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Characterization of Post-Transcriptionally Suppressed Transgene Expression That Confers Resistance to Tobacco Etch Virus Infection in Tobacco

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Tobacco lines expressing transgenes that encode tobacco etch virus (TEV) coat protein (CP) mRNA with or without nonsense codons give rise to TEV-resistant tissues that have reduced levels of TEV CP mRNA while maintaining high levels of transgene transcriptional activity. Two phenotypes for virus resistance in the lines containing the transgene have been described: immune (no virus infection) and recovery (initial systemic symptoms followed by gradual recovery over several weeks). Here, we show that at early times in development, immune lines are susceptible to TEV infection and accumulate full-length CP mRNA. Therefore, immune lines also exhibit meiotic resetting, as is seen in the recovery lines, providing molecular evidence for a common mechanism of gene silencing and virus resistance in both cases. We also investigated the characteristics of two sets of low molecular weight RNAs that appear only in silenced tissue. One set has nearly intact 5′ ends, lacks poly(A) tails, and is associated with polyribosomes; the second set contains the 3′ end of the mRNA. Treating silenced leaf tissue with cycloheximide resulted in decreased levels of full-length mRNA and an increase in the levels of the low molecular weight RNAs, supporting a cytoplasmic decay mechanism that does not require ongoing translation. Surprisingly, mRNA from the transgene containing nonsense codons was associated with more ribosomes than expected, possibly resulting from translation from a start codon downstream of the introduced translational stop codons. We present a hypothesis for transgene/viral RNA degradation in which RNA degradation occurs in the cytoplasm while in association with polyribosomes.

INTRODUCTION

The transfer of genes into plants is a common practice in a variety of plant species. However, in many transformation experiments, a significant percentage of plants containing an intact transgene fails to express it. Evidence exists for at least two mechanisms that may explain this phenomenon. The first, transcriptional silencing, is generally associated with methylation of the transgene promoter region and is indicated by a lack of transcriptional activity, as measured by run-on transcription (Park et al., 1996). The second, post-transcriptional gene silencing, is characterized by relatively high transcription activity and low or undetectable levels of mRNA and protein accumulation (Meins and Kunz, 1994; Mol et al., 1994; Jorgensen, 1995). Several models have been proposed for the induction of post-transcriptional silencing (PTS), including a "threshold" model in which PTS is triggered by a high level of an mRNA and an aberrant RNA model in which PTS is triggered by a critical level of truncated or otherwise altered mRNAs (Dougherty and Parks, 1995; Baulcombe, 1996). In either case, the mechanism can also work in *trans* to silence other highly homologous genes (Van Blokland et al., 1994). In addition, post-transcriptional gene silencing appears to require reinitiation of the mechanism after each generation, a process termed meiotic resetting (de Carvalho et al., 1992; Hart et al., 1992).

Recent evidence has shown that post-transcriptional gene silencing is one mechanism by which pathogen-derived resistance is conferred (Baulcombe, 1996). In the most studied system, tobacco plants are described that contain a "nontranslatable" tobacco etch virus (TEV) coat protein (CP) transgene. These plants are resistant to TEV infection (Lindbo and Dougherty, 1992b; Dougherty et al, 1994; Goodwin et al., 1996). This resistance is manifested as two distinct phenotypes, immunity or recovery. Immune plants do not support virus replication, do not accumulate transgene mRNA, and show Mendelian inheritance of

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resistance. Recovery plants initially are susceptible to TEV, accumulating full-length transgene mRNA, but eventually they develop resistance, at which point there is little transgene mRNA (Lindbo et al., 1993; Dougherty et al., 1994). The resistance induced in the recovery phenotype is reacquired after pathogen infection in the next generation. Resistant lines, recovery or immune, show a distinct pattern of degraded transgene mRNA (Goodwin et al., 1996).

Goodwin et al. (1996) genetically dissected an immune multicopy transgenic line carrying a nontranslatable construct into lines containing varying numbers of transgenes in an isogenic background. The immune lines exhibited higher transgene copy number (three, four, or six copies), increased transgene-specific transcription, and low steady state levels of full-length transgene RNA when compared with recovery lines (one or two copies).

We have further analyzed the tobacco lines containing TEV nontranslatable CP transgenes (Goodwin et al., 1996). We show that immune lines accumulate a high level of transgene mRNA at early times in development and are susceptible to TEV infection, as is found in plants exhibiting the recovery phenotype. We have also characterized the low molecular weight (LMW) RNAs that appear only in silenced tissue to determine how these RNA fragments are generated. We have shown that the 5′ fragments of the CP mRNAs are associated with ribosomes but that translational elongation is not a requirement for transgene degradation. Our data support a model in which two sets of discrete silencing-specific mRNA fragments are generated by specific endonucleolytic cleavage events, perhaps while associated with ribosomes.

RESULTS

Developmental Expression of the 2RC Transgene and TEV Resistance

To accurately compare the expression of the 2RC transgene with viral symptoms, we analyzed mRNA patterns at various stages of development in plants containing different transgene copy numbers. Transgenic lines exhibiting a recovery phenotype (line 17 with two copies and line 18 with one copy) had a relatively high level of the full-length 2RC mRNA at all time points examined in the absence of viral infection (data not shown). Figure 1A shows that a reduction in full-length 2RC mRNA and the accumulation of obvious LMW RNAs do not occur in the absence of virus (compare lane U0 with lane U35). However, upon virus infection, the amount of full-length 2RC mRNA began to decline at 15 days postinoculation (DPI) and declined to approximately the same level in each line (approximately seven- and threefold reduction in lines 17 and 18, respectively). Accumulation of LMW 2RC RNAs could be observed during the decline of the full-length RNA (Figure 1A). Histone mRNA levels did not change significantly in these experiments (Figure 1C).

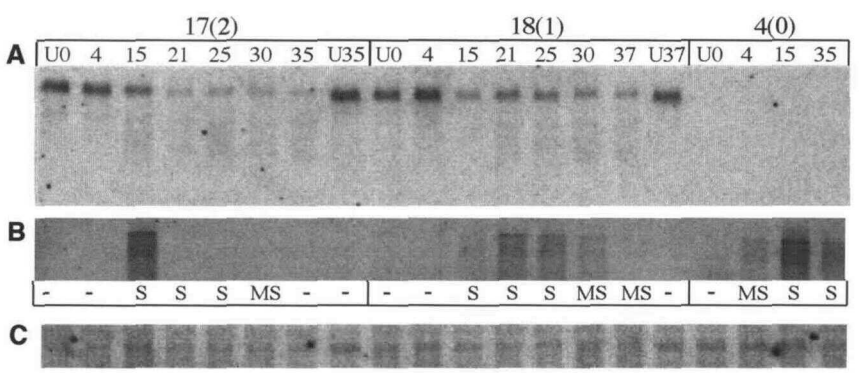

Figure 1. TEV Viral Symptoms Persist after CP Transgene Silencing and Elimination of Viral RNA.

