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Stochastic and Nonstochastic Post-Transcriptional Silencing of Chitinase and β-1,3-Glucanase Genes Involves Increased RNA Turnover—Possible Role for Ribosome-Independent RNA Degradation

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Stochastic and nonstochastic post-transcriptional gene silencing (PTGS) in *Nicotiana sylvestris* plants carrying tobacco class I chitinase (*CHN*) and β-1,3-glucanase transgenes differs in incidence, stability, and pattern of expression. Measurements with inhibitors of RNA synthesis (cordycepin, actinomycin D, and α-amanitin) showed that both forms of PTGS are associated with increased sequence-specific degradation of transcripts, suggesting that increased RNA turnover may be a general feature of PTGS. The protein synthesis inhibitors cycloheximide and verrucarin A did not inhibit degradation of *CHN* RNA targeted for PTGS, confirming that PTGS-related RNA degradation does not depend on ongoing protein synthesis. Because verrucarin A, unlike cycloheximide, dissociates mRNA from ribosomes, our results also suggest that ribosome-associated RNA degradation pathways may not be involved in *CHN* PTGS.

INTRODUCTION

Transgenes introduced into plants frequently inactivate expression at the RNA level of homologous endogenous genes and transgenes in *trans* (for an overview, see Meyer, 1995). This phenomenon is called gene silencing and is a type of epigenetic modification, that is, the silent state is usually stable but is not permanent (Meins, 1996). Two forms of silencing have been identified. Transcriptional silencing results from a marked decrease in transcription and hypermethylation of the genes affected. These genes show a high level of meiotic heritability (Matzke et al., 1989; Meyer et al., 1993). Post-transcriptional gene silencing (PTGS) appears to be a less stable process and usually is not meiotically heritable. As determined by results from nuclear run-on transcription experiments, silent and high-expressing genes are transcribed at similar rates, and silencing is not usually associated with hypermethylation of the genes affected (Ingelbrecht et al., 1994; Smith et al., 1994; van Blokland et al., 1994; de Carvalho Niebel et al., 1995; reviewed in Depicker and Van Montagu, 1997).

PTGS is developmentally regulated and strongly influenced by as yet poorly defined environmental factors (Hart et al., 1992; Brandle et al., 1995; Palauqui et al., 1996). In some cases, PTGS is stochastic, that is, the incidence of silencing is variable in a genetically homogeneous population of plants (Hart et al., 1992), whereas in other cases, all plants in the population show a silent phenotype (de Carvalho et al., 1992). Both forms of PTGS result from the interaction of similar transcribed sequences, depend on the number of transgene loci (Vaucheret et al., 1997), and usually (Herget et al., 1990; English et al., 1997; Vaucheret et al., 1997), but not always (van Blokland et al., 1994), require continuous transcription of the genes affected. Analysis of spatial patterns of silencing in individual plants (Jorgensen, 1995; Kunz et al., 1996), grafting experiments with silent and high-expressing plants (Palauqui et al., 1997), and studies of systemic silencing triggered by a local signal (Voinnet et al., 1998) strongly suggest that there is a silencing-inducing principle (SIP), that is, a *trans*-acting, sequence-specific silencing signal that can move from cell to cell or over longer distances in the plant.

The nature of these silencing-inducing signals and the mechanism for PTGS are not known. Most current models include an autoregulatory component to account for stability, production of special or aberrant RNAs for sequence specificity, and a mechanism for increased degradation of specific transcripts (reviewed in Baulcombe, 1996; Meins, 1996). Although altered RNA metabolism is a central feature of these models, direct measurements of RNA degradation have only been reported for nonstochastic silencing of the *Nicotiana plumbaginifolia* β-1,3-glucanase *gn1* in one transgenic line of tobacco (Jacobs et al., 1997).

To date, it is not clear whether the broad range and stability

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of PTGS phenotypes can be accounted for by the same mechanism. We addressed this issue by comparing RNA degradation associated with stochastic and nonstochastic forms of PTGS affecting transgenes with unrelated transcribed sequences. The experimental system we chose was *N. sylvestris* transformed with coordinately regulated, plant defense–related tobacco class I chitinase (*CHN*) and β-1,3-glucanase (*GLU*) genes under the control of the cauliflower mosaic virus 35S RNA promoter (Neuhaus et al., 1991; Hart et al., 1992; Kunz et al., 1996; Kunz, 1997). PTGS of the *CHN* transformants occurs stochastically at the six- to 10-leaf stage of development, persists throughout vegetative growth, and is then reset to the high-expressing state during seed development. Similar >10-fold reductions in RNA content and developmental regulation of silencing were found for otherwise identical constructs in which the *CHN* cDNA was replaced with a *GLU* cDNA. The novel feature of these *GLU* transformants is that silencing is not stochastic, that is, all homozygous seedlings show silencing, and that the plants consistently show a decreasing gradient of silencing in leaves from the bottom to the top of the plant.

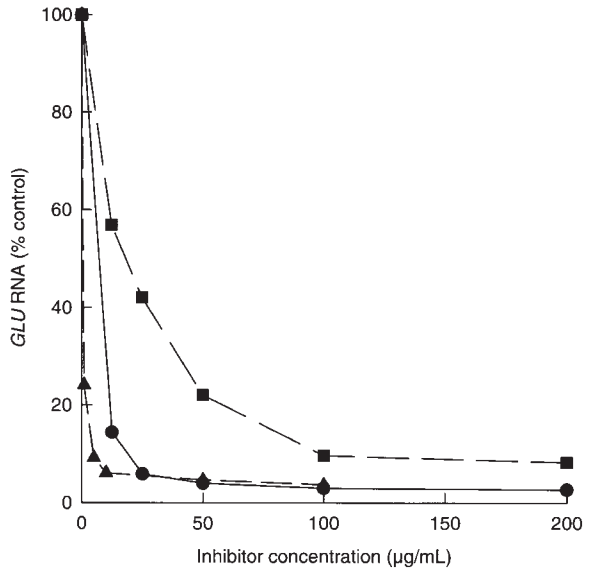

Figure 1. Inhibition of *GLU* RNA Accumulation by Cordycepin, α-Amanitin, and Actinomycin D.

Discs excised from mature *N. sylvestris* leaves were incubated for 5.5 hr with cordycepin (filled circles), actinomycin D (filled squares), and α-amanitin (filled triangles). Total RNA (20 μg) prepared from the discs was analyzed by RNA gel blot hybridization with a probe for *GLU* RNA. *GLU* RNA content was measured by using a PhosphorImager to quantify signals normalized for the 18S rRNA loading standard and is expressed as the percentage of the value obtained with control discs incubated for 5.5 hr without inhibitors. *GLU* RNA content of the control discs increased 11-fold in 5.5 hr.

In this study, we show that stochastic *CHN* PTGS as well as nonstochastic *GLU* PTGS, which differ in incidence, stability, and spatial pattern of expression, are associated with increased RNA degradation. Results of experiments with protein synthesis inhibitors suggest that degradation of *CHN* RNA targeted for PTGS does not require ongoing protein synthesis and that ribosome-associated RNA degradation pathways may not be involved in *CHN* PTGS.

