses.

(A) *SRS4* gene construct and transcripts. The *SRS4* construct was used in the transformation of *Petunia hybrida* var Mitchell. The cauliflower mosaic virus 35S promoter (CaMV P35S) was fused to the transcribed sequence of *SRS4*, as described in Methods. The TATA

in the greatest detail in this manuscript. The similarity of low molecular weight RNA products in soybean and transgenic petunia also demonstrated that the CaMV promoter had not significantly altered the production of *SRS4* low molecular weight RNAs. Figure 3 shows that transgenic petunia plants contained a few low molecular weight RNAs, which were occasionally in much lower concentrations in independent

Figure 1. (continued).

box of CaMV P35S (TATA) is located 25 nucleotides upstream of the introduced HindIII site. This site is located seven nucleotides upstream of the start of *SRS4* transcription in soybean and transgenic petunia. The three exons are indicated as open boxes. The nontranslated leader, introns, and 3′ trailer are shown as dark lines. Two polyadenylation regions are indicated by plus signs (+) (Shirley et al., 1990). In vivo *SRS4* mRNA is produced in soybean and transgenic petunia plants. The full-length RNA is 790 nucleotides long and consists of three joined exons. Two polyadenylation regions are indicated by plus signs. The poly(A) tail, heterogeneous in length and ranging in size from 20 to 200 nucleotides, is indicated by $(AAA)_n$. The *SRS4* RNA synthesized in vitro is longer than the in vivo mRNA by 15 nucleotides at the 5′ end. It is polyadenylated at the 3′ most polyadenylation site identified, and the poly(A) tail contains ~20 A residues, followed by 24 nucleotides of vector multilinker sequence.

(B) DNA probes. The DNA fragments and oligonucleotides used as probes of RNA gel blots and in primer extension and S1 nuclease analysis are shown, and their locations within the *SRS4* sequence are indicated beneath the *SRS4* transcript. The full-length cDNA and 5′ and 3′ fragments **(A** and **B)** were used as probes on RNA gel blots. S1 probes D, E, and F were labeled at the 3′ end of the nonsense strand, indicated by asterisks. For detailed descriptions, see Methods.

(C) Transcripts identified by RNA gel blot. The major *SRS4* RNA fragments identified are pictured (FL, full-length *SRS4* mRNA; A through P). Their approximate locations within the *SRS4* mRNA are indicated by a dark line. Oligonucleotides to which they hybridize are indicated by asterisks, corresponding to oligonucleotides 1 to 6 in **(B)** and in Table 1. FL and A retain their poly(A) tails, signified by a stippled line. Following the letter designations of the mRNAs are the plants in which low molecular weight RNAs of this size were identified. S, soybean; T, transgenic petunia; s, RNAs of low or variable abundance in soybean.

(D) Primary extension analysis of 5′ ends. The 5′ ends of distal low molecular weight RNAs were identified by primer extension from oligonucleotides 2, 3, and 5, which were 5′ end-labeled, as indicated by asterisks. Dark lines indicate the size of the primer extension products. The 5′ ends of fragments identified by primer extension are shown, numbered relative to the start of *SRS4* transcription.

(E) Partial S1 analysis of 3′ ends. The 3′ ends of some proximal low molecular weight RNAs were identified by S1 nuclease analysis. The results of S1 nuclease analysis with S1 Probe D are shown. Dark lines indicate the size of some of the S1 products. The 3′ ends of these fragments are numbered relative to the start of *SRS4* transcription. Stippled lines indicate that S1 nuclease analysis does not identify the 5′ ends of these fragments. Following the S1 nuclease products are the plants in which low molecular weight RNAs of this size could be identified. S, soybean; T, transgenic petunia.

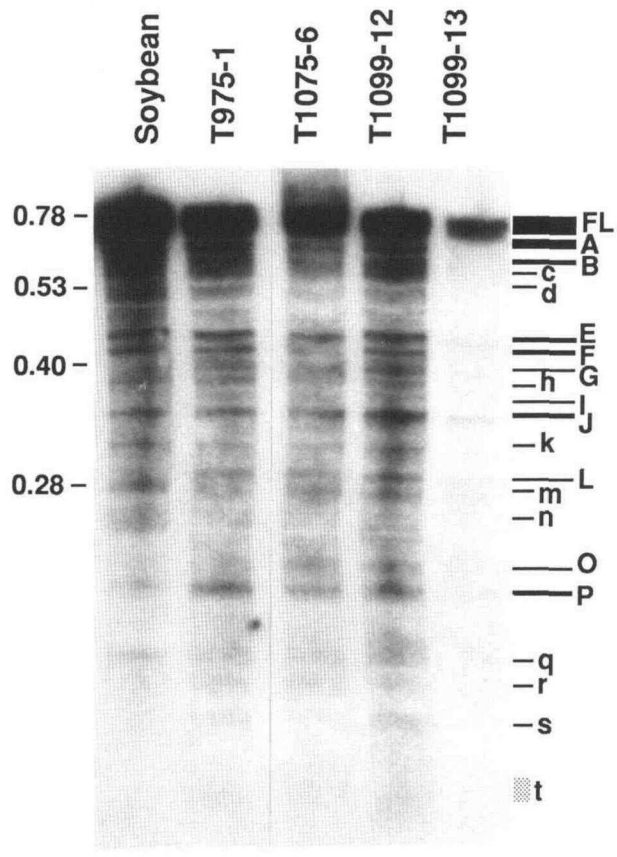

Figure 2. Detection of *SRS4* Low Molecular Weight RNAs in Soybean and Transgenic Petunia by Polyacrylamide RNA Gel Blot.

RNA isolated from soybean (2.5 μg) and transgenic plant lines T975-1 (10 μg), T1075-6 (20 μg), T1099-12 (5 μg), and T1099-13 (15 μg) was subjected to analysis on polyacrylamide RNA blots and probed with 5′ Probe A (see Figure 1B). The mobility of molecular weight markers is shown at the left in kilobases. The *SRS4* RNAs detected are designated at the right. FL, full-length *SRS4* mRNA; A to t, low molecular weight RNAs; upper case letters, RNAs that are discussed in the text; lower case letters, RNAs that are not discussed in the text.

preparations of soybean RNA (RNAs G, L, O, P, and s). The *SRS4* cDNA probes did not cross-hybridize to the native petunia *rbcS* RNAs.