(A) RNA gel blots of the 2RC mRNA during TEV infection of recovery lines. The tobacco lines 17, 18, and 4 were inoculated with TEV NC155A. Total RNA (5 μg) from samples of young, rapidly expanding leaves after TEV infection at the days postinoculation as indicated was analyzed using a transgene-specific *tml* 3′ untranslated region probe (see Methods). The numbers in parentheses indicate the transgene copy number in each line. The numbers above each lane indicate the days postinoculation at which the samples were taken. U0, U35, and U37 indicate the mock-inoculated controls at the days postinoculation indicated.

(B) RNA gel blots of TEV genomic RNA during TEV infection of recovery lines. A duplicate of the gel shown in **(A)** was hybridized with a virus-specific oligonucleotide probe (3421A) that hybridizes with the TEV gene P3. Symptoms of viral infection are indicated at the bottom: (−), asymptomatic; S, systemic symptoms; MS, mild systemic symptoms.

(C) A duplicate of the gel blot shown in **(A)** probed with a histone probe as a control for loading.

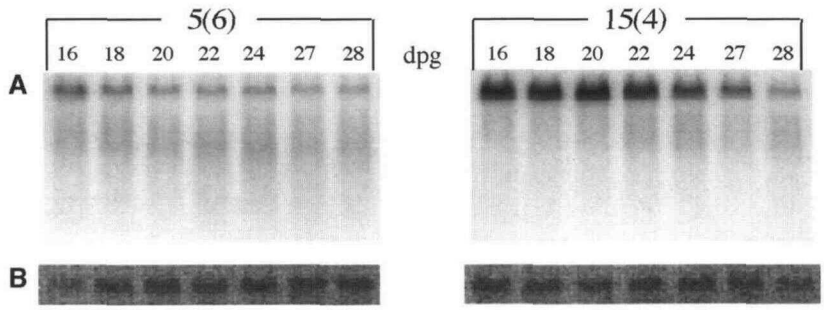

Figure 2. Intact CP mRNA Is Detected during Early Development of Immune Lines.

(A) RNA gel blots of the 2RC mRNA over a developmental time course from 16 to 28 DPG. Total RNA (5 μg) from immune lines 5 (six copies; 5(6)) and 15 (four copies; 15(4)) at the days postgermination indicated were analyzed by RNA gel blot hybridization with a TEV coat protein DNA probe (see Methods).

(B) A duplicate of the gel blot shown in **(A)** probed with a histone probe as a control for loading (see Methods).

A duplicate blot was used to examine virus accumulation within the same individual leaves (Figure 1B). In the inoculated two-copy line, virus RNA was detected in young noninoculated leaves at 15 DPI, corresponding with the onset of transgene mRNA degradation. No virus was detected in newly emerged noninoculated leaves at later times. For the inoculated one-copy line, virus RNA was detected in young, noninoculated leaves at 15 DPI, peaked between 20 and 25 DPI, and then declined between 30 and 37 DPI. In both lines, chlorotic virus–like symptoms were evident in sampled leaves in the absence of detectable viral RNA (Figure 1B). However, these symptoms did not progress, and eventually no symptoms were observed in the younger leaves at later time points in the two-copy line. In contrast, chlorotic virus–like symptoms were observed on the youngest leaves at the latest time point in the one-copy line, becoming localized to small sectors. For comparison, viral RNA in a nontransgenic line was detected at 4 DPI in noninoculated leaves, persisted throughout the time course, and was always accompanied by severe viral symptoms (Figure 1B, line 4).

Transgenic lines exhibiting the immune phenotype (line 5 with six copies and line 15 with four copies) had a low level of full-length mRNA and an increased level of LMW RNAs when whole-leaf samples were harvested between 27 and 50 days postgermination (DPG) (data not shown; Goodwin et al., 1996). However, Figure 2 shows that at very early developmental time points, a relatively high level of the full-length 2RC mRNA was seen, and there was less of a LMW RNA smear, suggesting that the silencing mechanism had not been triggered at this early time point.

In line 5 (six-copy line) at 16 DPG, the level of the full-length mRNA was relatively low, and the LMW RNAs were already present; however, further reduction of the full-length message was seen at subsequent time points (Figure 2A), whereas the control mRNA levels did not change significantly (Figure 2B). Line 15 (four-copy line) had a higher level of full-length 2RC mRNA at 16 DPG that did not begin to diminish until significantly later than did line 5 (Figure 2A). We have observed variations in the time course for onset of silencing in these lines (data not shown), which is most likely due to variations in growth conditions between independent experiments similar to the variation observed with other systems (Elmayan and Vaucheret, 1996; Pang et al., 1996). However, we were always able to detect significant amounts of full-length mRNA in immune lines at the earliest time points.

Because the four- and six-copy immune lines (lines 15 and 5, respectively) did not appear to be silenced at early time points, we asked whether these lines would develop a systemic infection when challenged with virus at these early times. Plants from each line (zero, one, two, four, and six copies) were inoculated at either 20 or 28 DPG and scored for symptoms 5 days later. In this experiment, silencing of the six-copy line was initiated after 20 DPG (data not shown). Four- and six-copy plants inoculated at 28 DPG showed no symptoms, whereas the zero-, one-, and two-copy lines showed viral symptoms (data not shown). In contrast, all lines inoculated at 20 DPG showed systemic symptoms 5 DPI (data not shown). However, the four- and six-copy lines that initially exhibited symptoms at 5 DPI showed no symptoms on newly emerging leaves and no detectable virus at later times (data not shown). This recovery occurred more rapidly in the four- and six-copy lines than in the one- and two-copy lines. Therefore, resistance to virus infection coincides with reduced full-length transgene mRNA and the appearance of discrete LMW RNAs in plant lines exhibiting both recovery and immune phenotypes (one- and two-copy lines and four- and six-copy lines, respectively).

Identification and Characterization of the LMW 2RC RNA Fragments

The LMW RNA fragments associated with the silenced phenotype initially observed by Goodwin et al. (1996) were

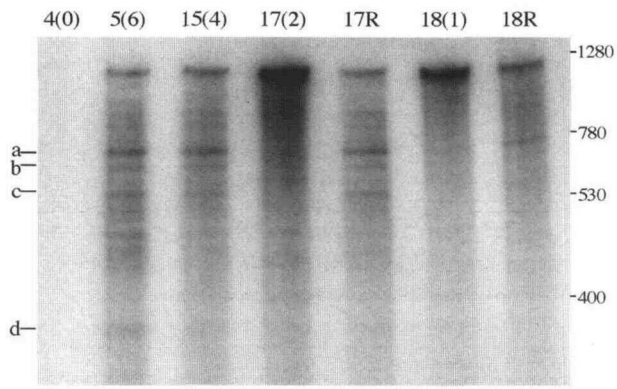

Figure 3. LMW CP RNAs from Silenced Tissue.