RESULTS

Increased Degradation of *CHN* RNA Is Associated with *CHN* PTGS in Two Independent *CHN* Transformants

Rates of RNA degradation in tissues were estimated from the decrease in mRNA content with time in leaf discs treated with cordycepin, α-amanitin, and actinomycin D, which have been shown to inhibit plant RNA synthesis (Seeley et al., 1992; Newman et al., 1993; Peters and Silverthorne, 1995; Zhu et al., 1995; Börgre et al., 1997). We verified that these inhibitors were effective in our experimental system. Under our standard conditions, which markedly increase transcription of *GLU* genes (Vögeli-Lange et al., 1994), the *GLU* RNA content of untransformed *N. sylvestris* leaf discs increased ~10-fold after 5.5 hr (data not shown). Figure 1 shows that over the same time period, the three inhibitors inhibited *GLU* RNA accumulation in a dose-dependent fashion. Concentrations of inhibitors giving ~90% inhibition of *GLU* RNA accumulation were used in subsequent turnover experiments.

Our initial experiments were performed with the well-characterized 35S–*CHN* transformant SSC2.3, which carries two full-length copies and one truncated copy of T-DNA at a single locus, shows a high incidence of stochastic silencing in plants homozygous for the transgene, and never shows silencing in hemizygous plants (Kunz et al., 1996; Kunz, 1997). The homozygous, vector control transformant SCIB2 treated with ethylene to induce high-level expression of host *CHN* and *GLU* genes (Vögeli-Lange et al., 1994) was used to investigate host *CHN* and *GLU* RNA stability. Leaf discs were incubated in the presence of 150 μg/mL cordycepin, and RNA content was measured using RNA gel blot hybridization with a probe that allowed detection of both host and transgene *CHN* RNA. The phenotype of leaf tissues was verified by immunoblot analyses. Silent tissues are defined as tissues with the same or lower antigen content as comparable tissues of control wild-type or SCIB2 plants in the same experiment. Plants with one or more silent leaves are designated silent plants. Tissues showing antigen levels at least 10-fold higher than those in controls are designated high-expressing tissues. Where indicated, the phenotype of leaf discs was confirmed by measurements of RNA.

Figure 2A shows representative RNA gel blots obtained with homozygous high-expressing and silent SSC2.3 sibling

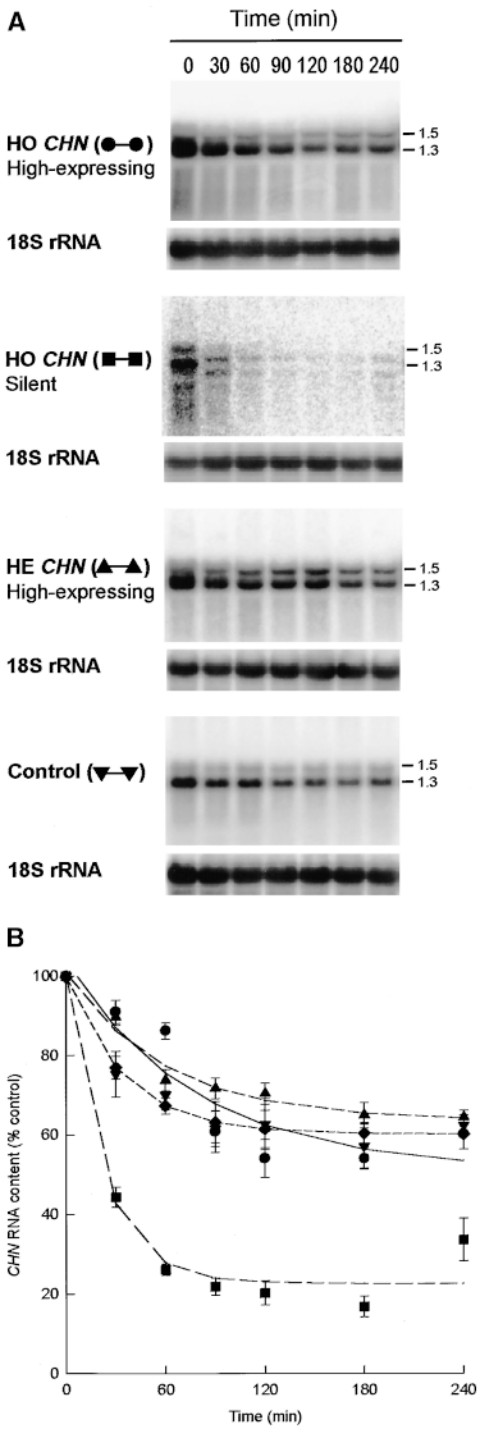

Figure 2. *CHN* RNA Degradation in Cordycepin-Treated Leaf Discs from Silent, High *CHN*-Expressing, and Vector Control Plants.

Discs excised from leaves of high *CHN*-expressing and silent SSC2.3 homozygous plants (HO *CHN*), of high *CHN*-expressing hemizygous SSC2.3 plants (HE *CHN*), and of ethylene-treated, vector control SCIB2 plants (Control) were incubated with 150 μg/mL cordycepin. Total RNA (20 μg) prepared from the discs was analyzed by RNA gel blot hybridization with a probe for *CHN* RNA that detects both the transgene- and host gene–encoded transcripts. *CHN* RNA content was measured by using a PhosphorImager to quantify signals normalized for the 18S rRNA loading standard and is expressed as the percentage of the zero-time value. Results are shown for single leaves obtained in three independent experiments.

(A) RNA gel blot hybridizations obtained with one representative set of leaf discs. Blots were hybridized with the *CHN* RNA probe, stripped, and then rehybridized with an 18S rRNA probe. Note that signal strength on different blots cannot be compared. The blot shown for silent tissues was scanned with the PhosphorImager at high sensitivity to show the less-abundant RNA bands. Approximate lengths (in kilobases) of selected *CHN* RNA bands are indicated at right.

(B) Time course for *CHN* RNA degradation. Average *CHN* RNA contents obtained with three sets of discs and expressed as the percentage of the zero-time value are plotted versus incubation time in the presence of cordycepin. Note that the values obtained with the vector control plants represent host gene–encoded *CHN* RNA. The degradation constants estimated from the data are shown in Table 1, experiment 1. Error bars are ±SE. Filled circles and the solid line indicate high *CHN*-expressing homozygous SSC2.3; filled squares and dashed line show silent homozygous SSC2.3; filled triangles and dashed line represent high *CHN*-expressing hemizygous SSC2.3; and inverted filled triangles and dashed line indicate homozygous vector control SCIB2.

plants, a high-expressing hemizygous SSC2.3 plant, and a vector control SCIB2 plant. The average *CHN* RNA content of the homozygous high-expressing leaf discs from three different plants was ~28-fold higher than that of the silent leaf discs (data not shown). RNA gel blots of samples from silent tissues were overexposed to detect low levels of *CHN* RNAs. Inspection of lanes loaded with zero-time samples showed that for each genotype, at least two transcripts hybridized strongly with the *CHN* probe, as reported earlier (Neuhaus et al., 1991). These transcripts decreased far more rapidly for RNA from silent tissues than those for RNA from high-expressing tissues or for host gene *CHN* RNA from vector control tissues. The size of these major transcripts obtained with silent tissues appeared to shift to a slightly smaller size early in the experiment. A similar shift also was observed with transcripts of the neomycin phosphostransferase II (*NPTII*) gene, which did not exhibit silencing (data not shown). This might be due to effects of cordycepin on polyadenylation reported in animal studies (Kuznetsov and Musajev, 1990). In addition, several smaller transcripts of low abundance were consistently detected in RNA prepared from silent tissues but faintly or not at all in RNA prepared from high-expressing tissues or from tissues of the vector control plant.