A polyadenylated *SRS4* transcript generated in vitro (Figure 1A) was used in reconstruction experiments to show that low molecular weight RNAs identified were not artifacts generated during the RNA isolation procedure. In vitro transcribed *SRS4* RNA migrated in a polyacrylamide gel as two molecules with apparent molecular weights of 760 and 630 nucleotides (Figure 3). The higher molecular weight RNA had a similar mobility to full-length *SRS4* mRNA from soybean and transgenic petunia. The other RNA appeared to be the result of early termination of the in vitro transcript at a site within the *SRS4* gene (data not shown). The *SRS4* RNA synthesized in vitro was added to leaf homogenate from

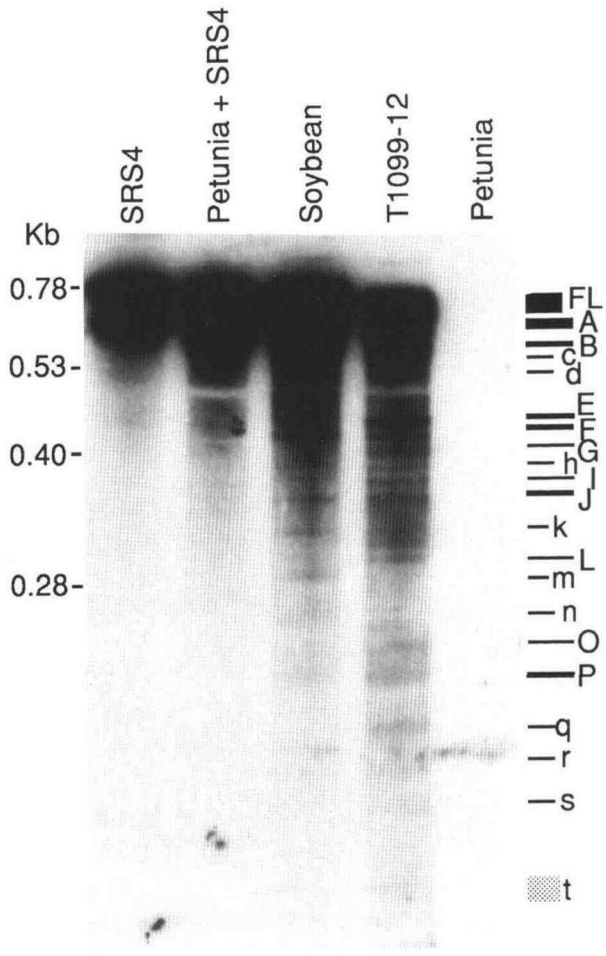

Figure 3. The *SRS4* Low Molecular Weight RNAs Are Not Generated In Vitro.

SRS4 RNA transcribed in vitro (*SRS4*; 0.1 μg), transgenic petunia reconstruction RNA (Petunia + *SRS4*; 5 μg), RNA isolated from soybean (Soybean; 2.5 μg), transgenic petunia strain 1099-12 (T1099-12; 5 μg), and nontransformed petunia (Petunia; 5 μg) were separated by electrophoresis through a polyacrylamide gel, electroblotted to a nylon membrane, then probed with a cDNA clone of soybean RNA *SRS4* (cDNA; see Figure 1B). This autoradiogram was overexposed relative to the one in Figure 2 to demonstrate that no discrete fragments are generated from the RNA purified from petunia extracts synthesized in vitro. The mobility of molecular weight standards is shown at the left. Low molecular weight RNAs shown at the right are defined in Figure 2.

nontransformed petunia (Petunia + *SRS4*) and total RNA was isolated. Although the *SRS4* transcript synthesized in vitro was degraded slightly during the RNA isolation procedure, there were no discrete low molecular weight RNAs produced that were similar to those detected in soybean or transgenic petunia RNA.

EDTA Releases *SRS4* mRNA and Low Molecular Weight RNA Degradation Products from Polysomes

Polysome-bound mRNA can be distinguished from RNA bound in ribonuclear particles (RNP) by its ability to be released from polysomes with EDTA treatment (Penman et al., 1968). To determine whether the *SRS4* low molecular weight RNA products were bound to polysomes, we investigated the distribution of the low molecular weight products in polysomes from soybean seedling leaves prepared with and without EDTA. Polyacrylamide RNA gel blot analysis of soybean polysomal RNAs, shown in Figure 4, revealed the *SRS4* low molecular weight RNAs in high concentrations in

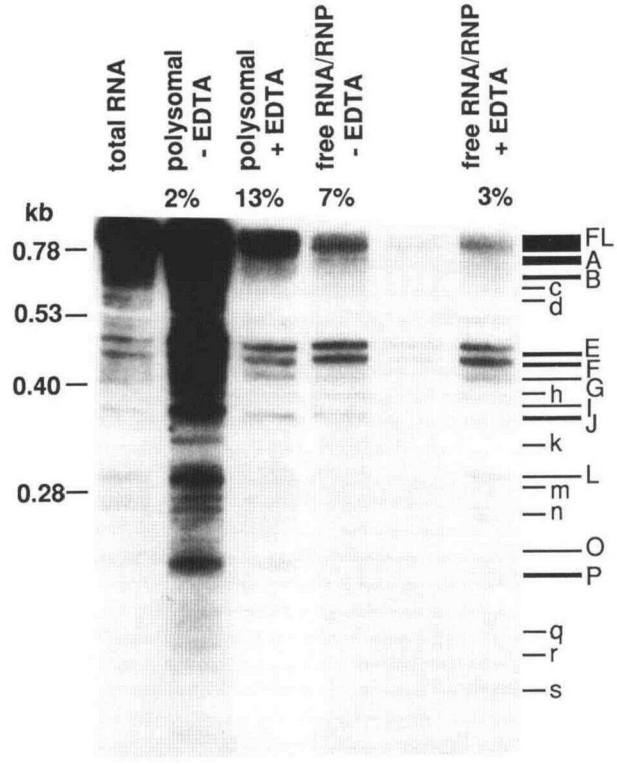

Figure 4. EDTA Release of *SRS4* mRNA Degradation Products from Soybean Polysomes.