Detection of specific 5′ CP RNA fragments on high-resolution polyacrylamide RNA gel blots. Total RNA (5 μg) from lines 4, 5, 15, 17, and 18 was analyzed on a polyacrylamide gel and hybridized with the TEV CP DNA probe. Lanes 17R and 18R are RNA samples isolated from the corresponding lines after recovery from TEV NC155A infection. The letters at left indicate a subset of specific LMW products that were detected with a 5′ specific oligonucleotide probe, as described in the text. The numbers in parentheses indicate the transgene copy number for each line. Molecular length standards are indicated at the right in bases.

examined in more detail on high-resolution RNA gel blots. Figure 3 shows that a set of discrete LMW TEV CP RNA fragments can be observed only in RNA samples derived from silenced (virus-resistant) tissue (lines 5, 15, 17R, and 18R). Duplicate blots were probed with oligonucleotides that are complementary to different regions of the transgene mRNA to determine the regions from which the various LMW RNAs were derived. Oligonucleotides complementary to sequences within the 5′ untranslated region (7A) of the CP RNA and within the first one-third of the coding region (8712A) hybridized with the full-length CP mRNA and to fragments a, b, c, and d (data not shown), whereas those complementary to sequences within the last one-third of the coding region (9107A and 9274A) hybridized with the full-length mRNA as well as to a nonspecific LMW smear (data not shown).

Identification of the 3′ LMW RNA fragments via hybridization is complicated by variations in poly(A) tail length and/or the use of multiple poly(A) addition sites that prevent resolution of discrete bands on RNA gel blots (see Shirley et al., 1990). To address this possibility, we specifically removed the extreme 3′ region of the 2RC mRNAs by oligonucleotide-dependent RNase H cleavage at a specific site at the 3′ end of the coding region of 2RC mRNA (see Methods). The resulting RNA samples from silenced line 5 and nonsilenced line 17, now containing homogenous 3′ ends, were analyzed as shown in Figure 4A. Because the silenced tissue had approximately five- to sevenfold less CP mRNA compared with the nonsilenced tissue, five times more RNA was loaded for the silenced RNA samples to facilitate comparison. In the absence of the oligonucleotide, RNase H had little if any effect (Figure 4A; compare [−] with [+] lanes). However, in the presence of the oligonucleotide, RNase H treatment produced a number of specific LMW RNA fragments in addition to a distinct band at the size expected for full-length mRNA (arrow). Some of these fragments were detected only in the

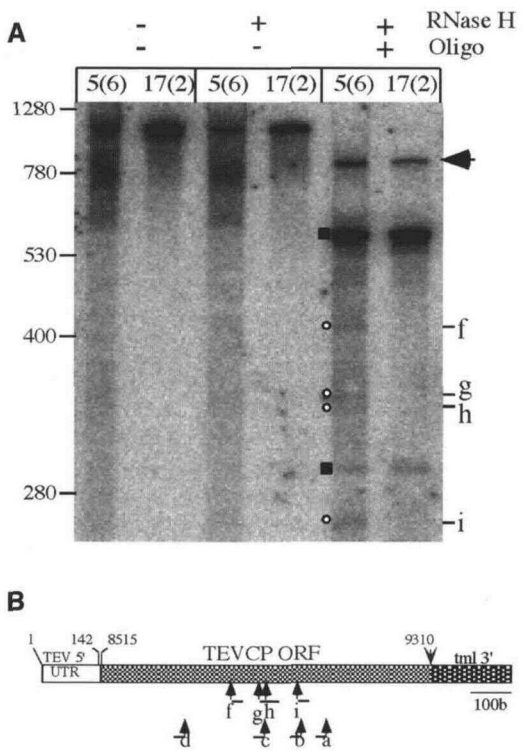

Figure 4. Silencing-Specific 3′ RNA Fragments from Silenced Tissue.

(A) Detection of specific 3′ RNA fragments on polyacrylamide RNA gel blots after cleavage of heterogenous 3′ ends by oligonucleotide-directed RNase H cleavage. Total RNA from the silenced line 5 (10 μg) and nonsilenced line 17 (2 μg) was hybridized with oligonucleotide (Oligo) TEV9274A and digested with RNase H ([+ +] lanes). Duplicate samples without the oligonucleotide digested with RNase H ([+ −] lanes) and total RNA samples ([− −] lanes) were run as controls. The samples were analyzed on high-resolution polyacrylamide gels, blotted, and probed with a 95-bp DNA fragment complementary to sequences upstream of TEV9274A, allowing detection of specific fragments from the 3′ end of the CP mRNA. The numbers in parentheses indicate the transgene copy number for each line. The arrow indicates the expected fragment after digestion of the full-length mRNA with the oligonucleotide. The circles and letters at right represent the silencing-specific fragments, and the black boxes represent fragments shared by the silenced and nonsilenced tissues. The molecular length standards are shown at left in bases.

(B) Physical map of the 5′ and 3′ LMW RNA fragments. The approximate positions of the 5′ ends of RNA fragments f to i within the TEV CP mRNA are indicated by arrows. The 3′ ends of the 5′ fragments, a to d, are also indicated. ORF, open reading frame; UTR, untranslated region.

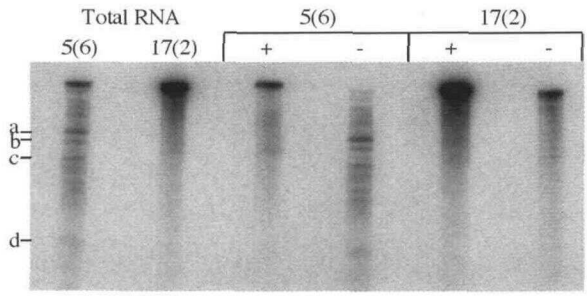

Figure 5. Silencing-Specific LMW RNAs Lack Polyadenylated Tails.

Total RNA from lines 5 and 17 was separated by oligo(dT)–cellulose chromatography into poly(A)$^+$ and poly(A)$^-$ fractions. Total (5 μg) poly(A)$^+$ (~0.1 μg) and poly(A)$^-$ (5 μg) RNAs were analyzed on a polyacrylamide gel blot and probed with the TEV CP DNA probe. The numbers in parentheses indicate the copy number of the line. The (+) or (−) indicates the poly(A)$^+$ and poly(A)$^-$ fractions, respectively. The letters a to d at left indicate the LMW RNA fragments identified in Figure 3.

silenced sample (Figure 4A, open circles, bands f to i). These bands define a set of silencing-specific fragments containing 3′ sequences from 2RC mRNA. Several additional bands were found in RNA from both silenced and nonsilenced tissues (Figure 4A, black boxes). These fragments may have been generated by an independent decay mechanism because they are present in both silenced and nonsilenced tissues. Several of the 5′ LMW fragments described above roughly fit with several of the 3′ fragments, and we were able to reconstruct the full-size mRNA of the transgene (Figure 4B). Additional 5′ LMW RNAs were detected that may be contiguous with the 3′ fragments f and g (data not shown). Proportionally sized fragments were detected in RNA from silenced tissue of fully translatable FL lines, suggesting cleavage at the same sites within the body of the TEV CP region of the mRNA (data not shown).

