Extensive 3′ modification of plant small RNAs is modulated by helper component-proteinase expression

H. Alexander Ebhardt*, Emily P. Thi*, Ming-Bo Wang†, and Peter J. Unrau*‡

*Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6; and †Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, P.O. Box 1600, Canberra ACT 2601, Australia

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RNA silencing is an evolutionarily conserved process in eukaryotes that represses gene expression by using 21- to 24-nt guide RNAs to mediate mRNA cleavage or translational inhibition. Plants have two distinct groups of silencing-associated small RNAs (smRNAs): the micro RNAs (miRNAs) and the small interfering RNAs (siRNAs). A recent report by Yu *et al.* [Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R. & Chen, X. (2005) *Science* 307, 932–935] has shown that plant miRNAs are modified at their 3′ termini with a methyl group. Here, we show that a large fraction of all silencing-associated smRNAs in tobacco are modified; this modification occurs on the 2′ hydroxyl of the terminal ribose and significantly reduces the cloning efficiency of these modified smRNAs. Expression of the strong silencing suppressor P1/helper-component proteinase results in a marked decrease in the 3′-terminal modification of viral siRNAs but does not significantly affect the modification of endogenous miRNAs and 24-nt siRNAs. The differential modification mediated by helper-component proteinase expression implies that exogenous and endogenous smRNAs are processed through independent pathways that are isolated by subcellular compartmentalization and/or the association with distinct Dicer complexes. The degree of terminal modification may play an important role in regulating the extent to which primary smRNA signals can be amplified by RNA-dependent RNA polymerases.

RNA silencing | silencing suppressor | cucumber mosaic virus | Y-satellite

Plants have three overlapping but distinct RNA silencing pathways that involve small interfering RNAs (siRNAs) and micro RNAs (miRNAs) (1). In the siRNA-related pathways, perfectly complementary dsRNAs are cleaved into siRNAs having two distinct size classes, 21–22 and 24 nt, by RNase III-type Dicer enzymes (2). These siRNAs are then incorporated into RNA-induced silencing complexes (RISCs) that guide the specific degradation of RNA in the cytoplasm (and possibly the nucleus) and/or cytosine methylation of DNA in the nucleus (1, 3, 4). miRNAs are predominately 21–22 nt in length and are formed by the Dicer-mediated cleavage of hairpin-structured RNAs (5, 6). Both siRNAs and miRNAs play important roles in the control of spatial and temporal expression of key regulatory genes and can act through RISC-mediated mRNA cleavage (6, 7). The different pathways leading to the biogenesis of siRNAs and miRNAs in plants involve multiple protein factors that include the Dicer-like proteins (7, 8) and RNA-dependent RNA polymerases (9). A recent study has revealed that the 3′-terminal nucleotides of miRNAs in plants are methylated at their 2′ or 3′ hydroxyls by HEN1 (10), a protein previously shown to be required for the accumulation of miRNAs and sense transgene-derived siRNAs in *Arabidopsis* (11). It remains unclear, however, whether siRNAs are also methylated in plants, because HEN1 fails to methylate perfectly complementary duplex siRNAs *in vitro* (10).

RNA silencing, and the siRNA pathway in particular, is a natural antiviral defense mechanism in plants (12). Consequently, many plant viruses encode proteins that can suppress RNA silencing by a variety of mechanisms. These silencing suppressors have become important tools in the elucidation of RNA silencing pathways in plants because they act differentially on the silencing machinery. One of the first silencing suppressors to be studied is the P1/helper component-proteinase (P1/HC-Pro, hereafter referred to as HC-Pro) from potyviruses (13). Unlike several other viral silencing suppressors that inhibit silencing by sequestering small RNAs (smRNAs), HC-Pro does not bind to smRNAs yet strongly inhibits mRNA cleavage directed by both siRNAs and miRNAs in plants (14, 15). HC-Pro differentially affects the accumulation of miRNAs and siRNAs derived from sense, inverted-repeat, or viral amplicon transgenes (14–17). A recent study demonstrates that HC-Pro enhances the accumulation of 21- to 22-nt-long virus-encoded siRNAs during infection with a cucumber mosaic virus (CMV) Y-satellite (Y-Sat) RNA (18).