Figure 2B shows quantitative measurements of RNA content in the two major bands expressed as the percentage of the zero-time value and normalized for the 18S rRNA loading

standard. The 18S rRNA signals did not change appreciably over the period of the experiment (data not shown). The results are the averaged values obtained with single leaves from different plants in three independent experiments. First-order degradation constants were estimated by fitting curves of RNA content versus time to an exponential function. Table 1, experiment 1, shows that the degradation constants for *CHN* RNA in high-expressing hemizygous and homozygous SSC2.3 transformants and for host *CHN* RNA in the vector control transformant were similar. This indicates that in high-expressing tissues, transgene dose does not affect *CHN* RNA degradation and that transgene and host gene *CHN* RNAs are similar in stability. In contrast, the rate of *CHN* RNA degradation in silent tissues was ~3.5 higher than the rate in homozygous, high-expressing tissues. Thus, *CHN* RNA degradation was substantially increased in cordycepin-treated tissues showing *CHN* silencing.

The cordycepin–chase experiments were repeated with the independent *CHN* transformant SSC2.4, carrying two full-length T-DNA copies at a single locus, which is not linked to the SSC2.3 transgene locus (Kunz, 1997). Comparison of the degradation constants obtained with SSC2.3 and SSC2.4 (Table 1, experiments 1 and 2) shows that rates of *CHN* RNA degradation in silent and high-expressing leaves of the two transformants were very similar. These results suggest that neither the increase in RNA degradation asso-

Table 1. First-Order Degradation Constants Estimated for *CHN*, *GLU*, and *NPTII* RNA

					Degradation Rates	
Experiment	Inhibitor	Genotype[a]	State[b]	RNA	$k \times 10^2$ min^{-1} [c]	Silent/High[d]
1	Cordycepin	HO SSC2.3	High	*CHN*	1.28 ± 0.75 (7)	
			Silent	*CHN*	4.49 ± 1.18 (7)	3.50
		HE SSC2.3	High	*CHN*	1.66 ± 0.36 (7)	
		HO SSC2.3	High	*NPTII*	1.78 ± 0.17 (8)	
			Silent	*NPTII*	1.35 ± 0.21 (8)	0.75
		HE SSC2.3	High	*NPTII*	0.97 ± 0.16 (10)	
		HO SCIB2	Wild type	Host *CHN*	1.60 ± 0.60 (7)	
				Host *GLU*	0.18 ± 0.096 (12)	
2	Cordycepin	HO SSC2.4	High	*CHN*	1.46 ± 0.87 (7)	
			Silent	*CHN*	6.45 ± 3.38 (7)	4.4
3	Cordycepin	HO SSC2.3	Variable			
			High	*CHN*	0.68 ± 0.08 (5)	
			Silent	*CHN*	3.74 ± 0.93 (5)	5.5
4	Cordycepin	HE SSG7.2	High	*GLU*	0.41 ± 0.13 (12)	
		HO SSG7.2	Silent	*GLU*	1.14 ± 0.15 (12)	2.78
		HE SSG7.2	High	*NPTII*	1.02 ± 0.16 (11)	
		HO SSG7.2	Silent	*NPTII*	0.92 ± 0.20 (12)	0.89
		HO SCIB2	Wild type	Host *GLU*	0.28 ± 0.15 (12)	
				NPTII	1.20 ± 0.15 (12)	
5	Cordycepin	HO SSG7.2	Variable			
			High	*GLU*	0.64 ± 0.10 (6)	
			Silent	*GLU*	1.32 ± 0.08 (6)	2.1
6	α-Amanitin	HO SSC2.3	High	*CHN*	0.61 ± 0.15 (15)	
			Silent	*CHN*	3.70 ± 0.65 (10)	6.1
7	Actinomycin D	HO SSC2.3	High	*CHN*	0.51 ± 0.30 (5)	
			Silent	*CHN*	1.63 ± 0.41 (5)	3.2
		HE SSC2.3	High	*CHN*	1.21 ± 0.28 (5)	
8	Actinomycin D	HE SSG7.2	High	*GLU*	0.59 ± 0.07 (4)	
		HO SSG7.2	Silent	*GLU*	1.01 ± 0.13 (4)	1.71

[a] Independent *CHN* (SSC2.3 and SSC2.4), *GLU* (SSG7.2), and vector control (SCIB2) transformants homozygous (HO) or hemizygous (HE) for the transgene are indicated.

[b] Whether leaf tissue was expressing the silent or high-expressing transgene phenotype was determined from immunoblot tests. Variable indicates SSC2.3 plants showing the silent and high-expressing phenotype in adjacent leaves and SSG7.2 plants showing the high-expressing phenotype in upper leaves and the silent phenotype in lower leaves.

[c] First-order degradation constants $k \pm$SE for the number of data points within parentheses were estimated by fitting an exponential curve to RNA content versus time. The data were obtained from two or three experiments.

[d] Ratio of degradation rate constants obtained for high-expressing and silent tissues.

ciated with silencing nor the rates of RNA degradation are unique features of the SSC2.3 T-DNA.

Increased Degradation of *CHN* RNA Is Also Correlated with *CHN* PTGS in a Single Plant

To examine the relationship between RNA degradation and silencing in the same plant, we exploited the finding that in some SSC2.3 plants, adjacent leaves can exhibit either a uniformly high-expressing or a uniformly silent phenotype (Kunz et al., 1996). Figures 3A and 3B show that increased *CHN* RNA degradation is strictly correlated with silencing. In leaf discs from four adjacent leaves alternatively exhibiting the silent (L1 and L3) and the high-expressing (L2 and L4) phenotypes, only those from L1 and L3 showed increased RNA degradation. The average RNA degradation rate of silent leaves was 5.5-fold higher than that of high-expressing leaves (Table 1, experiment 3). These results show that variable silencing within the same plant as well as uniform silencing are associated with a substantial increase in the rate of *CHN* RNA degradation.

Nuclear Run-on Transcription Assays with *GLU* Transformants

We chose the 35S–*GLU* transformant SSG7.2 to examine RNA degradation in a transformant that exhibits nonstochastic silencing. This line carries two complete T-DNA copies at a single locus (Kunz et al., 1996; Kunz, 1997). Nuclear run-on transcription experiments were performed to determine whether SSG7.2 exhibits PTGS. Leaf tissues of a known *GLU*-conferred phenotype were pooled from two or three hemizygous SSG7.2 plants, homozygous SSG7.2 plants, and wild-type plants. Total RNA for measuring steady state mRNA and nuclei for run-on transcription assays was prepared from the same pool. Hybridization signals obtained using probes that detect both transgene and host *GLU* RNA (Neuhaus et al., 1991) were quantitated using a PhosphorImager and normalized relative to signals for the ribulose-1,5-bisphosphate carboxylase small subunit (*SSU*) gene.