One set of the LMW RNAs identified has intact 5′ ends; thus, they could be the products of premature termination of transcription, or they could result from endonucleolytic cleavage of the mRNA. To aid in distinguishing between these two possibilities, the polyadenylation status of the 5′ LMW RNAs was examined. RNA from lines 5 (six copies) and 17 (two copies) was separated into poly(A)$^+$ and poly(A)$^-$ RNA fractions by oligo(dT)–cellulose chromatography to determine whether a poly(A) tail on the LMW RNAs was present or absent. Figure 5 shows that the discrete 5′ LMW RNAs were only observed in the poly(A)$^-$ fraction in the immune line (cf. line 5, [−] and [+] lanes). Full-length mRNA was detected in the poly(A)$^+$ fraction only. A nonspecific smear of hybridizing RNA was also detected in the poly(A)$^+$ fraction along with the full-length mRNA (Figure 5, line 5, [+] lane), which may correspond to 3′ LMW RNAs. In the recovery line (17), most of the full-length mRNA was in the poly(A)$^+$ fraction. Some nearly full-length mRNA was detected in the poly(A)$^-$ fraction from this line as well as a few discrete LMW RNAs. However, these LMW RNAs differed in both size and abundance from those in the silenced line (5, six copies). The sizes of the 5′ and 3′ LMW RNAs, as measured on polyacrylamide gels (Figures 3 and 4), and the absence of poly(A) tails on the 5′ LMW species (Figure 5) support a model in which an endonucleolytic cleavage of full-length 2RC mRNA generates 5′ and 3′ fragments.

Distribution of Transgene RNAs in Polysomal Fractions in Silenced and Nonsilenced Tissue

Transgene silencing that leads to virus resistance may be a cytoplasmic process involving mRNA degradation (see Lindbo et al., 1993; Smith et al., 1994). In addition, studies of eukaryotic mRNA decay have suggested a role for the cytoplasmic process of translation in the mRNA degradation process (Ross, 1988; Sachs, 1993). Because the 2RC construct analyzed here generates a translatable mRNA and the first nonsense codon in the 2RC mRNA is positioned such that a four–amino acid polypeptide would be produced, we looked at the distribution of the CP RNAs in association with ribosomes in silenced and nonsilenced tissues. In addition to the 2RC lines, tobacco lines containing fully translatable TEV CP constructs, FL3.3 and FL24.3, were used for comparison. These lines are silenced only after infection with TEV (Lindbo et al. 1993) in a manner analogous to the 2RC recovery lines 17 and 18. Figure 6 shows polysome analysis of 2RC and FL lines. The recovered (silenced) tissue from FL3.3 and 2RC 17 had ~15- and sevenfold less full-length mRNA, respectively, compared with uninfected tissue, and had similar smears of LMW RNA fragments (Figures 6C and 6D, Total lanes). Much of the full-length mRNA was associated with fewer ribosomes after silencing (Figures 6C and 6D; cf. Uninfected and Recovered lanes). However, a significant amount of the LMW RNA fragments was found associated with the larger polysomes in silenced tissue (Figures 6C and 6D, Recovered; fractions 5 to 7 included polyribosomes with more than two ribosomes per mRNA). The ratio of the abundance of the LMW RNAs to intact mRNA in silenced tissue was greater in the polysome fraction than it was in total RNA samples (3.48 ± 0.67 for polysome versus 2.36 ± 0.36 for total RNA), showing that the majority of the LMW RNAs were associated with ribosomes. Figure 6B shows ethidium bromide–stained gel to show loading.

To show that the 2RC full-length and LMW RNAs were indeed associated with ribosomes, EDTA (which dissociates ribosomes by chelating magnesium ions required for their association with mRNAs; Penman et al., 1968) was added to the sample before centrifugation. After this treatment, a majority of the full-length 2RC mRNA and LMW products were found in the top fractions of the gradient. These contained free RNA and ribosomal subunits (Figure 6E, Recovered). Similar results were seen with the silenced line 2RC 15 and with an

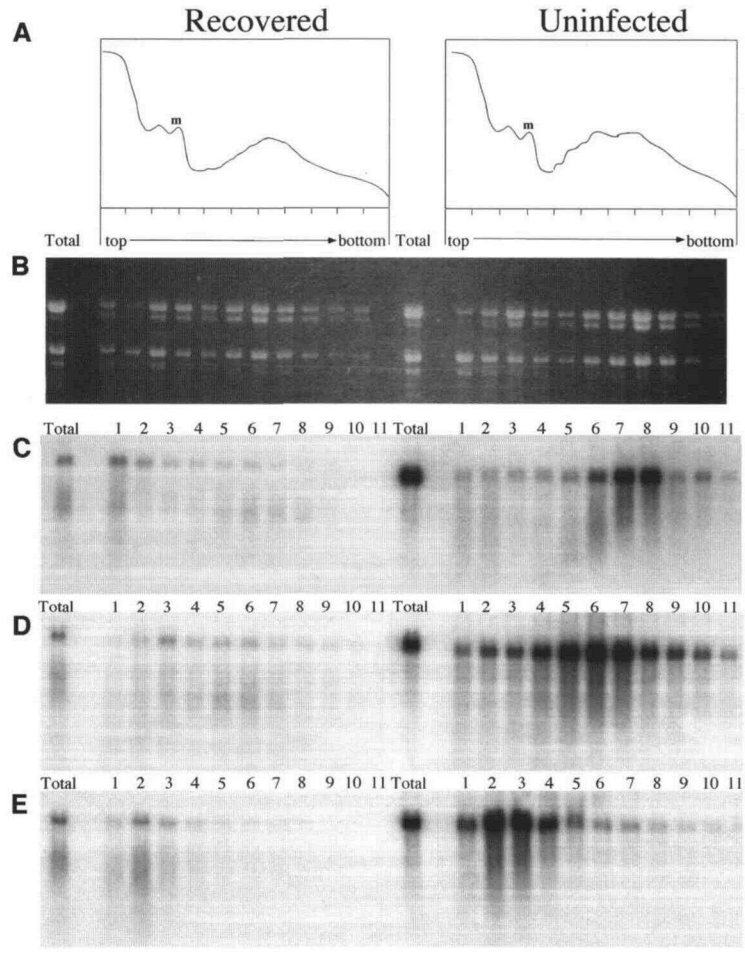

Figure 6. Translatable and Nonsense Codon–Containing Transgene mRNAs Are Associated with Ribosomes in Nonsilenced Tissue, and a High Proportion of LMW RNAs Are Associated with Ribosomes in Silenced Tissue.