In the current study, we combine HC-Pro expression with CMV and Y-Sat infection to investigate viral and endogenous smRNA biogenesis in tobacco. Infection of wild-type tobacco by Y-Sat and its helper virus, CMV, results in leaf yellowing (18). These Y-Sat-specific symptoms are suppressed by the expression of HC-Pro despite the presence of increased levels of Y-Sat RNA, suggesting that the yellowing symptoms are due to the silencing of host genes directed by Y-Sat siRNAs (18). Because CMV and Y-Sat infection is associated with the accumulation of high levels of Y-Sat-derived smRNAs that are further enhanced by HC-Pro expression (18), the combination of HC-Pro expression with CMV/Y-Sat infection provides an ideal system through which to study viral smRNAs and RNA silencing under the influence of HC-Pro. Here, we report that a substantial fraction of all viral and endogenous smRNAs are modified at their 3′ termini and that the expression of HC-Pro significantly modulates the extent of this modification for viral but not endogenous smRNAs.

Materials and Methods

smRNA Isolation from Tobacco. The HC-Pro$^+$ and HC-Pro$^-$ tobacco plants described in ref. 18 were prepared as follows. Five weeks after mock inoculation or infection with CMV plus Y-Sat, ≈0.5 g of leaf tissue from HC-Pro$^+$ and HC-Pro$^-$ tobacco was collected and ground into fine powder with a pestle and mortar in the presence of liquid nitrogen and sand. The powder was transferred to a precooled tube and suspended quickly with a mixture of 0.5 ml of extraction buffer (100 mM LiCl/1% SDS/10 mM EDTA/100 mM Tris, pH 9) and 0.5 ml of phenol that was preheated in boiling water. After the tube cooled down, 0.5 ml of chloroform was added, and the mixture was extracted by inverting the tube for 20 min. The tube

Abbreviations: CIP, calf intestinal alkaline phosphatase; CMV, cucumber mosaic virus; miRNA, micro RNA; HC-Pro, P1/helper-component proteinase; PAP, poly(A) polymerase; PNK, polynucleotide kinase; siRNA, small interfering RNA; smRNA, small RNA; Y-Sat, Y-satellite RNA.

‡To whom correspondence should be addressed. E-mail: punrau@sfu.ca.

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was centrifuged, and the supernatant was transferred to a fresh tube and extracted again with 0.5 ml of chloroform. Nucleic acids were precipitated from the final supernatant by using 0.1 vol of 3 M sodium acetate and 3 vol of ethanol, pelleted by centrifugation, air-dried, and dissolved in 0.73 ml of water. To remove large RNA molecules, the nucleic acid solution was mixed with 0.19 ml of 4 M NaCl and 0.58 ml of 13% polyethylene glycol 8000, stored on ice for 30 min, and centrifuged for 20 min at 4°C. The supernatant was transferred to a fresh tube, and the smRNA species were precipitated with 0.1 vol of 3 M sodium acetate and 3 vol of ethanol at −20°C for 1 h, followed by centrifugation at 4°C for 20 min at 12,000 × *g*.

smRNA Labeling and Handling. A small fraction of the isolated RNA was 5′ end-labeled at 37°C for 15 min by using 0.33 μM [γ-^{32}P]ATP (PerkinElmer), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 50 mM Tris·HCl (pH 7.9), and 0.5 units/μl polynucleotide kinase (PNK) (New England Biolabs). smRNAs were eluted from polyacrylamide gel slices overnight in 300 mM NaCl at 4°C. The resulting RNA was precipitated by the addition of 12.5 μg/ml glycogen (Ambion, Austin, TX) and 2.5 vol of ethanol. Samples were placed at −96°C for 60 min before pelleting the labeled RNA at 12,000 × *g* for 30 min.

Calf Intestinal Alkaline Phosphatase (CIP) Treatment of RNA. RNA samples (≈0.1 pmol) were incubated in 100 mM NaCl/10 mM $MgCl_2$/1 mM DTT/50 mM Tris·HCl, pH 7.9 at 25°C/0.16 units/μl CIP (EC 3.1.3.1, New England BioLabs) for 30 min at 37°C. After incubation, the samples were phenol- and chloroform-extracted. RNAs were precipitated with ethanol as described above.

Sodium Periodate Treatment of RNA. Radiolabeled RNA was incubated in 10 mM Hepes, pH 7.0/100 mM sodium periodate at 22°C for 10 min. An equal volume of formamide loading dye (supplemented with 5 mM EDTA) was added, and β-elimination was facilitated by heating at 99°C for 30 min. The reaction was typically run on a 20% denaturing polyacrylamide gel.