Figures 4A and 4B show that although the steady state *GLU* RNA content in silent leaves was 14.9-fold lower than that in high-expressing leaves, the template activity of *GLU* genes in these tissues differed by less than ~20%. This indicates that *GLU* genes in silent and high GLU tissues are transcribed at approximately equal rates. Hence, the markedly lower steady state *GLU* RNA content of silent tissues is not due to differences in transcription. Because the run-on assays and RNA gel blot hybridizations do not distinguish between transgene and host gene *GLU* RNA, we cannot exclude the possibility that silencing of the host genes occurs transcriptionally. However, this seems unlikely because the amount of nascent *GLU* RNA in silent tissue is approximately equal to that of high-expressing tissues in which the host genes are expressed. Taken together, the results suggest that *GLU* transgenes, and probably their host homologs, are silenced by a post-transcriptional mechanism. As described earlier for *CHN* (Kunz et al., 1996), nascent antisense *GLU* transcripts were detected in high-expressing, silent, and untransformed tissues.

Increased Degradation of *GLU* RNA Is Associated with *GLU* PTGS

We used cordycepin–chase experiments to measure the rates of *GLU* RNA degradation in discs prepared from silent homozygous SSG7.2 leaves, high-expressing hemizygous SSG7.2 leaves, and vector control SCIB2 leaves treated with ethylene to induce GLU accumulation. Three independent experiments were performed, and average values obtained for single leaves from different plants were used to estimate RNA degradation rates. Representative RNA gel blots in Figure 5A show that one abundant and several smaller, less abundant *GLU* RNAs are present in the leaf discs. Note that RNA gel blots with samples of RNA from silent tissues were overexposed and that signal strengths on different blots are not comparable. In contrast to the *CHN* transformants, the smaller *GLU* RNAs were sometimes detected in hemizygous high-expressing leaves and in leaves of vector control plants as well. With increasing incubation time, the *GLU* RNA content decreased more rapidly in silent leaf discs than in high-expressing or vector control leaf discs (Figure 5B). Table 1, experiment 4, shows that the rate of *GLU* RNA degradation was ~2.8-fold higher in silent tissues than in high-expressing tissues. A similar 2.1-fold higher rate was found for high-expressing and silent leaves on the same homozygous SSG7.2 plant, which differed 3.2-fold in *GLU* RNA content (Table 1, experiment 5). Although the differences in degradation rates in silent and high-expressing tissues were not as pronounced as for *CHN* transformants, the results show that increased RNA degradation also is correlated with *GLU* PTGS in *GLU* transformants.

Degradation Rate of *NPTII* RNA Is Not Altered in Silent and High-Expressing Transformants

The T-DNA of the *CHN* and *GLU* transformants contains a second expressible gene, *NPTII*, which we used as a kanamycin resistance marker. All homozygous SSC2.3 and SSG7.2 seedlings are kanamycin resistant, indicating that *NPTII* is expressed in both silent and high-expressing plants. Moreover, silent and high-expressing leaf tissues have very similar steady state *NPTII* RNA contents, as determined from RNase protection assays of RNA from entire leaves and quantitation of zero-time RNA samples from leaf discs (data not shown). Cordycepin–chase experiments with leaf discs indicate that the degradation rates of *NPTII* RNA

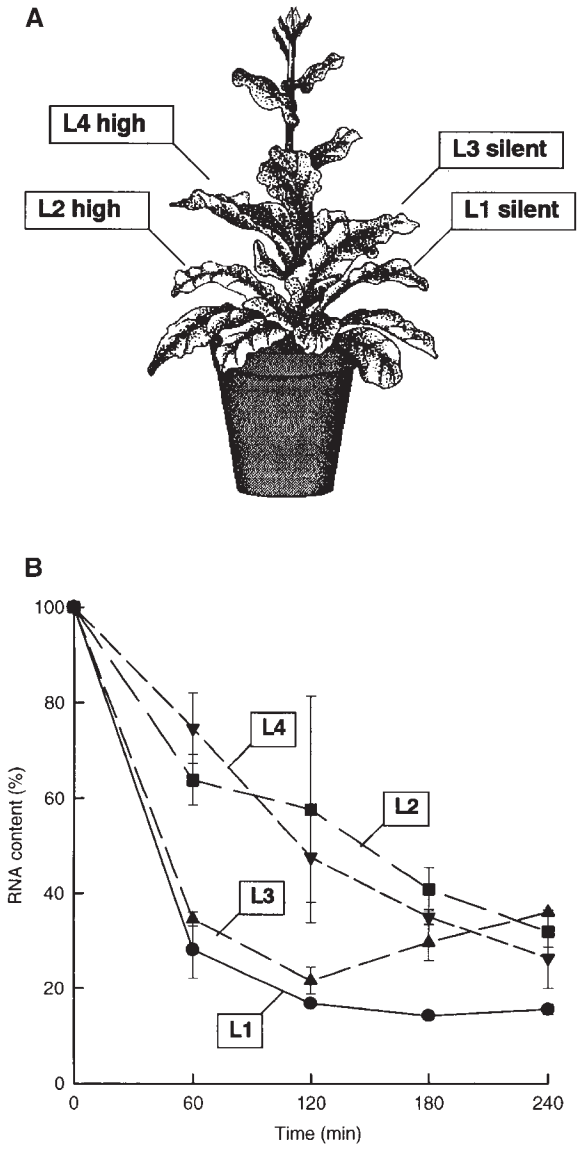

Figure 3. *CHN* RNA Degradation in Cordycepin-Treated Leaf Discs from Silent and High *CHN*-Expressing Leaves on the Same Plant.

Leaf discs excised from four adjacent leaves of an SSC2.3 plant showing spatial patterns of *CHN* gene silencing were incubated with 150 μg/mL cordycepin. *CHN* RNA contents in two sets of discs from each leaf were measured as described for Figure 2.

(A) A diagram showing the relative positions and CHN phenotype of leaves used for measurements of *CHN* RNA degradation. L1 to L4 represent the position of the leaf sampled, counting from the bottom of the plant.

(B) Time course of *CHN* RNA degradation in leaf discs from silent (L1 and L3) and high *CHN*-expressing (L2 and L4) leaves. Filled circles and filled triangles represent silent leaves; filled squares and filled inverted triangles correspond to high-expressing leaves from a single plant. Average *CHN* RNA contents obtained for replicate sets of leaf discs and expressed as the percentage of the zero-time value are plotted versus incubation time in the presence of cordycepin. The degradation constants estimated from the data are shown in Table 1, experiment 3. Error bars represent ±SE.

are very similar in high-expressing and silent SSC2.3 and SSG7.2 transformants and in vector control SCIB2 transformants (Table 1, experiments 1 and 4). Taken together, these results show that the steady state levels and stability of RNA transcribed from a gene with an unrelated sequence are not affected by silencing of *CHN* and *GLU* genes present in the same T-DNA at the same locus.