Polysomes were fractionated on sucrose gradients. Total RNA (5 μg) and one-fourth of the RNA from each fraction were analyzed on RNA gel blots. The fractions are numbered 1 to 11 from top to bottom of the gradient, respectively.

(A) A_{254} traces from the FL recovery line containing the fully translatable FL3.3 construct. The monosome peak, identified by two methods, is indicated by the m (see Methods).

(B) Ethidium bromide–stained gel from the experiment with line FL3.3.

(C) RNA gel blot for line FL3.3 probed with the TEV CP cDNA (see Methods).

(D) RNA gel blot from recovery line 17 containing the nonsense codon–containing 2RC construct and probed with the TEV CP cDNA (see Methods).

(E) RNA gel blot from recovery line 17 incubated with 100 mM EDTA before fractionation and probed with the TEV CP cDNA (see Methods).

independent transformant, FL24.3, containing the FL construct and exhibiting the recovery phenotype (data not shown).

The CP mRNA from line 2RC 17 was not expected to be associated with more than two ribosomes, because the 2RC mRNA was predicted to have a 5′ untranslated region of 142 nucleotides and encode only four amino acids before reaching the first nonsense codon. However, most of the intact mRNA from the nonsilenced 2RC line was found in fractions 5 to 7, which includes polyribosomes with more than two ribosomes per mRNA (Figure 6D, Uninfected). Supporting this assertion, the 2RC mRNA was shifted to the top of the gradient after the addition of EDTA (Figure 6E, Uninfected). These data indicate that ribosomes were likely bound to the 2RC mRNA beyond the premature stop codons. Analysis of the 2RC mRNA sequence revealed a second initiation codon in good translational context (Kozak, 1991) at codon 47,

perhaps explaining the association of this mRNA with polysomes. Initiation of translation from this AUG would generate a truncated TEV CP with an expected size of ~24 kD. Such a protein band could be detected on protein gel blots using an anti-CP antibody (data not shown).

Cycloheximide Differentially Affects the CP mRNA in Silenced and Nonsilenced Lines

As an alternative approach to examining the role of translation in the silencing mechanism, tissues were treated with the translation elongation inhibitor cycloheximide (CHX). The 2RC lines 5, 15, and 17 were examined for the effect of CHX on the mRNA phenotype. Figure 7A shows that in the nonsilenced line (17, two copies), treatment with CHX resulted in an increase in the abundance of full-length mRNA compared with untreated tissue. CHX also caused the appearance of discrete LMW RNAs (Figure 7A, line 17 with two copies, lane CHX). These fragments differed in size and abundance compared with LMW RNAs from silenced samples (Figure 7A; cf. line 17 with lines 5, 15, and 17R with CHX).

In all silenced tissue, treatment with CHX resulted in a slight but reproducible reduction (approximately twofold) of the full-length mRNA level and a large increase in the abundance of particular silencing-specific LMW RNAs (Figure 7A, lines 5, 15, and 17R). These LMW RNAs differed in abundance from those found in untreated silenced tissue in that several of the smaller RNA fragments were enriched compared with untreated samples (e.g., fragments d and e). Fragment e was also seen on other blots (e.g., long exposures of the blot shown in Figure 3). These results suggested that in silenced tissue, blocking translation elongation with CHX promoted the degradation of full-length mRNA into the same LMW RNA fragments seen during silencing. Analogous results were obtained after CHX treatment of uninfected and recovered tissue from the fully translatable line FL3.3 (data not shown). In addition, treatment with CHX did not affect histone mRNA, showing the specificity of this effect (Figure 7B).

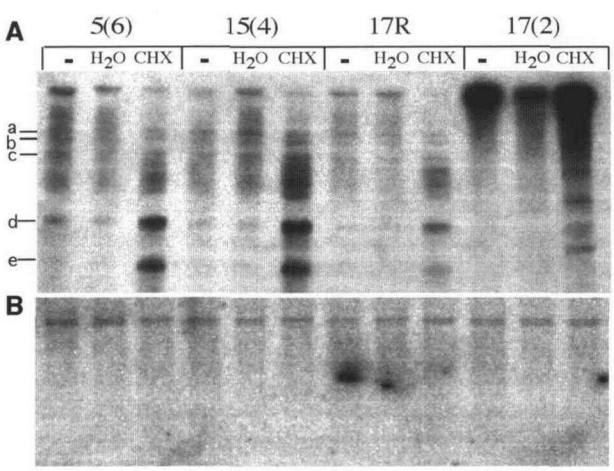

Figure 7. CHX Induces RNA Degradation and Increases the Abundance of Some Silencing-Specific LMW RNAs.

(A) Plants from the indicated 2RC lines treated with CHX to inhibit translation. The immune lines (5 and 15) and a recovery line before and after TEV infection (17 and 17R, respectively) were treated as follows: (−), no treatment; H_2O, injected with water; CHX, injected with 1 mg/mL CHX. The injected areas were harvested after 24 hr, and total RNA was isolated. RNAs (5 μg) were analyzed on polyacrylamide gel blots with the TEV CP DNA probe (see Methods). Specific LMW products discussed in the text are labeled a to e. Products a, b, c, and d correspond to products shown in Figures 3 and 5. The numbers in parentheses indicate the copy number.

(B) A duplicate gel blot as shown in **(A)**, probed with a histone probe as a control for loading.

DISCUSSION

Post-Transcriptional Gene Silencing and Development of Resistance

We have shown that both the immune phenotype and the recovery phenotype previously described in transgenic tobacco (Dougherty et al., 1994; Goodwin et al., 1996) are manifestations of the same process in that all lines tested were susceptible to TEV at early developmental stages. Furthermore, lines containing more transgene copies induced silencing or recovery more rapidly, consistent with previous results showing the phenotype to be dependent on the transgene copy number (Elmayan and Vaucheret, 1996; Goodwin et al., 1996). The primary difference between the two phenotypes was the necessity for the presence of TEV to initiate silencing in plants showing the recovery phenotype. Thus, as with other post-transcriptional gene silencing examples, the immune lines exhibit the characteristic meiotic resetting, as is found in the recovery lines. Resetting may result from a lack of transgene expression in the apical meristems. Studies with the cauliflower mosaic virus 35S promoter fused to a β-glucuronidase reporter gene have shown that it is at best poorly transcribed in the apical meristem of tobacco (Benfey et al., 1989). Because all organs, including the floral organs, are derived from meristematic tissue, the seed giving rise to the next generation would be derived from tissues that were never silenced.

Lack of silencing in the meristem could also explain our observation that viruslike symptoms were present when virus was no longer detected in "recovery" plants. Many viruses do not enter meristematic tissue (Matthews, 1991); however, the virus can replicate in a set of cells just below the apical meristem (Gilbertson and Lucas, 1996). As these infected cells continue to develop, silencing would be initiated so that virus replication would be reduced or eliminated. Plants silenced by this mechanism could show morphological symptoms resulting from prior exposure to virus, perhaps due to persistence of certain viral proteins (Pascal et al., 1993). If

meiotic resetting is the result of low level of transcription in the meristem, then transgenes driven by strong promoters that are active in the meristem should not exhibit meiotic resetting and give rise to stable viral resistance.