RNA samples were subjected to polyacrylamide blot analysis and probed with 5′ Probe A (see Figure 1B). The first control lane contains 2 μg total RNA from soybean. Subsequent lanes contain 5 μg of polysomal RNA fraction (see Methods) prepared without EDTA (– EDTA), the equivalent fraction prepared with EDTA (+ EDTA), the free RNA/ribonuclear particles (RNP) supernatant prepared without EDTA (– EDTA), and the equivalent fraction prepared with EDTA (+ EDTA). The percentages were calculated from the fraction that 5 μg represents from each of the four samples (see Methods). The mobility of molecular weight markers is shown at the left and the characterized low molecular weight RNAs are shown at the right and defined in Figure 2.

the polysomal pellet, much higher than found in the supernatant fraction containing free RNA and RNPs. Clearly, the low molecular weight RNAs were in substantially higher concentrations in the polysomal pellet prepared without EDTA than found in total RNA. The 5 μg of polysomal pellet RNA showed *SRS4* low molecular weight products at ~10 times the concentration found in 5 μg of the supernatant RNA (see Methods). Because the majority of the RNA fractionated with the polysomal pellet (~78%), only 2% of the total polysomal pellet RNA was loaded onto the gel relative to 7% of the total supernatant RNA. Therefore, when normalized to the total amount of RNA in each fraction, there were ~35 times more of the low molecular weight RNAs in the pellet (~97%) than in the supernatant (~3%).

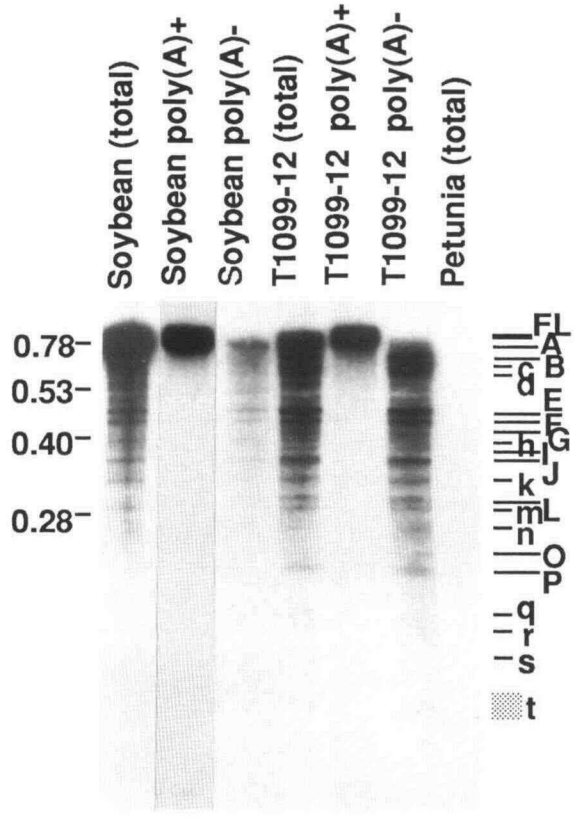

Figure 5. Low Molecular Weight RNAs Lack Poly(A) Tails.

RNA isolated from soybean seedling leaves ([total]; 2.5 μg) and leaves of transgenic petunia T1099-12 ([total]; 5 μg) was separated into poly(A)$^+$ [Soybean poly(A)$^+$; 0.2 μg and T1099-12 poly(A)$^+$; 0.05 μg] and poly(A)$^+$-depleted [Soybean poly(A)$^-$; 2.5 μg and T1099-12 poly(A)$^-$; 5 μg] fractions, and polyacrylamide RNA gel blots were performed on all the fractions. Total RNA from nontransformed petunia (Petunia [total]) was included as a negative control for hybridization. This RNA gel blot was probed with 5′ cDNA Probe A (see Figure 1B). The mobility of molecular weight standards is indicated at the left in kilobases. The low molecular weight RNAs shown at the right are defined in Figure 2.

When a polysomal pellet was prepared in the presence of EDTA, a significant portion of low molecular weight products was shifted into the free RNA or RNP fraction. The EDTA-treated polysomal pellet, as expected, contained a much smaller proportion of the RNA (~19%) than did the EDTA-treated polysomal supernatant (~81%). Thus, 13% of the EDTA-treated polysomal pellet was loaded relative to 3% of the EDTA-treated polysomal supernatant. The lanes with 5 μg of RNA from each of these fractions contained equivalent amounts of the low molecular weight products. In other words, ~80% of the degradation products were found in the free RNA and RNP supernatant fraction prepared with EDTA. This dramatic shift of low molecular weight RNAs from the polysomal pellet prepared without EDTA (97%) to the EDTA-treated polysomal supernatant suggests that the low molecular weight fragments of *SRS4* RNA mimic the behavior of mRNA on polysomes.

SRS4 Low Molecular Weight RNAs Do Not Retain Their Poly(A) Tail

To determine whether low molecular weight RNAs retained their poly(A) tails, and hence whether 3′ *SRS4* transcript sequences were retained, total cellular RNA was isolated from leaves of soybean seedlings, transgenic plant T1099-12, and nontransformed petunia, and was separated into poly(A)$^+$ and poly(A)$^+$-depleted fractions. The RNA gel blot was probed with 5′ *SRS4* Probe A (Figure 1B). Figure 5 shows the spectrum of low molecular weight RNAs detected in total RNA from leaves of soybean seedlings and transgenic petunia T1099-12. The poly(A)$^+$ RNA fractions of soybean and T1099-12 retained primarily the full-length *SRS4* mRNA and no detectable low molecular weight RNAs. In contrast, the majority of the low molecular weight RNAs were present in the poly(A)$^+$-depleted RNA fraction of soybean and T1099-12. This result suggests that the poly(A) tail might be removed early in the metabolism of *SRS4* mRNA in soybean and transgenic petunia.

Sequences from the 5′ End of *SRS4* mRNA Are More Prevalent in the Low Molecular Weight RNA Fraction

RNA gel blots were probed with cDNA fragments from the 5′ and 3′ portions of the *SRS4* mRNA (Figure 1B) to begin to map the sequences present in each low molecular weight RNA. Figure 6 shows the result of hybridization with a probe to the 5′ portion of *SRS4*. This probe hybridized to the same *SRS4* low molecular weight RNAs detected with an *SRS4* cDNA probe in soybean and T1099-12 (compare with Figure 3A). In contrast, a probe to the 3′ portion of *SRS4* hybridized only to the largest of the low molecular weight RNAs. Thus, RNAs that retained the 5′ portion of the *SRS4* mRNA were overrepresented in the population of low molecular weight

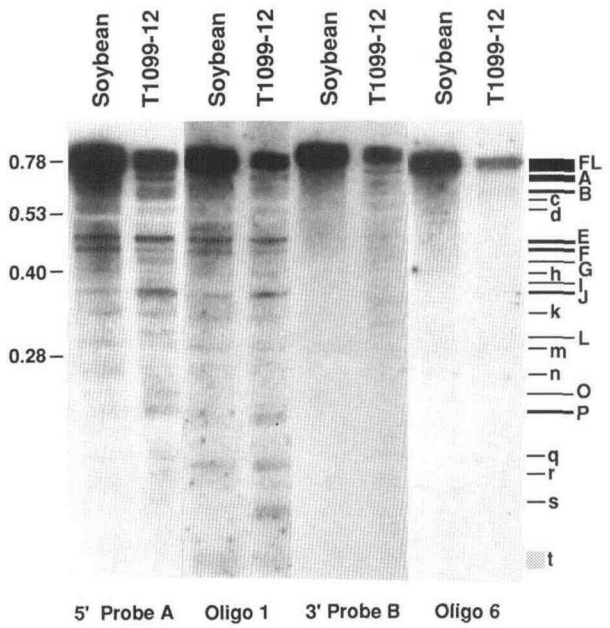

Figure 6. Degradation Proceeds in a Net 3′-to-5′ Direction.