Studies of RNA Degradation Using the RNA Synthesis Inhibitors α-Amanitin and Actinomycin D

Measured RNA degradation rates can depend on the inhibitor of RNA synthesis used (Grayson and Berry, 1973; Tilghman et al., 1974). Therefore, we examined the effects of two other inhibitors, α-amanitin and actinomycin D, which differ from cordycepin in their modes of action (reviewed in Belasco and Brawerman, 1998). The concentration used, 100 μg/mL, was shown to inhibit *GLU* RNA synthesis by >90% and was in the range employed in earlier studies of plant RNA degradation (Newman et al., 1993). The results obtained for the *CHN* transformant SSC2.3 with α-amanitin and cordycepin were similar (Table 1, experiments 1 and 6). Based on values from two independent experiments, the rate of *CHN* RNA degradation measured with α-amanitin was approximately sixfold higher in silent tissue than in high-expressing tissue. Rates of *CHN* RNA degradation measured with actinomycin D in three experiments were lower than those measured with either cordycepin or α-amanitin and were ~2.3-fold higher in silent than in high-expressing tissues (Table 1, experiment 7). In the case of actinomycin D treatment of SSG7.2 leaf discs, *GLU* RNA degradation rates were ~1.7-fold higher in silent tissues than in high-expressing tissues (Table 1, experiment 8).

Silencing-Specific Degradation of *CHN* RNA Does Not Require Translation or Binding of the RNA to Ribosomes

If silencing-specific RNA degradation depends on either labile protein factors or ongoing protein synthesis, then treatment with inhibitors of protein synthesis should increase the steady state level and decrease the degradation rates of silenced transcripts. To investigate this possibility, we treated leaf discs from silent leaves of homozygous SSC2.4 plants with combinations of cordycepin and cycloheximide, which inhibit polypeptide chain elongation (Pestka, 1971). Cycloheximide was used at a concentration of 10 μg/mL, which

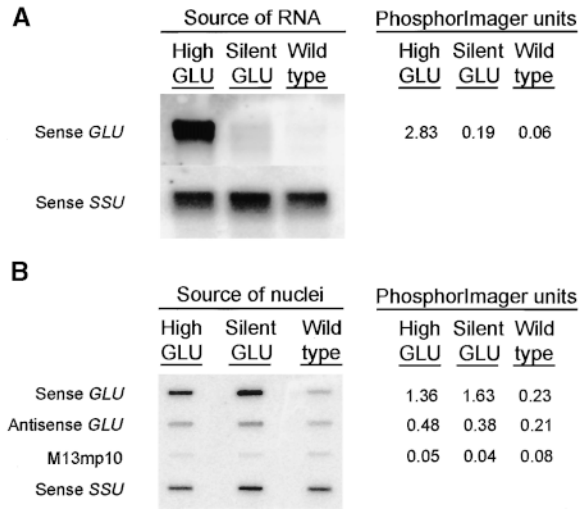

Figure 4. RNA Gel Blot Analysis and Slot Blot Hybridization of Nuclear Run-on Transcription Assays of Leaf Tissues from High *GLU*-Expressing and Silent SSG7.2 Plants.

Leaves from high *GLU*-expressing hemizygous SSG7.2 plants (High GLU), silent homozygous SSG7.2 plants (Silent GLU), and untransformed plants (Wild type) were pooled to prepare total RNA for RNA gel blot hybridization analysis and to isolate nuclei for nuclear run-on transcription experiments. The intensities of hybridization signals normalized for the *SSU* RNA signals and expressed in arbitrary PhosphorImager units (see Methods) are shown at right.

(A) RNA gel blot hybridization analysis of *GLU* RNA prepared from high *GLU*-expressing, silent, and untransformed tissues. Total RNA (20 μg) was hybridized using probes that detect transgene- and host gene–encoded sense *GLU* RNA and sense *SSU* RNA.

(B) Nuclear run-on transcription analysis. Slot blot hybridization was performed with nuclear RNA using probes that detect sense and antisense *GLU* RNA and sense *SSU* RNA.

we verified as inhibiting GLU and CHN accumulation by >95% in leaf discs of *N. sylvestris* (data not shown). *NPTII* RNA content was measured as an internal control for a T-DNA–encoded transcript that shows similar stability in silent and high-expressing *CHN* transformants (see Table 1, experiment 1).

In our initial experiment, we measured the time course of *GLU*, *NPTII*, and *CHN* RNA accumulation in leaf discs treated with cycloheximide over a 12-hr period. Steady state levels of RNA transcribed from host *GLU* genes, which are not affected by *CHN* silencing (Kunz et al., 1996), and RNA transcribed from silent *CHN* genes increased by <50% during the course of the experiment (data not shown). In contrast, steady state *NPTII* RNA content increased by up to ~5.5-fold in the same samples. Thus, cycloheximide treatment markedly increased *NPTII* RNA accumulation but had only slight effects on the accumulation of *GLU* and *CHN* RNAs.

To detect effects of cycloheximide on RNA degradation, we pooled leaf discs from a single, mature leaf expressing the silent *CHN* phenotype. One set of discs was treated with cordycepin alone; the other one was treated with cordycepin and cycloheximide. Figure 6 shows that the kinetics of *CHN* RNA degradation with and without cycloheximide treatment (Figure 6A) and *NPTII* RNA degradation without cycloheximide treatment (Figure 6B) were very similar. However, cycloheximide treatment greatly delayed degradation of *NPTII* RNA (Figure 6B). Although cycloheximide was effective under the conditions used, as indicated by the inhibition of *NPTII* RNA degradation, cycloheximide did not inhibit *CHN* RNA degradation. This strongly suggests that ongoing translation is not required for the silencing-related degradation of *CHN* RNA.

To determine whether association with ribosomes is required for *CHN* RNA degradation, we used another potent inhibitor of eukaryotic protein synthesis, verrucarin A, which dissociates mRNA from ribosomes (Wei and McLaughlin, 1974; Cannon, 1976; Fresno and Vazquez, 1979). Under our standard conditions, 100 μg/mL verrucarin A effectively blocked the synthesis of GLU and CHN proteins in leaf discs (data not shown). Figure 7A shows that neither cycloheximide nor verrucarin A increased the steady state *CHN* RNA content of leaf tissues exhibiting *CHN* gene silencing and that the degradation rates of *CHN* RNA in the presence of cordycepin were comparable for the two inhibitors. We also verified that verrucarin A treatment, like cycloheximide, stabilized the *NPTII* RNA used as an internal control (Figure 7B). These results suggest that silencing-specific degradation of *CHN* RNA does not depend on association of the RNA with ribosomes.