Poly(A) Polymerase (PAP) Extension. Radiolabeled RNA were incubated in Ambion's buffer conditions by using 10 mM ATP and 0.5 units/μl PAP (EC 2.7.7.19, Ambion) for 30 min at 37°C.

smRNA Ligation and Cloning. RNA samples isolated from tobacco plants were cloned as described in ref. 19 by using the 3′-adenylated DNA adaptor (5′-AppGAAGAGCCTACGACGA) (20) and the 5′ RNA adaptor (5′-rAUCGUAGGCACCUGAAA). Ligations were performed in 50 mM Hepes, pH 8.3/10 mM $MgCl_2$/3.3 mM DTT/10 μg/ml BSA/8.3% glycerol by using 4 units/μl T4 RNA ligase (GE Healthcare) for 90 min at room temperature unless otherwise specified. The adenylated DNA oligonucleotide was used at 20 μM, and the RNA adaptor was used at 31.5 μM in the presence of ATP.

Analysis of smRNA Sequence. Applied Biosystems sequencing chromatograms were manually reviewed by using CHROMAS (www.technelysium.com.au/chromas.html). All files having unambiguous sequence within the cloning region were subject to a cgi-script, which automatically searches for the 5′ and 3′ primer sequences, excises the cloned inserts, and deposits the annotated sequences into a MySQL database.

Gel-Shift Isolation of smRNAs. A sample lacking large RNA fractions is essential with this protocol. The following DNA oligonucleotides with a terminal biotin (3′-BiotinTEG-CPG, Glen Research, Sterling, VA) and complementary to particular smRNAs were synthesized by using standard DNA phosphoramidite chemistry and were used for the streptavidin gel shift assay ("B" indicates biotinylation): MIR166 probe (antisense sequence is in italics), 5′-*GGGGAATGAAGCCTGGTCCGA*TTB; MIR168 probe, 5′-*GTCCCGACCTGCACCAAGCGA*TTB; Y-Sat probe (nucleotides 193–173 of Y-Sat), 5′-*ATGCAGAGCTGAAAAAGTCAC*TTB; Y-Sat antisense probe (nucleotides 173–193 of Y-Sat), 5′-*GTGACTTTTTCAGCTCTGCAT*TTB; and random control sequence, 5′-ATGAGCGGAGATAGGCTGGTTCTB. The smRNA populations were dephosphorylated and labeled to high specific activity with PNK before being mixed with an excess of the biotinylated DNA (typically 2.5 μM) in 20 mM Tris-borate, pH 8/25 mM NaCl. The mixture was heated to 90°C for 1 min, slowly cooled to room temperature, and then incubated with an excess of streptavidin (Sigma) for 5 min. The streptavidin-shifted RNA:DNA duplexes were then separated away from other smRNA species in a 10% nondenaturing polyacylamide gel, which contained 90 mM Tris-borate (pH 8) and 100 mM NaCl. This method has the following advantages over the conventional Northern blot hybridization approaches: (*i*) The relative amount of a particular smRNA can be directly measured by comparing the signal intensity of the RNA:DNA duplex with that of the total smRNA population. (*ii*) The potential difficulty in detecting short RNA species by Northern hybridization is overcome by performing the hybridization in a solution containing a large excess of biotin-labeled DNA probe. For instance, as little as 1–3 fmol of smRNA can be detected by a conventional Northern blot (21), but the streptavidin gel shift assay outlined here is 10–100 times more sensitive. (*iii*) The smRNA in the shifted RNA:DNA duplex can be recovered for further biochemical analysis.

Results

Labeling of smRNA Populations. smRNAs were extracted from uninfected and CMV/Y-Sat-infected tobacco plants with or without a transgene expressing HC-Pro (referred to as HC-Pro$^+$ or HC-Pro$^-$ plants, respectively). As described in ref. 18, the HC-Pro$^+$ and HC-Pro$^-$ plants were siblings from the same hybrid parent obtained by pollinating Wisconsin 38 tobacco with pollen from a transgenic HC-Pro plant. RNA extracts were first precipitated in the presence of polyethylene glycol 8000 to remove large RNAs.