RNA isolated from soybean (2.5 μg) and transgenic plant T1099-12 (5 μg) was subjected to polyacrylamide blot analysis. Blots were probed with 5′ Probe A (see Figure 1B), oligonucleotide 1 (Oligo 1; see Figure 1B and Table 1), 3′ Probe B (see Figure 1B), and oligonucleotide 6 (Oligo 6; see Figure 1B and Table 1). The mobility of molecular weight standards is indicated at the left in kilobases. The low molecular weight RNAs shown at the right are defined in Figure 2.

RNAs relative to those that retained the 3′ portion of the *SRS4* mRNA. This result suggests that 5′ (proximal) *SRS4* RNA degradation products are more stable than 3′ (distal) *SRS4* RNA degradation products.

RNA gel blots were then probed with the six oligonucleotide probes shown in Table 1 that are complementary to regions spanning the *SRS4* mRNA sequence, allowing us to map more accurately these low molecular weight RNA fragments (Figure 1). Representative blots are shown in Figure 6. An oligonucleotide probe complementary to nucleotides 1 to 17 of the *SRS4* mRNA hybridized to most of the low molecular weight RNAs detected with the 5′ probe. This result shows that most of the low molecular weight RNAs retain their 5′ ends. Only four low molecular weight RNAs (out of 21 detected) did not hybridize to oligonucleotide 1 (RNAs G, I, L, and O) and presumably lack their extreme 5′ ends. An oligonucleotide complementary to nucleotides 735 to 751, which maps immediately upstream of the first poly(A) site, hybridized only to the full-length *SRS4* mRNA in soybean and to the full-length *SRS4* mRNA and low molecular weight RNA A in T1099-12. Oligonucleotides 2, 3, 4, and 5, respectively, reacted with successively fewer bands, showing patterns intermediate between oligonucleotides 1 and 6 (Thompson, 1990). The results of oligonucleotide probing of these products are summarized in Figure 1C.

Detection of Distal RNA Degradation Products

If the proximal low molecular weight RNAs described above were generated, at least in part, by endonucleolytic cleavage of the *SRS4* mRNA, it might be possible to detect the low abundance distal *SRS4* RNA fragments by primer extension of oligonucleotides from the 3′ end of these RNAs. Oligonucleotides 2, 3, 4, 5, and 6 were end-labeled and used to direct primer extension on *SRS4* RNA from leaves of soybean seedlings, T1099-12, and petunia. A representative result is shown in Figure 7. *SRS4* RNA synthesized in vitro and the transgenic petunia RNA reconstruction were used as controls for degradation of the *SRS4* transcript during RNA isolation or primer extension and for premature termination by reverse transcriptase. The full-length *SRS4* mRNA transcribed in vitro was larger than the mRNA from soybean or T1099-12 by 15 nucleotides, as described in Methods. There was some degradation of the *SRS4* mRNA synthesized in vitro (or premature termination by reverse transcriptase) at nucleotides 58 and 59 in the control lanes. However, soybean and T1099-12 shared novel *rbcS* primer extension products terminating at nucleotides 4, 7, 49, and 50. The data from this experiment and other primer extension experiments are summarized in Figure 1D and Table 2.

Primer extension from these five oligonucleotides identified 11 primer extension products clustered in three regions internal to the full-length RNA: nucleotides 49/50, 186 to 193, and 525/528. These products represent three regions in which *SRS4* RNA fragments have 5′ termini. The regions do not correspond to previously identified splice sites (Grandbastien et al., 1986) and therefore probably did not result from primer extension directed by *SRS4*-splicing intermediates. There is no obvious sequence similarity in the RNA immediately surrounding these sites as determined by a visual comparison of the sequences (Table 2). The most predominant 5′ end detected by primer extension, at nucleotides 49/50, contains the consensus determined from apolipoprotein II mRNA degradation, 5′-AAU-3′ (Binder et al., 1989). RNAs G, L, and O, which did not hybridize to oligonucleotide 1, hybridized to oligonucleotides 2 and 3 on RNA gel blots. Thus, the 5′ ends

Table 1. Oligonucleotide Sequence

Oligo No.	Nucleotides	Sequence
1	1–17	5′-ATCTGGCAGCAGAAGAA-3′
2	126–142	5′-TGGCCTCAAGTCCATGG-3′
3	222–249	5′-GTTGGCAAGAAGAAGTTT-3′
4	353–369	5′-AGCACGGTTTCGTGTAC-3′
5	604–621	5′-GCATTTGTAGCCGCCAC-3′
6	735–751	5′-AGAACTAATGAATAAGC-3′

The oligonucleotides used to probe polyacrylamide RNA gel blots and in primer extension analysis are shown in the first column. The nucleotides to which they are complementary are indicated in the second column. The sequence of *SRS4* to which each oligonucleotide is complementary is shown in the third column.