DISCUSSION

Increased Degradation of Silenced Transcripts Is a General Feature of PTGS

At present, it is unclear whether the wide range of phenotypes reported for PTGS have a common mechanism (Que et al., 1997). We compared two extreme forms of PTGS affecting structurally unrelated transcripts in the same experimental system. Our most important conclusion is that silencing-specific increases in RNA degradation are features of both stochastic *CHN* PTGS and nonstochastic *GLU* PTGS, which differ in incidence, stability, and spatial pattern of expression. Direct measurements of RNA turnover were made with three inhibitors—cordycepin, actinomycin D, and α-amanitin. This was important because each of these inhibitors has untoward effects, including transcript stabilization (Fritz et al., 1991; Seeley et al., 1992; Li and Foley, 1996; Rajagopalan and Malter, 1996), which can affect estimates of degradation rate. For example, as reported for other

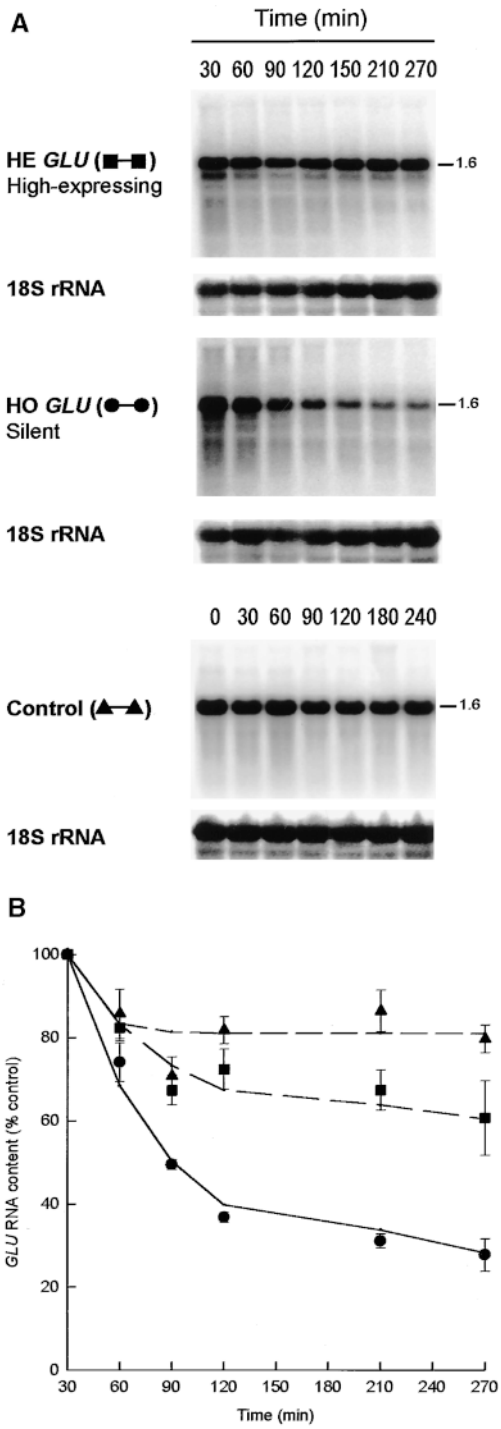

Figure 5. *GLU* RNA Degradation in Cordycepin-Treated Leaf Discs from Silent, High *GLU*-Expressing, and Vector Control Plants.

Discs excised from leaves of high *GLU*-expressing hemizygous SSG7.2 plants (HE *GLU*), of silent homozygous SSG7.2 plants (HO *GLU*), and of ethylene-treated, vector control SCIB2 plants (Control) were incubated with 150 μg/mL cordycepin, and *GLU* RNA content was measured by using a probe that detects transgene- and host gene–encoded *GLU* RNA, as described for Figure 2. *GLU* RNA content is expressed as the percentage of the value obtained 30 min after cordycepin was added. Results are shown for single leaves of different plants obtained in three independent experiments.

(A) RNA gel blot hybridizations for one representative experiment. Blots were hybridized with the *GLU* RNA probe, stripped, and then rehybridized with an 18S rRNA probe. Note that the signal strength on different blots cannot be compared. The blot shown for silent tissues was scanned with the PhosphorImager at high sensitivity to show the less abundant RNA bands. Approximate lengths (in kilobases) of selected *GLU* RNA bands are indicated at right.

(B) Time course of *GLU* RNA degradation. Average *GLU* RNA contents obtained with three sets of discs and expressed as the percentage of the 30-min value are plotted versus incubation time in the presence of cordycepin. Note that the values obtained with the vector control plants represent host gene–encoded *GLU* RNA. The degradation constants estimated from these data are shown in Table 1, experiment 4. Error bars represent ±SE. Filled squares indicate high *GLU*-expressing hemizygous SSG7.2; filled circles represent silent homozygous SSG7.2; and filled triangles show homozygous vector control SCIB2.

RNAs (Grayson and Berry, 1973; Tilghman et al., 1974), we found that measurements of *GLU* and *CHN* RNA degradation with cordycepin gave higher rates than those obtained with actinomycin D. The key point is that independent of the inhibitor used, RNA degradation was consistently increased for silenced *GLU* and *CHN* transgenes. We also confirmed these results by comparing silent- and high-expressing regions of the same plant and verified that the effect was specific by monitoring the degradation of *NPTII* RNA expressed from the same T-DNA.

Our findings, taken together with estimates of RNA stability obtained directly for *gn1 GLU* RNA in tobacco (Jacobs et al., 1997) and indirectly for *chsA* RNA in petunia, tobacco etch virus coat-protein RNA in tobacco (Tanzer et al., 1997), and tomato *ACC* synthase RNA in tomato (Lee et al., 1997), lead to the conclusion that increased RNA degradation is a general feature of PTGS.

The important question that arises is whether the measured degradation rates can account for the decrease in steady state RNA levels found. If rates of RNA synthesis are comparable in silent- and high-expressing tissues, as suggested from the results of nuclear run-on transcription experiments, then the fold reduction of steady state RNA in silent tissues should be similar to the fold increase in RNA degradation rates. This was not the case. The PTGS-related reduction in steady state *CHN* RNA level was approximately eightfold higher than was predicted from degradation rates. Substantial discrepancies, 2.1 to 5.9 fold, also have been reported by Jacobs et al. (1997) for *gn1 GLU* PTGS in tobacco, suggesting that this may be a more general phe-

nomenon. It appears either that measurements employing inhibitors underestimate RNA destabilization in silent tissues or that other factors, for example, compartmentation, inefficient processing, or nucleocytoplasmic transport, contribute to the reduction of steady state RNA levels. These possibilities could be investigated by pulse–chase measurements of RNA degradation rates in the nuclear and cytoplasmic compartments.

Possible Mechanisms for PTGS

Several major models have been proposed for PTGS (reviewed in Meins and Kunz, 1995; Baulcombe, 1996; Johnson et al., 1998). According to the ectopic pairing model, certain arrangements of T-DNA insertions can pair ectopically with other genes of similar sequence, resulting in increased degradation of transcripts produced by the interacting genes. This model could account for the finding that DNA methylation, which is a consequence of ectopic pairing, is sometimes associated with PTGS (Ingelbrecht et al., 1994; English et al., 1996; Jones et al., 1998). It also might account for the puzzling observation that promoterless transgenes, which presumably are not transcribed or only weakly transcribed, also can trigger PTGS (van Blokland et al., 1994; English et al., 1996; Voinnet et al., 1998).