To determine the phosphorylation status of the smRNA populations within each sample, RNA was labeled by exchanging the terminal 5′ phosphate (assumed to be present on most smRNAs) with PNK and [γ-^{32}P]ATP. When CIP was used before phosphorylation, labeling intensity was increased by ≈2.5-fold, consistent with the higher labeling efficiency that is obtainable by direct phosphorylation of a 5′ hydroxyl by using PNK. If RNA were 3′ phosphorylated, a change in the RNA mobility pattern would be observable after CIP-PNK treatment. Because this change was not observed (see Fig. 4, which is published as supporting information on the PNAS web site), we concluded that 3′ phosphates were not predominant, consistent with the established cleavage mechanism of the RNase III-type Dicer complex (2, 9, 22).

Only the 21- to 22-nt smRNAs from Virally Infected HC-Pro$^+$ Plants Are Significantly Sensitive to Periodate. Fig. 1*A* shows the smRNA distribution of all four plant samples as judged by 5′ radiolabeling of the smRNAs (Fig. 1*A*, lanes 3, 5, 7, and 9) and periodate treatment (Fig. 1*A*, lanes 4, 6, 8, and 10). All samples contained significant and apparently similar levels of 24-nt-long smRNAs. Uninfected plants contained predominantly 24-nt-long smRNA with low but detectable levels of 21- to 22-nt-long RNA (Fig. 1*A*, lanes 3 and 5). Infected plants showed an increased level of 21- to 22-nt smRNA that was further enhanced by HC-Pro expression (Fig. 1*A*, lanes 7 and 9). Interestingly, two shorter RNA products ≈15 nt in size were present in uninfected RNA samples.

Periodate was used to examine the availability of the 2′ and 3′ hydroxyls found on the 3′-terminal ribose of the four smRNA populations. If both 2′ and 3′ hydroxyls are unmodified, periodate oxidizes them to form an unstable dialdehyde that leads to the eventual β-elimination of the terminal nucleoside and an ≈2-nt

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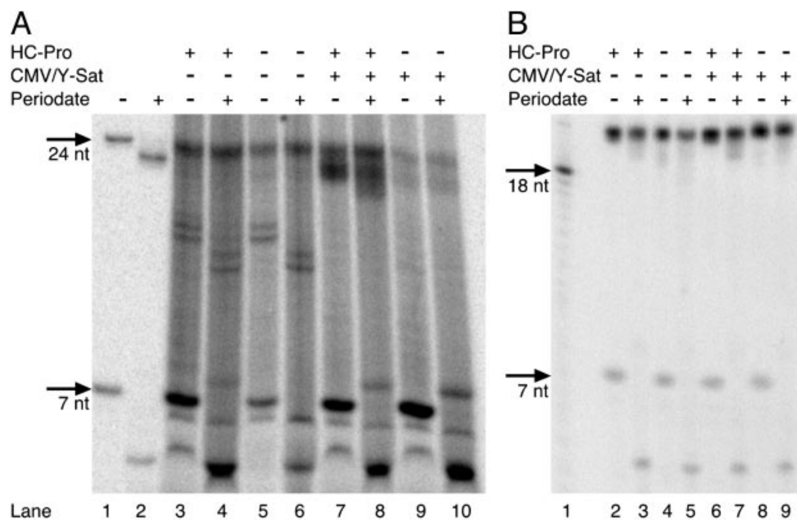

Fig. 1. General resistance of smRNAs to periodate treatment. (*A*) smRNAs were radiolabeled with PNK (lanes 3, 5, 7, and 9) and then subjected to periodate treatment (lanes 4, 6, 8, and 10). Only RNA from virally infected HC-Pro$^+$ plants showed significant sensitivity to periodate (lane 8). A synthetic 7-nt internal control RNA was added to all RNA fractions before chemical treatment to confirm the efficient periodate-mediated elimination of the terminal nucleoside from unmodified RNA. A 24-nt RNA marker reacts to completion when exposed to periodate, shifting downward by ≈2 nt in mobility (lanes 1 and 2). Note that the ≈15-nt double bands in the two uninfected samples are also fully sensitive to periodate (lanes 3–6). (*B*) The 24-nt-long RNAs from both the infected and uninfected plant samples were gel-purified and subjected to periodate treatment separately. Quantification of cleavage products indicates that cleavage in uninfected plants is weakly dependent on HC-Pro expression (7% and 5% in lanes 3 and 5, respectively, after background subtraction using the − periodate lanes). In infected plants, HC-Pro effects a 2-fold increase in periodate cleavage (12% and 6% in lanes 7 and 9, respectively).

downward mobility shift (10). The 21- to 24-nt smRNAs in all four plant RNA samples showed high levels of resistance to periodate (Fig. 1*A*, lanes 4, 6, and 10). Only 21- to 22-nt smRNAs from virally infected HC-Pro$^+$ plants were found to have an obvious sensitivity to periodate (Fig. 1*A*, lane 8).