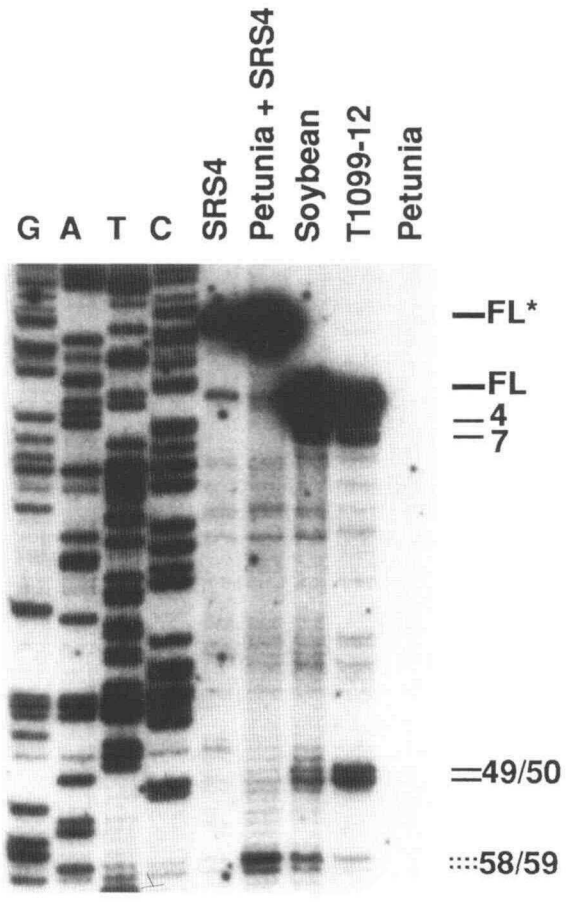

Figure 7. Determination of 5′ Ends of Distal *SRS4* RNA Fragments.

Primer extension analysis from oligonucleotide 2 (see Figures 1B and 1D, and Table 1) was performed on in vitro synthesized *SRS4* RNA (*SRS4*; 0.19 μg), the *SRS4* reconstruction described in the text (Petunia + *SRS4*; 20 μg), RNA isolated from soybean (10 μg), transgenic plant T1099-12 (20 μg), and nontransformed petunia (20 μg). A sequence ladder of plasmid pDMT27 is also shown. FL, the full-length *SRS4* mRNA from soybean and transgenic petunia; FL*, the *SRS4* mRNA produced by SP6 polymerase in vitro; the numbers at the right indicate the nucleotide number (relative to the in vitro start of *SRS4* transcription) for the 5′ ends of the primer extension products. The dotted lines indicate 5′ ends that may have been generated during RNA purification.

of these RNAs may be at nucleotides 49/50. A similar comparison of oligonucleotide hybridization data with primer extension data suggested that RNA I may have its 5′ end between nucleotides 186 and 193. A weak 5′ end was detected at nucleotides 525/528 and could correspond to a distal fragment generated during formation of low molecular weight RNA E. However, 5′ ends corresponding to distal fragments produced from endonucleolytic cleavages that may have generated the proximal low molecular weight RNAs were not detected.

S1 Analysis of 3′ Ends Internal to the Transcript

We have shown that the majority of the low molecular weight RNAs detected on RNA gel blots must have 3′ ends internal to the full-length *SRS4* mRNA, and oligonucleotide probing was used to give approximate 3′ end points for some of these RNAs. If some of these 3′ ends were generated by the same endonucleolytic events that gave rise to 5′ ends internal to the *SRS4* RNA, it might be possible to align adjacent fragments (e.g., *lac* RNAs; Subbarao and Kennell, 1988). Limited S1 analysis was performed to further map 3′ ends. S1 probes D, E, and F (Figure 1B) were labeled at the 3′ end of their nonsense strands and hybridized with RNA from soybean and T1099-12. The probes were hybridized with nontransformed petunia RNA and with the Petunia + *SRS4* reconstruction as controls for cross-hybridization of the probe with petunia *rbcS* RNA or degradation of the *SRS4* transcript during isolation or S1 treatment. Some of the 3′ ends detected with S1 probe D mapped to regions potentially corresponding to the 3′ ends of low molecular weight RNAs G, I, J, L, O, and P, but no 3′ end was detected by S1 nuclease analysis adjacent to the 5′ end at nucleotides 525/528 (Table 2). Representative data are shown in Figure 1E. Thus, comparison of 5′ and 3′ termini within the *SRS4* RNAs did not distinguish between endonucleolytic cleavage and 5′ to 3′ exonuclease activity as the mode of degradation at these regions.

Table 2. Location and Sequence Context of 5′ Ends of Distal *SRS4* RNA Fragments

nt	Sequence Context	Strong/Weak
4	ATC**T** GGCAGC	**S/T**
7	ATCTGG**C** AGCAGA	**S/T**
26/27	AGTAGT**T**GAGAACT	T [poly(A)$^+$ only]
49/50	AGCAAA**T**GGCTTCC	**S/T**
186/187	CTCCAT**T**GCTAGCA	**S [total, poly(A)$^+$]**
193	GCTAGC**A** ACGGTG	**S/T**
194	CTAGCA**A** CGGTGG	**Petunia + *SRS4***
525	CGGATT**C** GACAAC	S/T
528	ATTCGA**C** AACGTT	S

The positions of the 5′ termini within the *SRS4* RNA as determined by primer extension are indicated in the first column (nt). The sequence surrounding the last nucleotide incorporated into the primer extension product is indicated in the second column. The sequence is written from 5′ to 3′, and the last nucleotide incorporated is in bold letters. The relative intensities of primer extension products are indicated in the third column, with stronger products in bold type and weaker products in lightface type. The normal start of transcription of *SRS4* is nucleotide 1 and represents the majority of 5′ ends. Products that are detected by primer extension of soybean RNA are labeled S, and those detected from transgenic plant RNA are labeled T. **S**, strong in soybean; **T**, strong in T1099-12; S, weak in soybean; T, weak in T1099-12. Homology to an animal consensus sequence is underlined in 49/50.

DISCUSSION

We have identified fragments of the soybean *rbcS SRS4* RNA in soybean seedlings and, under the control of a heterologous promoter, in transgenic petunia. We suggest that these fragments are specific in vivo RNA degradation products. If the low molecular weight RNAs identified are generated as the result of pausing by RNA polymerase, aberrant transcription termination, or altered splicing of the *SRS4* mRNA, these products have been subsequently loaded onto polysomes. It seems more likely that the low molecular weight RNAs are generated from full-length mRNA already loaded onto polysomes. In animals, the degradation of tubulin (Gay et al., 1989) and histone mRNAs (Ross and Kobs, 1986; Marzluff and Pandey, 1988) is intimately linked to translation on polysomes. The degradation products of *SRS4* mRNA are similar in soybean and transgenic petunia, implying that the nucleases that act upon *SRS4* RNA are similar in these two highly diverged species of plants. By one interpretation, the fragments with intact 5′ mRNA sequences are simply more stable than fragments containing 3′ sequences. Degradation of *SRS4* mRNA could proceed by a combination of endonucleolytic cleavage and exonucleolytic digestion (see models below).