The Silencing-Specific LMW RNAs

Several models for a mechanism for post-transcriptional gene silencing predict the presence of aberrant RNAs (such as prematurely terminated messages) as well as the degradation of mRNAs (Flavell, 1994; Dougherty and Parks, 1995; English et al., 1996). The silenced state is characterized by reduced levels of full-length mRNA and the appearance of specific LMW RNA fragments. We characterized these specific LMW RNAs present in silenced tissues containing either the TEV 2RC transgene or the TEV full-length transgene. These LMW RNAs consist of both the 5′ and 3′ portions of the CP transgene, although special techniques are required to visualize the 3′ fragments. The relative sizes of some of the 5′ and 3′ LMW RNAs described here and by Goodwin et al. (1996) are consistent with contiguous fragments being produced from endonucleolytic cleavage of the full-length mature RNA. Endonucleolytic cleavages have also been implicated in the silencing-specific degradation of the chalcone synthase *chsA* mRNA in petunia (Metzlaff et al., 1997).

Although we have provided a body of evidence consistent with the view that most of the discrete LMW RNAs are generated by degradation of the full-length mRNA, some of the remaining LMW RNAs (identified as a smear) may represent the aberrant RNAs that have been postulated to trigger the silencing mechanism (Baulcombe and English, 1996). Alternatively, the LMW fragments generated by degradation of the full-length mRNA may themselves act as the aberrant RNA, inducing further degradation and perpetuating the silenced state.

Cytoplasmic Decay of the TEV CP mRNAs

The polysomal distribution of the TEV CP mRNAs suggests a role for translation in the silencing process. In nonsilenced lines, the polysomal fractions contained primarily full-length mRNA for both the FL and the 2RC transgenes. In contrast, the polysome fractions of silenced tissue contained primarily LMW mRNAs, with some of the full-length RNA being shifted toward the top of the gradient. The appearance of these LMW products in the silenced tissue is due most likely to the specific degradation of the TEV CP transgene mRNAs by an endonucleolytic RNase while associated with the polyribosomes. This would explain the enrichment of LMW RNAs in the polysome fractions, the presence of 5′ and 3′ fragments, and the lack of polyadenylated tails on 5′ fragments. Alternatively, the LMW RNAs could arise from premature termination of transcription, with these species being more efficiently recruited into the polysomal fraction than full-length mRNAs. This possibility seems unlikely in light of the fact that the full-length RNA is almost entirely polyadenylated and therefore should be more efficiently recruited onto polysomes than are nonpolyadenlyated RNAs (reviewed in Gallie, 1996).

The role of the ribosome in post-transcriptional gene silencing was analyzed further by use of the peptidyl transferase inhibitor CHX. Treatment of silenced tissue with CHX caused a marked increase in the abundance of the LMW RNAs associated with silencing. Therefore, CHX did not appear to inhibit the activity of the putative endoribonuclease in the silenced and nonsilenced lines, although it is possible that the increase in abundance of LMW RNA fragments resulted from the loss of activity of a labile or CHX-sensitive nuclease involved in the next step(s) of RNA decay (Brawerman, 1993). Because samples were taken at 24 hr after the addition of CHX, most if not all of the CP mRNA would have been newly synthesized. Thus, CHX would be expected to block elongation of the first ribosome. This would result in a largely "naked" RNA within the cytoplasm that would have increased susceptibility to silencing-specific ribonucleases. Therefore, the smaller LMW RNA fragments observed may be the result of increased access by the silencing-specific nucleases to the mRNA because of the absence of ribosomes. Alternatively, a ribosome bound near the 5′ end of the mRNA may protect the 5′ end from degradation, resulting in the apparent increase in the smaller 5′ specific degradation products (i.e., Figure 7, fragments d and e). In addition, viral RNA decay could occur during translation when the positive-strand RNA is exposed to cytoplasmic endonucleases by the passage of ribosomes.

If exposing a silenced mRNA enhances its degradation, the addition of other translation inhibitors, such as puromycin, which causes premature chain termination and the release of ribosomes from mRNAs, or pactamycin, which prevents translation initiation, should also destabilize mRNA targeted for silencing and may cause the accumulation of LMW RNAs.

The LMW fragments could have also been the result of CHX-induced cryptic splicing of the FL and 2RC mRNAs, as has been shown for T cell receptor–β mRNA splicing (Qian et al., 1993a, 1993b) and interleukin-2 mRNA processing in mammalian cells (Gerez et al., 1995). However, sequence analysis of the 2RC mRNA found no cryptic splice sites, making this an unlikely possibility. The appearance of a different set of discrete LMW RNAs in the nonsilenced tissue after CHX treatment may have been the result of a different degradation mechanism or altered action of the same ribonuclease proposed for silenced tissue.

Our results with CHX are consistent with a cytoplasmic site-specific RNA degradation mechanism in silenced tissues. We suggest that once ribosomes are loaded onto the target RNA, ongoing translation (peptide bond formation) is not required for degradation of the RNA; in fact, it is likely that blocking the loading of multiple ribosomes actually enhances the silencing-specific degradation mechanism.

Identification of the 2RC mRNA in association with the polysomal fractions was initially surprising, because we as-

sumed that ribosomes would disassociate from the message after encountering the stop codons at codon positions 5, 6, and 9. However, we identified a second open reading frame starting from a natural initiation codon in good translational context (Kozak, 1991) that could produce a 24-kD protein. A 24-kD protein that cross-reacted with the TEV CP antibody was detected in nonsilenced tissue from the 2RC transgenic lines, TEV-infected tissue, and in nonsilenced tissue from lines containing the fully translatable construct. These results indicate that translation to produce this truncated protein most likely occurs in each of these cases. Malpica et al. (C.A. Malpica, J.H. Fitchen, D.M. Stark, C.M. Fauquet, and R.N. Beachy, submitted manuscript) have also observed a truncated protein in TEV-infected plants. Furthermore, it has been reported that efficient replication of the TEV genome requires translation to codon 189 and the region between codons 210 and 246 within the CP gene (Mahajan et al., 1996). The internal start codon for the 24-kD protein is upstream of these required regions, and thus, translation from this start codon may enhance replication of the TEV genome during virus infection. Therefore, a role for this protein in the resistance exhibited by the recovery lines cannot be ruled out.