Because the uncleaved 21- to 22-nt RNA species could mask the periodate cleavage products of the 24-nt RNA population, the 24-nt RNAs from virally infected and uninfected samples were purified from a denaturing gel and subjected to periodate treatment in the absence of the 21- to 22-nt smRNAs. As shown in Fig. 1*B*, only a small proportion (5–12%) of the 24-nt smRNAs was susceptible to periodate-induced cleavage, regardless of their origin (lanes 3, 5, 7 and 9). The expression of HC-Pro slightly increased the proportion of periodate-sensitive 24-nt smRNAs in infected and uninfected plants (lane 7 vs. lane 9 and lane 3 vs. lane 5), but the major fraction remained resistant to periodate (internal controls consisting of a radiolabeled 7-nt synthetic RNA confirmed that periodate treatment and subsequent β-elimination were nearly quantitative for all treatments). Taken together, these results indicated that HC-Pro expression specifically increased the periodate sensitivity of the 21- to 22-nt smRNA population in the virally infected HC-Pro$^+$ plants (Fig. 1*A*, lane 8), whereas the 24-nt smRNAs were only weakly affected.

Periodate-Resistant smRNAs Are Substrates for T4 RNA Ligase but Not PAP. Recently, Yu *et al.* (10) showed that plant miRNAs in *Arabidopsis* are modified with a methyl group that is added to either the 2′ or 3′ hydroxyl group of the terminal ribose by an enzyme called HEN1. Although our data do not directly prove that a methyl group is present on the tobacco smRNAs, they are entirely consistent with the observation of Yu *et al.* (10) (Fig. 1). RNA blocked at a terminal 3′ hydroxyl are not substrates for enzymes such as T4 RNA ligase (23–25) or PAP (26) that require a free terminal 3′ hydroxyl group. However, it is possible that these enzymes can use 2′-modified RNA as substrates. We therefore tested the substrate specificity of the modified plant smRNAs for these two enzymes to identify the modified hydroxyl.

T4 RNA ligase was capable of ligating an adenylated DNA oligonucleotide to a synthetic 7-nt terminal 2′-*O*-methyl (*O*-Me)-modified RNA (see lanes 5–7 in Fig. 5, which is published as supporting information on the PNAS web site). The modification reduced the ligation rate by 2- to 3-fold relative to that with an unmodified 7-nt RNA of identical sequence (39% ligation yield for modified RNA and 86% ligation yield for unmodified RNA after 1 h of reaction; Fig. 5, lanes 8–10). Therefore, plant smRNAs modified by a 2′-*O*-Me group should be competent, but not optimal, substrates for T4 RNA ligase. In contrast, PAP failed to add a poly(A) tail to the same synthetic 2′-*O*-Me RNA (Fig. 5, lanes 1–4), precluding the use of this enzyme in mapping the location of the modified terminal hydroxyl in plant smRNAs. Consistent with this finding, 24-nt plant smRNAs were resistant to extension by PAP (data not shown).

Radiolabeled 21- to 24-nt smRNAs from virus-infected HC-Pro$^+$ or HC-Pro$^-$ plants, together with the four gel-purified samples of 24-nt smRNAs (the same as used in Fig. 1*B*), were tested for their ability to serve as substrates for T4 RNA ligase. As shown in Fig. 2*A*, the smRNAs from the infected HC-Pro$^+$ and HC-Pro$^-$ plants formed two slower-moving RNA–DNA ligation products corresponding to the initial 21- to 22-nt and 24-nt smRNA populations (lanes 5–12). For the viral-infected HC-Pro$^+$ samples, the ligation products corresponding to the 21- to 22-nt smRNAs were significantly enriched relative to that of the 24-nt smRNA population (Fig. 2 *A*, lanes 6–8, and *B Left*). In contrast, no significant enrichment was observed in the virus-infected HC-Pro$^-$ samples for either of the two RNA–DNA ligation populations (Fig. 2*A*, lanes 10–12, and *B Right*). The ligation bias for the 21- to 22-nt smRNAs in the virus-infected HC-Pro$^+$ plants was well correlated with their periodate sensitivity, consistent with the unmodified smRNAs being better substrates for T4 RNA ligase.