Promoters have been shown to influence post-transcriptional processes, including RNA maturation (Sisodia et al., 1987). *rbcS* promoters are controlled by light in a complex manner and thus could complicate the analysis of *SRS4* mRNA degradation products. In addition, an *rbcS* promoter and a particular nuclear environment might be required for normal *rbcS* RNA degradation in a transgenic host. Therefore, the *SRS4* gene was fused to a constitutively expressed promoter for analyses in transgenic plants. The major *SRS4* mRNA degradation products identified in RNA isolated from soybean seedling leaves were also present in RNA from the leaves of petunia plants transformed with this *SRS4* construct. It has been shown that the stability of *rbcS* mRNA is modulated by light in soybean (Shirley and Meagher, 1990; Thompson and Meagher, 1990), but not in petunia (Thompson and Meagher, 1990). However, the overall similarity of the *SRS4* mRNA degradation products between soybean and transgenic petunia suggests that the primary pathway for degradation of *rbcS* mRNAs is shared between soybean and petunia and is not affected by transcription from a different RNA polymerase II promoter or by the position of insertion in the genome.

Poly(A) Tail Removal Is an Early Step in *SRS4* mRNA Degradation

The poly(A) tail, together with poly(A)-binding proteins, has been proposed to act as a barrier to 3′ to 5′ exonuclease action in eukaryotes (Bernstein and Ross, 1989; Jackson and Standart, 1990). The poly(A) tail has also been implicated in reinitiation of protein translation (Jackson and Standart, 1990). Studies of RNA degradation of c-*myc* (Brewer and Ross, 1988), apolipoprotein II (Binder et al., 1989), and β-globin (Albrecht et al., 1984) have shown that degradation products of these RNAs are more prevalent in the poly(A)$^+$-depleted fraction of RNA, implying that they have lost their poly(A) tails early in the RNA degradation process. In a similar fashion, the major *SRS4* RNA fragments detected, even the largest ones, have already lost their poly(A) tails, as seen by a comparison of poly(A)$^+$ RNA with poly(A)$^+$-depleted RNA (Figure 5). The lack of a poly(A) tail in these low molecular weight RNAs may have the double effect of allowing increased degradation while preventing the reinitiation of translation of the fragmented RNA.

Proximal *SRS4* RNA Fragments Are More Stable than Distal *SRS4* RNA Fragments

Considering the various results obtained for the net direction of degradation of bacterial and vertebrate mRNAs, it was essential to characterize the *SRS4* RNA sequences retained in the *SRS4* low molecular weight RNA fraction in soybean and in a transgenic host. The low molecular weight RNAs comprise a set of nested fragments lacking increasing amounts of the 3′ end of *SRS4* mRNA. Clearly, 3′ *SRS4* RNA sequences are being degraded in preference to 5′ sequences (see models below). It is unclear from these studies whether poly(A) removal is a separate step or merely the result of endo- or exonucleolytic action initiating near the 3′ end of the mRNA.

Although distal *SRS4* RNA degradation products seem to be in low abundance relative to 5′ end products, primer extension analysis allowed us to identify a few internal 5′ ends among *SRS4* RNA degradation products. S1 nuclease analysis was performed on the RNA fragments to determine whether 3′ ends of the major proximal *SRS4* RNA fragments (A to P) were adjacent to 5′ ends detected by primer extension. Although very few 5′ ends were detected, numerous 3′ ends of *SRS4* low molecular weight RNA fragments were easily observed. None of them mapped adjacent to 5′ ends identified above. It is therefore unclear whether the internal 5′ and 3′ ends detected result from endonucleolytic cleavage or exonucleolytic degradation that pauses at discrete sites in the molecule.

Models for *SRS4* mRNA Degradation

Any model for *rbcS* RNA degradation must be consistent with the observed overrepresentation of 5′ proximal *SRS4* RNA sequences. The degradation process results in the formation of a nested set of *SRS4* RNA fragments that are related to each other by increasing deletions from the 3′ end of the mRNA. Although most of these RNA fragments retain intact 5′ ends,

all of them have lost their poly(A) tails as a result of this degradation. Our data suggest three simple models for RNA degradation. In an exonuclease model, degradation would proceed by way of a 3′ to 5′ exonuclease whose progress is impeded at specific "hold-up points" (Ross and Kobs, 1986). In this model, sequence and/or structural signals impede the progress of the exonuclease to the extent that transient proximal *SRS4* RNA fragments are detectable. The lack of detectable 3′ *SRS4* RNA fragments is explained by the nature of the nucleolytic action. The presence of fragments such as RNAs G, I, L, and O, which lack 5′ RNA sequences, could be explained by the presence of a low background of endonuclease or 5′ to 3′ exonuclease activity.

In a scanning endonuclease model, degradation proceeds by way of endonuclease cleavages at discrete sites within the mRNA. These cleavage events proceed along the RNA by way of an unknown mechanism in a 3′ to 5′ direction, removing successively greater portions of the RNA. The endonuclease would scan to defined sequences and/or structures that direct cleavage. The nested set of proximal fragments produced are in general larger and therefore more easily detected by RNA gel blot analysis than the smaller distal fragments. Alternatively, the smaller distal fragments could be inherently less stable and rapidly degraded by exonucleases. In a stochastic endonuclease model, an endonuclease would have preference for specific recognition sites on intact RNA but would choose among these sites randomly. The stochastic model is different from the other two models in that the fragments would not be generated in any specific order. Degradation by the scanning model would give rise to small fragments that are removed in a stepwise manner, whereas degradation by the stochastic model would initially give rise to larger distal fragments. This stochastic endonucleolytic attack could produce 5′ low molecular weight RNA products indistinguishable from those produced in the exonuclease or scanning models if the larger distal fragments produced were inherently unstable. Our data do not distinguish among these three models. Clearly, distinguishing among these models will depend on the ability to monitor precursor/product relationships for the RNA molecules.

METHODS

Plant Material and Treatment

Soybean seeds (*Glycine max* var Pella '86) were germinated and grown as described (Shirley and Meagher, 1990). Seedling leaves were harvested after 8 days of growth.

The recombinant *Agrobacterium tumefaciens* strain GV3111SE/pTL11 was used to transform *Petunia hybrida* var Mitchell by way of the leaf disc cocultivation procedure (Horsch et al., 1985). Plants were regenerated and grown for 1 month in culture, after which they were grown under greenhouse conditions until leaves were harvested. Individual transformants were propagated vegetatively, and only young expanding leaves were harvested.