Proposed Mechanism of Gene Silencing

The data presented here and published previously suggest that post-transcriptional gene silencing is a normal mechanism of gene expression regulation. This mechanism does not appear to have any effect on mRNA transcription; thus, a nascent transcript would be capped, processed, and transported from the nucleus to the cytoplasm. The newly transcribed mature mRNA then associates with the protein synthesis machinery as it is transported into the cytoplasm. After association of the mRNA with the factors involved in cap binding, ribosome scanning, and other events preceding start codon recognition, translation is initiated at an appropriate AUG and is accompanied by the loading of multiple ribosomes. After translation is initiated, an endoribonuclease complex somehow recognizes and cleaves the mRNA targeted to be silenced, even in the presence of the translation machinery. Cleavage occurs at specific sites, perhaps enhanced by the pausing of ribosomes. The LMW RNAs thus generated would be further degraded by nonspecific exonucleases. Once silencing is initiated, maintenance of the silenced state is independent of the promoter and/or gene copy number. Although transcript recognition is most likely different, the degradation components of this mechanism may resemble components of other cytoplasmic RNA degradation mechanisms (Abler and Green, 1996).

If the silencing mechanism proceeds via this proposed process, a number of predictions can be made about the mRNAs targeted for silencing and the silenced RNA fragments. RNA in the nuclear fraction should be processed normally and exhibit no silencing-specific LMW RNAs, whereas cytoplasmic mRNA and all LMW RNAs should be free of introns. Cleavage of mRNA by a putative endoribonuclease should result in 5′ and 3′ RNA fragments with specific characteristics. The 5′ fragments should have a 5′ m^7G-cap structure (Reddy et al., 1992) and lack polyadenylated tails, whereas the 3′ fragments should lack a 5′ m^7G-cap structure and have polyadenylated tails. Both sets of fragments are, at least transiently, associated with ribosomes. Furthermore, mRNA targeted for silencing is cleaved at specific sites, and the internal ends of contiguous fragments should map to the same position within the full-length mRNA. Several of these predictions have been shown in the work presented here.

What Is the Biological Role for a Mechanism That Can Downregulate Specific mRNAs?

A number of possible functions have been proposed for gene silencing, including protection against RNA viruses (Sherwood, 1988) and against the potentially detrimental effects of active transposable elements (Flavell, 1994; Martienssen, 1996), and as a mechanism for controlling gene expression after aneuploidization, polyploidization (within the whole plant; Papp et al., 1996; Scheid et al., 1996), or polysomaty (within single cells; DeRocher et al., 1990; Galbraith et al., 1991). Alternatively, gene silencing may regulate mRNA levels from multigene families and/or duplicated genes within plant genomes.

The degradation of a soybean small subunit mRNA (a member of a plant multigene family; Dean et al., 1989) in both soybean and transgenic petunia exhibits some remarkable parallels to the PTS-specific degradation. Specific degradation products have been identified that clearly result from endonucleolytic degradation (Thompson et al., 1992; Tanzer and Meagher, 1994). The 5′ degradation products are associated with the polysomal fraction in vivo, suggesting a ribosome-associated degradation mechanism (Thompson et al., 1992). However, in vitro data indicate that neither translation nor intact ribosomes are required for the specific degradation mechanism (Tanzer and Meagher, 1994, 1995). In addition, it has been suggested that silencing of the petunia *chsA* gene may involve activation of an existing *chsA* mRNA decay pathway (Metzlaff et al., 1997). These similarities suggest that the silencing-specific degradation of 2RC mRNA may occur via a mechanism that is involved in the decay of at least some endogenous mRNAs.

METHODS

Transgenic Tobacco Plants and Virus Isolate

The 2RC tobacco lines were derived from a single, immune transformant and contain one, two, four, or six copies of the 2RC transgene (Goodwin et al., 1996). The 2RC transgene contains three nonsense

codons at the fifth, sixth, and ninth codon position of the tobacco etch virus coat protein (TEV CP) sequence (Lindbo and Dougherty, 1992a). The TEV FL–containing tobacco lines were generated as described in Lindbo and Dougherty (1992a). The virus strain TEV NC155A was propagated through *Nicotiana tabacum* cv Burley 21. All plants were grown in the greenhouse.

Sampling and RNA Isolation

Virus recovery was monitored in transgenic lines containing different copy numbers of the 2RC gene. Multiple plants of each line were inoculated with TEV. Leaves 3 and 4, numbered from the soil, of 40-day-old plants were mechanically inoculated with sap from TEV-infected tissue diluted (1:3 [w/v]) in 0.05 M K_2PO_4, pH 7.2. The youngest leaf from individual plants was destructively sampled 4, 15, 21, 25, 30, and 35 (or 37 for line 18) days postinoculation (DPI). Three plants were sampled per time point, and each experiment was repeated at least three times. Leaf samples were also taken from control plants (nontransgenic) that had been inoculated with TEV. The youngest leaf was destructively sampled 4, 15, and 35 DPI. Total RNA was isolated from ground whole-leaf tissue, with 5 μg being analyzed per lane on RNA gel blots. The samples were analyzed for the presence of transgene and virus. The phenotype of the sampled tissue was also assessed at the time of harvest. The recovery phenotype was also monitored in the transgenic lines with four or six copies of the 2RC transgene in the absence of virus infection. Individual plants were destructively sampled 16, 18, 20, 22, 24, 27, and 28 days postgermination (DPG). At each time point, the two youngest leaves were taken from six individual plants. Ground tissue from the two leaves from an individual plant was used for RNA isolation. Total RNA was isolated by grinding tissue in liquid N_2 and resuspending the powder in 4% *p*-aminosalicylic acid–1% 1,5-naphthalenedisulfonic acid. This solution was phenol–chloroform extracted followed by lithium chloride precipitation, as described by Lagrimini et al. (1987). RNA was quantitated on a spectrophotometer (model UV-160; Shimadzu Corp., Kyoto, Japan). Poly(A)$^+$ and poly(A)$^-$ RNA were fractionated from 200 μg of total RNA, using the Poly(A) Quik mRNA isolation kit (Stratagene).

To detect 3′ fragments with specific 5′ ends, RNase H (Promega), which cleaves the RNA in the DNA/RNA hybrid, was used to remove the 3′ ends of the CP RNAs within a specific region dictated by an oligonucleotide, thereby generating homogenous 3′ ends on these RNAs. RNase H cleavage assays were performed as described previously (Carrazana and Pasieka, 1988; Tanzer and Meagher, 1994). An oligonucleotide, TEV9274A, complementary to the 3′ end of the CP coding region, was used to direct cleavage of the 2RC RNAs. The experiments were repeated three times, and similar results were seen in comparisons of other silenced and nonsilenced lines.

Gel Blot Analysis

RNA samples were electrophoresed on 1.2% agarose–formaldehyde gels and blotted to nylon membranes (GeneScreen Plus; New England Nuclear, Beverly, MA), as previously described (Ausubel et al., 1987), or on 5% polyacrylamide urea gels and blotted, as previously described (Thompson et al., 1992). For agarose–formaldehyde gels, ethidium bromide (final concentration of 40 μg/mL) was included in the RNA samples before heating at 65°C for 15 min to allow visualization of RNA levels in gels while UV illuminated. Samples from all blots, including those in Figures 1 and 3 to 6, were probed with a pea H1 histone cDNA probe (Gantt and Key, 1987) to confirm equal loadings of RNA from those samples. All experiments were repeated at least twice.