The ligation of the four gel-purified 24-nt smRNA samples yielded slower-moving and faster-moving ligation products (Fig. 2*A*, lanes 13–28), which together represent 60–70% of the total input material. This level of ligation greatly exceeded the amount of periodate-sensitive material found in each sample (5–12%; Fig. 1*B*), indicating that at least 50–65% of the smRNAs in each of the samples must therefore consist of modified ligation-competent smRNA. This result suggests that the terminal nucleotide modification occurs at the 2′ hydroxyl group of the modified smRNAs, although 3′ hydroxyl modification cannot be ruled out for a small fraction of the modified smRNAs.

Interestingly, the expected RNA–DNA ligation product (the slower-moving species) only accounted for 20–25% of the total ligation products, with the faster-moving species being the dominant product (Fig. 2*A*, lanes 14–28). This product was resistant to CIP treatment (data not shown), indicating that it was likely to be circularized RNA. Faster-migrating bands of even smaller sizes were also seen in lanes 6–8 and lanes 10–12, suggesting that circularization also occurred in 21- to 22-nt smRNAs. Significantly, the amount of circularized product was negligible for an unmodified control RNA (lanes 2–4, faint bottom bands), suggesting that the presence of the terminal modification biases ligation toward circularization of smRNAs, presumably by altering the relative efficiency

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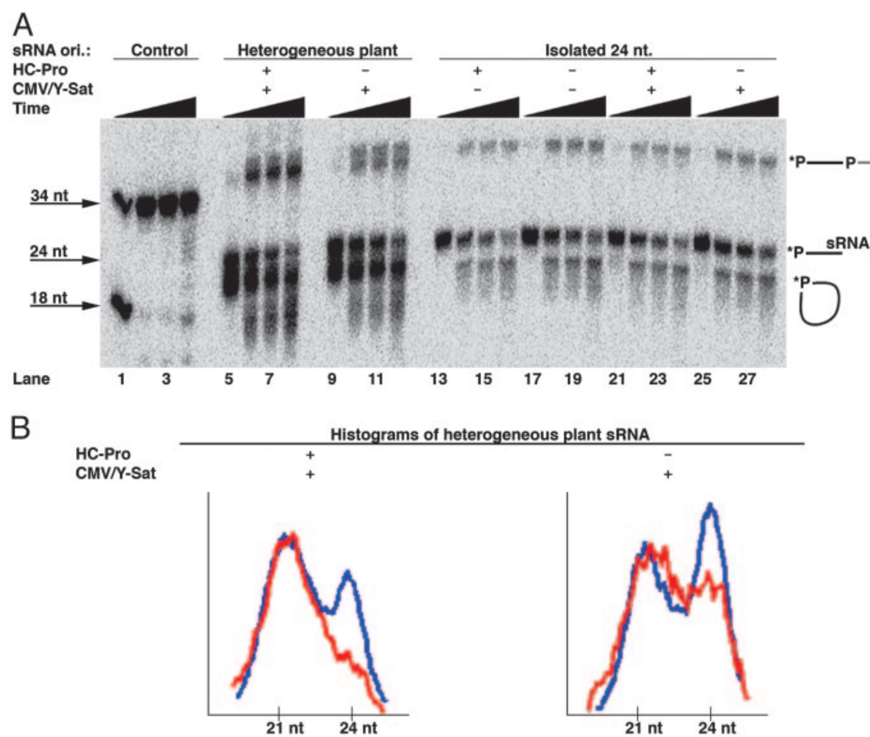