Plasmid Constructions

SRS4 *Plant Transformation Vector*

The plant expression vector pMON316 was obtained from Monsanto Corporation (St. Louis, MO) and has been described by Sanders et al. (1987). This vector contains a cauliflower mosaic virus promoter (P35S) to direct transcription of inserted sequences. Based on S1 nuclease mapping, transcription is reported to start 31 nucleotides downstream from the first adenine nucleotide in the TATATAA box of the P35S, at the first cytosine residue of the sequence CAC (Guilley et al., 1982; Odell et al., 1985). However, neither of these studies corrects for the difference in migration of chemical sequencing products and S1 nuclease products (Sollner-Webb and Reeder, 1979). When this correction was made, the transcription start site mapped to the adenine residue in the CAC sequence 32 nucleotides downstream of the TATA box. Oligonucleotide-directed mutagenesis of pMON316 was used, therefore, to insert a HindIII site 25 nucleotides downstream from the first A residue of the promoter TATA element for the 35S transcript of CaMV (P35S) and prior to the normal CaMV transcriptional start site to give plasmid pDMT17. The sequence of this mutagenic oligonucleotide is 5′-CATTTCATTAAGCTTTGGAGAGGA-3′. To remove the existing HindIII site downstream from the 3′ termination and polyadenylation signal of nopaline synthase, pMON316 was digested with HindIII and the ends filled in with the Klenow fragment of DNA polymerase and deoxynucleotide triphosphates. This molecule was recircularized, and the resultant plasmid, pDMT18, contained no HindIII sites. Plasmids pDMT17 and pDMT18 were digested with BstEII and EcoRI, and the fragments ligated together to form plasmid pDMT19. This vector contained the P35S with only the one new HindIII site.

The 1.5-kb *SRS4* soybean genomic sequence (Grandbastien et al., 1986) was subcloned on a HindIII fragment into the vector pTZ18R (Promega Biotech) to produce plasmid pTL3. The 5′ HindIII site is seven nucleotides upstream from the native *SRS4* transcriptional start site. Oligonucleotide-directed mutagenesis was used to insert a SalI site in *SRS4* 84 nucleotides downstream of the most distal polyadenylation region mapped by Shirley et al. (1990). The sequence of this oligonucleotide is 5′-TGTTAACCAGTCGACTCATTTAG-3′. The resultant plasmid, pTL7, was digested with SalI and recircularized to remove a portion of the multilinker and generate a plasmid, pTL8, which contained only one SalI site. pTL8 was digested with HindIII and SalI and ligated into the HindIII/SalI replacement region of pDMT19 to yield pTL11. This plasmid contained the promoter for the 35S transcript of CaMV fused to *SRS4* genomic coding sequence under the control of the *SRS4* 3′ termination and polyadenylation signals. The transcription start directed by P35S is predicted to begin at the same adenine nucleotide within the *SRS4* transcript as in soybean, now 32 nucleotides downstream from the first A of the P35S TATA box. This gene construct is shown schematically in Figure 1A. The plasmid pTL11 was introduced into *A. tumefaciens* strain GV3111SE (Fraley et al., 1985) by way of the triparental mating procedure of Comai et al. (1983), giving rise to the recombinant strain GV3111SE/pTL11.

SRS4 *cDNA Construct*

Several *SRS4* cDNA clones were isolated from a soybean cDNA library (Shirley et al., 1990). One of these clones, pL21, was digested

with NcoI and BstEII and ligated into the NcoI/BstEII replacement region of pTL8 to generate pDMT25. pDMT25 contained the entire transcribed sequence of *rbcS* RNA *SRS4*, flanked by seven nucleotides of untranscribed sequence at the 5′ end. The 3′ nontranscribed sequence included the sequence from the translation stop codon to 84 nucleotides downstream from the most distal polyadenylation site identified (Shirley et al., 1990).

SRS4 *RNA in Vitro Transcription Template*

Several *SRS4* cDNA clones were isolated from a soybean cDNA library (Shirley et al., 1990). In soybean seedling leaves, *SRS4* is polyadenylated at numerous sites concentrated in two regions separated by ~35 nucleotides. An *SRS4* cDNA clone that was polyadenylated at nucleotide 786, the most distal polyadenylation site identified, was used as the template for in vitro synthesis of *SRS4* RNA. This polyadenylated *SRS4* cDNA clone, pL10, was digested with BstEII and BamHI and ligated with the HindIII/BstEII fragment of pDMT25 into the HindIII/BamHI replacement site of the vector pSP64[poly(A)] (Promega Biotech) to generate the plasmid pDMT27. The plasmid pDMT27 was digested downstream from the *SRS4* sequence with BamHI and used as a template for SP6 polymerase–mediated in vitro synthesis of *SRS4* mRNA. The manufacturer's directions were followed except that the RNA was not treated with DNAse I after synthesis. The *SRS4* mRNA synthesized in vitro was not capped with 5′ ppG. This mRNA was identical to the *SRS4* mRNA synthesized in vitro except that 15 nucleotides of vector sequence were present at the 5′ end of the transcript synthesized in vitro. At the 3′ end of the RNA synthesized in vitro was a poly(A) tract of ~20 nucleotides (derived from the cDNA clone) followed by 24 nucleotides of vector sequence.

RNA Isolation

RNA was isolated from the leaves of petunia and soybean by a modification of the urea-phenol DNA extraction procedure of Shure et al. (1983), as described in Thompson and Meagher (1990). Poly(A)$^+$ RNA was isolated as described by Sambrook et al. (1989). Soybean and petunia RNA were also prepared by guanidine thiocyanate (Berry-Lowe and Meagher, 1985) and detergent phenol-chloroform (Dean et al., 1985) procedures in a few experiments (data not shown) to demonstrate that the low molecular weight RNA species were not dependent upon the purification method. All plants were grown on a 12-hr light/dark cycle, and leaves were harvested ~6 hr into the light cycle.

Reconstruction Control for *SRS4* RNA Degradation

To create the Petunia + *SRS4* RNA reconstruction, *SRS4* mRNA was transcribed in vitro. Immediately after homogenization of nontransformed petunia leaves, the *SRS4* RNA synthesized in vitro was added to the extract and total RNA was isolated as described. A total of 7.5 μg of RNA based on A_{260} from the in vitro synthesis of *SRS4* mRNA was added per gram of nontransformed petunia leaves. (One gram of leaves contains approximately 500 μg of RNA.)

RNA Gel Blotting and Probes

RNA gel blot analysis was done according to the method of Fitzwater et al. (1988), with the following refinements. The 6% polyacrylamide gels (0.75 mm thickness × 15 cm width × 13 cm length) were run for ~1 hr at 20 W. It should also be noted that when preparations of soybean seedling RNA are examined on standard agarose RNA gel blots, only a single broad band of RNA is resolved in the position of FL (Grandbastien et al., 1986). Initial data from our laboratory suggest that low molecular weight RNAs for transcripts other than *rbcS* are easily resolved on the electroblotted polyacrylamide gels.