Hybridization Probes and Oligonucleotides

The TEV CP DNA probe was generated by cutting pTC:FL (Lindbo and Dougherty, 1992a) with BamHI and gel purifying the resulting 800-bp fragment containing the TEV CP coding region corresponding to nucleotides 8515 to 9310 (Allison et al., 1986). The transgene-specific *tml* 3′ untranslated region DNA probe was generated by cutting pCGN1761 ENX with KpnI and XhoI and gel purifying the resulting 1200-bp *tml* 3′ untranslated region fragment. The resulting probe was specific for T-DNA sequences and did not detect viral RNA. The DNA probe used for the RNase H experiment was generated by digesting pTC:FL with AccI and NdeI and gel purifying the resulting 95-bp fragment corresponding to nucleotides 9062 to 9157 (Allison et al., 1986). A pea H1 histone cDNA probe was used as a hybridization control (Gantt and Key, 1987). DNA fragments were labeled by the random priming method, using a random priming kit (Gibco BRL). Hybridizations and washes were performed according to the method of Church and Gilbert (1984).

The synthetic oligonucleotides used in this report were made by Integrated DNA Technologies (Coralville, IA) and are as follows (5′ to 3′): 7A (TEV 5′ untranslated region; GTATATGTTGTGTTGAGATTTG), 3421A (TEV P3 gene; CCAATGCAAAACTTGTAGTACTCGAAAGCCTC), 8712A (TEV CP; TGGCTTGTATCCTAAAAGGTG), 9107A (TEV CP; GAAGTTAGCTCATAGAAGTCG), and 9274A (TEV CP; CCCCTAATAGTGTGTGCATGTTAC). Oligonucleotide numbering refers to the 5′ most nucleotide position within the full-length TEV clone; the TEV name indicates the homologous region within the TEV genome (Allison et al., 1986). Oligonucleotide probes were generated by transferring the γ-^{32}P from γ-^{32}P-ATP (New England Nuclear) to 10 pmol of oligonucleotide with T4 polynucleotide kinase (Promega). Hybridizations and washes were performed as described previously (Thompson and Meagher, 1990). Hybridizations were performed at the melting temperature −10 to 15°C.

Polysome Isolation

Polysomes were isolated by using modified methods previously described (Larkins and Davies, 1975). Frozen tobacco tissue was ground with a mortar and pestle in liquid nitrogen and resuspended in 10 mL of buffer A (200 mM potassium chloride, 50 mM magnesium chloride, 2 mM DTT, 200 mM Tris-HCl, pH 8.75, and 200 mM sucrose). The homogenate was centrifuged 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 centrifuged at 27,000*g* for 10 min at 4°C. The supernatant was layered onto a 15 to 55% linear sucrose gradient in buffer B (1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM DTT, 20 mM Tris-HCl, pH 8.75, and 200 mM sucrose) and centrifuged at 35,000 rpm (156,000*g* at the average radius) in a rotor (model SW41; Beckman, Palo Alto, CA) for 1.75 hr. Polysome gradients were fractionated by puncturing the bottom of the tube and forcing the contents through a UV-1 single path monitor (Pharmacia) with a 254-nm filter. One-milliliter fractions were collected after detection. RNA was isolated from 500 μL of each fraction by the addition of 20 μL of 0.5 M EDTA and 12.5 mL of 20% SDS and heated at 65°C for 5 min. These fractions were then phenol-chloroform extracted and precipitated with one-tenth volume of 3 M NaOAc, pH 5.0, and 2 volumes of 100% ethanol at −20°C overnight.

Samples were resuspended in 20 μL of water, and 5 μL was used for gel blot analysis.

For EDTA release experiments, EDTA (100 μM final concentration) was added (or an equal volume of water in control samples) after a 3000*g* spin in buffer A, as described above. The samples were incubated on ice for 15 min, followed by centrifugation at 27,000*g* for 10 min at 4°C. The supernatant was layered onto a 15 to 55% linear sucrose gradient in buffer B, followed by centrifugation, fractionation, and RNA isolation as given above.

The position of the monosome peak (Figure 6A, peak m) was identified by using two methods. First, the monosome peak was identified by cosedimentation with ferritin, which has a sedimentation coefficient of ~75S, thereby producing a peak between the 80S ribosome and 60S ribosomal subunit (Linder et al., 1981). Ferritin (100 μg; type I; Sigma) was added after the 3000*g* spin in buffer A with and without plant extract and carried through to fractionation, as described above. The position of the ferritin peak within the UV absorbance profile provided a reference for identification of the monosome peak. Second, the monosome peak was identified by treatment of polyribosomal extract with RNase A (Jackson and Larkins, 1976). RNase A (at 100 ng/mL, 300 ng/mL, and 1 μg/mL) was added to the extracts after the 3000*g* spin in buffer A above and incubated on ice for 30 min. The samples were treated and fractionated as given above. RNase A treatment shifted the polysomes into the monosome peak without affecting the 60S ribosomal subunit peak. The number of ribosomes within a particular polysome fraction was determined by the progressive shifting of the polysomes to monosomes when treated with increasing RNase A concentrations (Jackson and Larkins, 1976).

Quantitation and Sequence Analysis

Equivalent amounts of RNA, as determined by spectrophotometry, were loaded on gels. Equal loading of RNA samples was confirmed for all gel blots by visualization with UV light in the presence of ethidium bromide and by probing with the constitutively expressed H1 histone gene. The relative amount of specific RNAs was determined from the relative RNA intensity on RNA gel blots as measured using a PhosphorImager (model 445 SI; Molecular Dynamics, Sunnyvale, CA). Data from the PhosphorImager analysis were analyzed on IPLab Gel software version 1.5e (Signal Analytics Corp., Vienna, VA). For comparing ratios of low molecular weight (LMW) RNAs to full-length RNA in total and polysomal RNAs, we used four independent samples for each. The ratios of LMW RNAs to full-length mRNA from total RNA are compared with the ratios of LMW RNAs to full-length mRNA in polysomal fractions 5 and 6. The results are expressed as these ratios ±SD. The number and location of cryptic splice sites within the 2RC mRNA sequence were analyzed with BCM Gene Finder software (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html).

Cycloheximide Treatment

Cycloheximide (CHX; 1 mg/mL; Sigma) or water was injected into fully expanded tobacco leaves, as previously described (Uknes et al., 1993). It has been shown previously that at a concentration of 1 mg/mL, >99% of translation ceases in tobacco tissue (Uknes et al., 1993). Total RNA from the water-soaked tissue was isolated and analyzed on 1.2% agarose–formaldehyde and 5% polyacrylamide gels, as described above.

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