The RNA threshold model holds that there is a sensing mechanism for the total concentration of similar transcripts. When the cellular concentration of these transcripts exceeds a critical threshold, a sequence-specific machinery for degrading the RNA is activated (Lindbo et al., 1993; Smith et al., 1994). The biochemical switch model is a refinement of this model, which can account for the stability and potential reversibility of PTGS (Meins and Kunz, 1995; Kunz et al., 1996). This model holds that RNA concentrations above a critical threshold trigger the formation of a sequence-specific activator of RNA degradation, presumably a nucleic acid, which is maintained by a positive, autoregulatory feedback loop. It has been proposed that the activator is an “aberrant RNA” derived from the silenced genes, which is a self-replicating entity or is generated in a self-perpetuating fashion during degradation of target transcripts (Metzlaff et al., 1997). Although antisense mechanisms are an attractive explanation for the specificity of RNA degradation (Grierson et al., 1991; Mol et al., 1991), direct evidence for this hypothesis is still lacking. Low levels of antisense transcripts have been detected by nuclear run-on transcription assays, but we and others have not found a correlation between the abundance of these transcripts and the silent state (van Blokland et al., 1994; Kunz et al., 1996). Moreover, recent studies show that sense and antisense alleles of chalcone synthase transgenes in petunia generate markedly different patterns of silencing (Que et al., 1997).

We favor the biochemical switch model for several reasons. First, the model can account for variation in the incidence

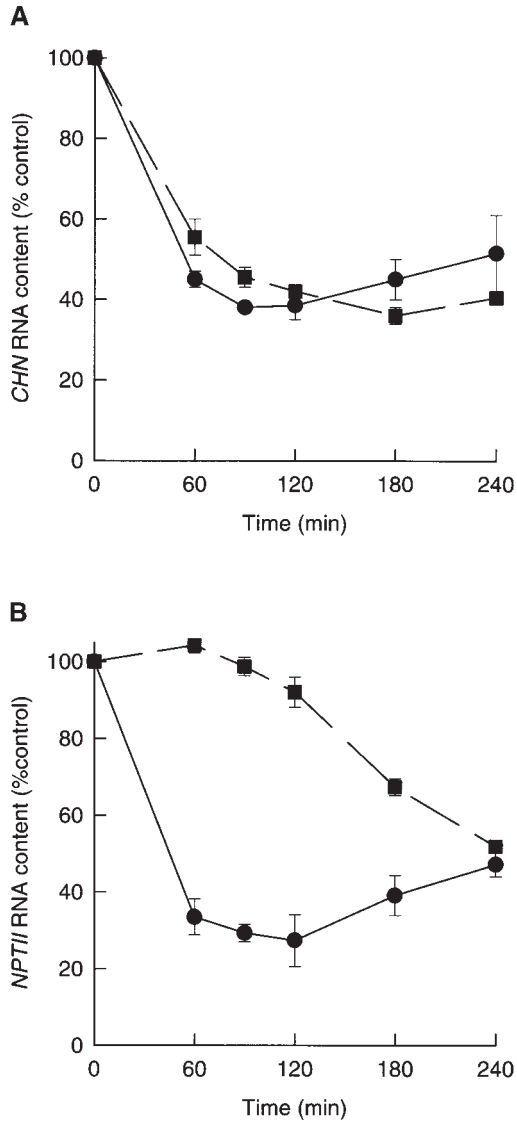

Figure 6. Effect of Treatment with Cycloheximide and Cordycepin on *CHN* and *NPTII* RNA Accumulation in Discs from Leaves Showing *CHN* Gene Silencing.

Discs excised from a single leaf of an SSC2.4 plant showing *CHN* gene silencing were incubated with 150 μg/mL cordycepin and 10 μg/mL cycloheximide. The leaf discs were preincubated in the inhibitors for 30 min before sampling. *CHN* and *NPTII* RNA content was measured as described for Figure 2, except that 10 μg of total RNA was applied to the gels. Results are shown as average values obtained with two sets of discs from the same leaf. Filled circles indicate cordycepin; filled squares represent cordycepin plus cycloheximide. Error bars indicate ±SE for two replicates.

(A) Time course of *CHN* RNA accumulation.

(B) Time course of *NPTII* RNA accumulation.

and stability of PTGS. According to this model, switching depends on the critical RNA threshold, which is likely to be influenced by the nature of the transcript, as well as the transcription levels of the genes affected. For genes such as *CHN* and *GLU*, which are subject to developmental regulation and induction by environmental stresses (reviewed in Meins et al., 1992), large, persistent developmental changes in RNA content could generate predictable spatial gradients of *GLU* PTGS in different leaves as well as uniform *CHN* PTGS within individual leaves. On the other hand, transient fluctuations in RNA content near the critical threshold in response to the environment could lead to stochastic *CHN* silencing of different leaves in the same plant and different plants in a genetically homogenous population. Second, positive autoregulation might amplify the silencing signal and account for the spread of silencing from cell to cell and over long distances in the plant (Palauqui et al., 1997; Voinnet et al., 1998). Finally, this model does not exclude the possibility that both ectopic gene pairing and critical thresholds generate "aberrant RNAs," which are then amplified by positive autoregulation.

A Novel Cytoplasmic Mechanism for Silencing-Related Degradation of *CHN* RNA?

There is evidence that PTGS-related RNA degradation starts with endonucleolytic cleavage at one or more sites followed by exonucleolytic degradation of the resultant fragments (Lee et al., 1997; Metzlaff et al., 1997; van Eldik et al., 1998). It has been proposed that this degradation pathway is then sustained by the pairing of these fragments or other "aberrant RNAs" with full-length transcripts (Metzlaff et al., 1997). Infection with RNA viruses, which have a cytoplasmic life cycle, can trigger PTGS, strongly suggesting that the transcripts are degraded in the cytoplasm (reviewed in Baulcombe, 1996). This conclusion is supported by reports that PTGS does not decrease full-length transcript levels in the nucleus (de Carvalho Niebel et al., 1995; Lee et al., 1997). Several major mechanisms for RNA degradation involve association of RNA with ribosomes, and it is often assumed that similar cytoplasmic mechanisms are operating in PTGS (reviewed in Johnson et al., 1998). Our cycloheximide studies and those of others (Jacobs et al., 1997; Tanzer et al., 1997; Hamilton et al., 1998) suggest that ongoing protein synthesis and rapidly turning over protein factors are not required. The significance of our verrucarin A studies is that, unlike cycloheximide, verrucarin A dissociates mRNA from ribosomes (Wei and McLaughlin, 1974; Cannon, 1976; Fresno and Vazquez, 1979). Verrucarin A did not affect either the stability or rate of degradation of *CHN* RNA in silent tissues. Thus, PTGS-related *CHN* RNA degradation may involve a novel cytoplasmic mechanism that does not require continuous association of the RNA with ribosomes. Further tests of this intriguing hypothesis and the possibility that it applies more generally are in progress.