The synthetic oligonucleotides used as probes and the sequences to which they hybridize are shown in Table 1. Oligonucleotides were labeled at the 5′ end with ^{32}P-γ-ATP and T4 polynucleotide kinase (Sambrook et al., 1989). Hybridization and wash solutions were as described in Thompson and Meagher (1990). The T_m of each oligonucleotide was determined by the formula 4°C per G or C residue plus 2°C per A or T residue of the oligonucleotide (Suggs et al., 1981). Hybridizations were performed overnight at the T_m of the oligonucleotide −10°C. Washes were for 2 × 10 min at room temperature, followed by 1 × 1 min at T_m −2°C. Filters were visualized by autoradiography.

DNA fragments used as probes are shown schematically in Figure 1B. The *SRS4* cDNA probe was derived from a HindIII/BamHI digest of plasmid pDMT25 and consisted of the entire transcribed sequence of *SRS4* plus seven nucleotides of nontranscribed 5′ sequence and 3′ nontranscribed sequence from the translation stop codon to 84 nucleotides downstream from the most distal polyadenylation site identified. The 5′ Probe A was the 5′ EcoRI/HindIII fragment of pDMT25 and contained the 5′ untranslated region (including seven nucleotides of 5′ untranscribed sequences), first exon, and most of the second exon of *SRS4*. The 3′ Probe B was the 3′ EcoRI/HindIII fragment of pDMT25 and contained some of the second exon, the third exon, and 3′ nontranscribed sequence from the translation stop codon to 84 nucleotides downstream from the most distal polyadenylation site identified. It should be noted that the *SRS4* cDNA probes (Figure 1) have the potential to cross-hybridize with RNA from the closely related *SRS1* gene in soybean (Shirley et al., 1990). Probes cDNA, A, and B were labeled, and filters were hybridized and washed as described (Thompson and Meagher, 1990). All hybridizations and washes with the soybean *rbcS* probes were performed at 60°C. Under these conditions, there was no hybridization with native petunia *rbcS* RNAs (Figure 3).

DNA fragments used as S1 probes are shown schematically in Figure 1B. S1 Probe D was generated by digestion of plasmid pDMT25 with NcoI, which cut at nucleotide 137 of the *SRS4* coding sequence (within the first exon), and BamHI, which cut in the multilinker 3′ to the gene. S1 Probe D was labeled by filling in the recessed NcoI end with the Klenow fragment of DNA polymerase and dCTP and ^{32}P-α-ATP. S1 Probe E was generated by digestion of plasmid pDMT27 with EcoRI, which cut at nucleotide 340 of the *SRS4* coding sequence (near the end of exon 2), and BamHI, which cut within the multilinker 3′ to the gene. S1 Probe E was labeled by filling in the recessed EcoRI end with the Klenow fragment of DNA polymerase and ^{32}P-α-ATP. S1 Probe F was generated by digestion of the cDNA probe (described above) with Fnu4HI, which cleaved the fragment at nucleotide 7 (within the first exon) and nucleotide 427 (within the third exon). S1 Probe F was labeled by filling in the recessed Fnu4HI end with the Klenow fragment of DNA polymerase and ^{32}P-α-TTP. This method labeled only the 3′ end of the non-sense strand of the Fnu4HI fragment spanning nucleotides 7 to 427 of *SRS4*.

RNA Characterization

Primer extension analysis was performed as described by Inoue and Cech (1985). Primer extensions were performed at T_m −5°C of the oligonucleotide primer. Sequence ladders were generated by dideoxy sequencing of the plasmid pDMT27 from the same oligonucleotide. S1 analysis was as described by Sambrook et al. (1989). Relative band intensities on dot blots and autoradiograms (Shirley and Meagher, 1990) were quantified using a computing densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA).

Polysome Isolation and EDTA Release

Polysomes were isolated by modifying methods previously described by Larkins and Davies (1975) and Ross and Kobs (1986). Frozen soybean tissue (10 pairs of leaves) was ground in a mortar and pestle in liquid nitrogen and resuspended in 10 mL of buffer A (1 mM potassium acetate, 50 mM magnesium acetate, 2 mM dithiothreitol, 200 mM Tris-HCl [pH 8.75], and 200 mM sucrose) in the absence or presence of 0.1 M EDTA. The homogenate was spun at 3000*g* for 10 min at 4°C to remove large debris. The supernatant was incubated with 0.1 volume of buffer A/10% Triton X-100 for 5 min on ice and spun at 27,000*g* for 10 min at 4°C to remove small debris. The polysomes were pelleted through a 30% sucrose cushion in buffer A at 35,000 rpm in a Beckman SW41 rotor (156,000*g* at the average radius) for 3 hr. The supernatant was saved, and the polysome pellet was resuspended in 0.3 mL of buffer A. RNA from the polysome pellet and the supernatant was precipitated with ethanol. The pellets were resuspended in 0.3 mL of lysis buffer (8.0 M urea, 0.35 M sodium chloride, 50 mM Tris-HCl [pH 7.5], 20 mM EDTA, and 2% sarcosyl) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1) two times and chloroform/isoamyl alcohol (24:1) one time. The RNA was precipitated with ethanol, resuspended in water, and stored with two volumes of ethanol. The percentage of the four samples loaded in the polyacrylamide gel blotted in Figure 4 was calculated from the amount that 5 μg represents from the 250 μg of polysomal RNA prepared without EDTA, 71 μg of supernatant RNA prepared without EDTA, 38 μg of polysomal RNA prepared with EDTA, and 167 μg of supernatant RNA prepared with EDTA, respectively. The total yield of RNA prepared without EDTA cannot be compared precisely to the amount prepared with EDTA because the yield of RNA from 10 leaf pairs varies. Relative band intensities were quantified using a computing densitometer (Molecular Dynamics).

ACKNOWLEDGMENTS

We thank Tricia Laurenson for construction of the pTL11 plasmid and Joann Davis for transgenic plant production. We also thank Caroline Ingle, Sidney Kushner, Lois Miller, Julie Senecoff, and Jeffrey Ross for constructive criticism and editing of this manuscript. This work was supported by Department of Energy Grant No. DE-FG09-86ER13543 and by a National Institutes of Health predoctoral training grant to D.M.T. This work serves as partial fulfillment of a Ph.D. degree in genetics to D.M.T. and M.M.T.

Received July 11, 1991; accepted November 12, 1991.

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