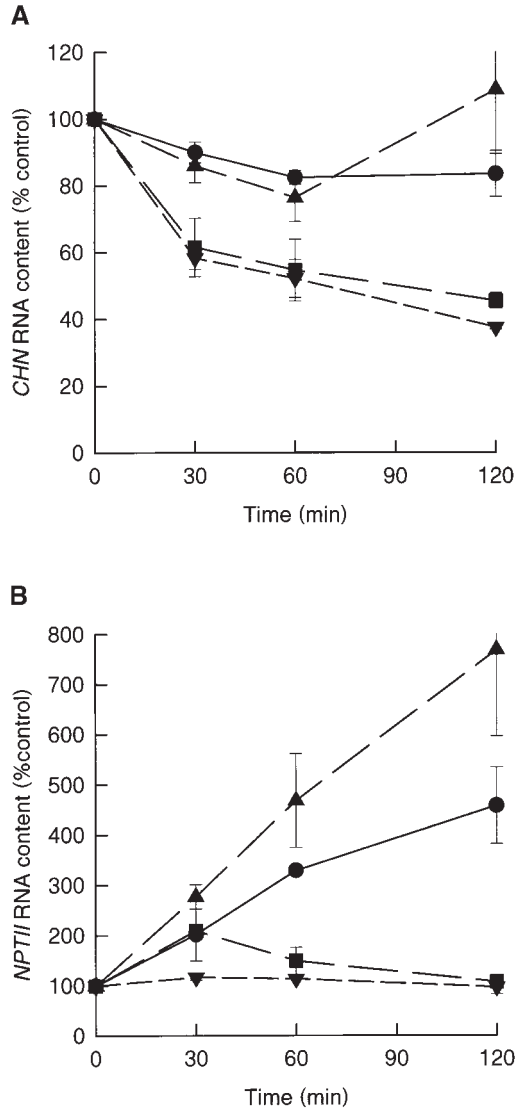

Figure 7. Comparison of Verrucarin A and Cycloheximide Effects on *CHN* and *NPTII* RNA Accumulation in Discs from Leaves Showing *CHN* Gene Silencing.

Discs excised from a single leaf of an SSC2.3 plant showing *CHN* gene silencing were incubated with 150 μg/mL cordycepin, 100 μg/mL verrucarin A, and 10 μg/mL cycloheximide. *CHN* and *NPTII* RNA content was measured as described for Figure 2, except that 50 μg of total RNA was applied to the gels. Results are shown as average values obtained with sets of leaf discs from two plants. Filled circles indicate cycloheximide; filled squares represent cycloheximide plus cordycepin; filled triangles denote verrucarin A; and filled inverted triangles show verrucarin A plus cordycepin. Error bars denote ±SE for two replicates.

(A) Time course of *CHN* RNA accumulation.

(B) Time course of *NPTII* RNA accumulation.

METHODS

Plant Material

The *Nicotiana sylvestris* plants used, the conditions used for promoting silencing, and the sampling leaves of mature, nonflowering, greenhouse-grown plants have been described previously (Kunz et al., 1996). The monogenic, independent transformants SSC2.3 and SSC2.4 carry the tobacco class I chitinase *CHN48* transgene; the monogenic transformant SSG7.2 carries a tobacco β-1,3-glucanase (*GLU*) transgene; and the monogenic transformant SCIB2 carries the empty vector T-DNA (Neuhaus et al., 1991; Kunz et al., 1996). Where indicated, host *GLU* and chitinase (*CHN*) genes were induced by incubating plants for 2 days in a chamber containing 20 ppm of ethylene (Keefe et al., 1990).

RNA Gel Blot Hybridization and Nuclear Run-on Transcription Assays

Total RNA was isolated from leaf tissue (100 to 200 mg) ground under liquid nitrogen by using the RNeasy Plant Mini Kit (Qiagen) and stored at −80°C. RNA blot hybridization was performed by standard methods (Sambrook et al., 1989). The DNA probes used were the 1.1-kb HindIII-BamHI insert of pCHN48, containing the full-length tobacco *CHN48* cDNA (Shinshi et al., 1990); the 1.2-kb HindIII-BamHI insert of pGLN17, carrying a hybrid tobacco *GLU36* and *GLU31* cDNA (Shinshi et al., 1988); the HindIII insert of pHB23, carrying the Tn*5* neomycin transferase *NPTII* gene (Bevan, 1984); and the 1.5-kb EcoRI insert of pRE30, carrying a tomato 18S rRNA gene (Schmidt-Puchta et al., 1989). Antisense riboprobes were synthesized with T3 or T7 RNA polymerase using α-^{32}P-CTP and linearized pBluescript KS+ plasmids (Stratagene, La Jolla, CA) containing a BamHI fragment of pSSU71 representing a petunia small subunit of ribulose-1,5-bisphosphate carboxylase (*SSU*) gene (Dean et al., 1992) and a PstI insert of pGL43 representing tobacco *GLU* (Shinshi et al., 1988). Exposure and quantitation of signals were conducted using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The signals obtained for the major hybridizing bands were added and then normalized for the 18S rRNA loading standard. The methods for the isolation of leaf nuclei, run-on transcription analysis, and preparation of M13 probes have been described previously (van Blokland et al., 1994; Kunz et al., 1996).

Measurement of RNA Degradation

Unless indicated otherwise, two or three independent experiments were performed, each with material from a single leaf. Discs (1 cm in diameter) excised from the lamella of a single, fully expanded leaf were collected in 50 or 100 mL of incubation buffer (15 mM sucrose, 1 mM KCl, 1 mM Pipes, and 1 mM sodium citrate, pH 6.5) and were incubated as described by Seeley et al. (1992). Linsmaier and Skoog (1965) medium rather than incubation buffer was used in experiments with translation inhibitors. Vacuum-infiltrated leaf discs were incubated for up to 4 hr with shaking (100 rpm) at room temperature under normal laboratory light conditions. Experiments with actinomycin D were conducted in a darkroom. Leaf discs removed immediately after vacuum infiltration and at intervals of 30 or 60 min were stored at −80°C. As indicated, the incubation buffer was supplemented with 150 μg/mL cordycepin (Sigma), 100 μg/mL α-amanitin (Sigma), and 10 μg/mL cycloheximide (Aldrich) added as 5 mg/mL stock solutions in water, with 100 μg/mL actinomycin D (Sigma) added as a 5 mg/mL stock solution in 80% (v/v) ethanol and with 100 μg/mL verrucarin A (Sigma) added as a 10 mg/mL stock solution in DMSO.

Estimation of RNA Degradation Rates

First-order rate constants for RNA degradation were estimated from the time course of RNA accumulation in leaf discs treated with the inhibitors indicated. RNA contents, expressed as a percentage of the control, that is, the first time point sampled, were fitted using the Marquardt-Levenberg algorithm (SigmaPlot software; Jandel Scientific, San Rafael, CA) to the exponential decay function $A + B\exp(-kt)$, where k is the degradation constant, t is time, and A and B are arbitrary constants. The degradation constants were estimated from the averaged data from two or three plants and are expressed ±SE for the number of data points indicated.

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