Fig. 2. RNA–DNA ligation with T4 RNA ligase. (*A*) The 21- to 24-nt smRNAs from infected HC-Pro$^+$ or HC-Pro$^-$ plants (lanes 5–12) or the 24-nt smRNAs from both infected and uninfected HC-Pro$^+$ and HC-Pro$^-$ plants (lanes 13–28) were gel-purified and ligated to a 16-nt adenylated DNA oligonucleotide. A synthetic 18-nt RNA control ligation is also shown (lanes 1–4). The ligation was analyzed at four time points for all of the samples: 1 min, 1 h, 2 h, and overnight. The slower-migrating species correspond to ligated RNA–DNA ligation products; the faster-migrating bands that emerge during ligation correspond to circularized RNA (only these bands remain labeled upon treatment with CIP, indicating the circular nature of the RNA). The RNA–DNA ligation product from infected HC-Pro$^+$ plants that corresponds to the 21- to 22-nt RNAs is significantly enriched (lanes 5–8) relative to the 24-nt RNAs. This effect does not occur in the absence of HC-Pro expression (lanes 9–12). The 24-nt RNAs from either infected or uninfected plants (HC-Pro$^+$ or HC-Pro$^-$) preferentially form self-ligated circular species, with a 3:1 ratio between this circular species and the DNA–RNA ligation products. In contrast, the ligation of the 18-nt control RNA (having the terminal sequence of . . . AAG-3′) is much faster and only gives a negligible amount of circular product (lanes 1–4). (*B*) Histograms showing the smRNA distribution before ligation (blue) or in the RNA–DNA ligated population (red) from viral-infected HC-Pro$^+$ plants (*Left*) or HC-Pro$^-$ plants (*Right*).

of trans and cis ligation. Thus, this result indicated that unmodified smRNAs preferentially form RNA–DNA products, whereas modified smRNAs preferentially form self-ligated species. It is unclear how self-ligation could occur in the absence of ATP, but ligation of any form was completely inhibited when the adenylated DNA oligonucleotide was omitted from the ligation reaction, suggesting that the enzyme was substantially deadenylated. This result indicates that adenylated DNA oligonucleotides can play a role in the adenylation of T4 RNA ligase enzyme (27).

Unmodified smRNAs in Infected Plants Resulting from HC-Pro Expression Are Predominantly of Viral Origin. The finding that modified plant smRNAs tend to form circles in the presence of an adenylated DNA oligonucleotide makes it possible that traditional cloning techniques [involving the ligation of smRNAs with an adenylated 3′ DNA adaptor (19)] could result in the underrepresentation of modified smRNAs in the cloned population. It is worth noting that only ≈15% of the modified 24-nt plant smRNAs shown in Fig. 2*A* were capable of forming a RNA–DNA ligation product, whereas synthetic unmodified RNAs typically give ligation yields of 50–100% after 90 min of reaction. A 3- to 6-fold enrichment of unmodified smRNA relative to modified smRNAs could therefore occur in the cloned population with current smRNA cloning techniques.

smRNAs that were 18–26 nt in size from all four plant samples were gel-purified and cloned with the conventional adaptor ligation/RT-PCR method (19), resulting in a total of 679 clones. The smRNA clones were classified into four groups: Y-Sat-derived, CMV-derived, endogenous (including miRNAs), and those that were likely to have resulted from degradation of tRNA and rRNA (Fig. 3).

The length distribution of the cloned smRNAs from the virus-infected HC-Pro$^+$ plant (Fig. 3 *Lower Right*) conformed very well with the results of periodate treatment (Fig. 1*A*, lane 8) and adenylated oligonucleotide ligation (Fig. 2*B Left*): only 21- to 22-nt RNAs were cloned in substantial numbers. This finding suggests that unmodified smRNAs are indeed significantly enriched, presumably during the 3′ adaptor ligation step of smRNA cloning. The majority of these 21- to 22-nt clones were derived from the Y-Sat and CMV genomes, arguing that the unmodified 21- to 22-nt smRNAs detected by periodate treatment were predominantly of viral origin.

Compared with the smRNA clones from the virus-infected HC-Pro$^+$ plant, the distribution of the 21- to 22-nt and 24-nt clones from the infected HC-Pro$^-$ plant (Fig. 3 *Lower Left*) was similar to that observed by 5′ end-labeling (Fig. 1*A*, lane 9) and was consistent with the similarly high levels of periodate resistance between the 21- to 22-nt and 24-nt smRNA populations in the absence of HC-Pro expression (Fig. 1*A*, lane 10). The distribution also coincided with the lack of strong ligation bias between the two size classes of smRNA (Fig. 2*B Right*).

The smRNA clones from uninfected HC-Pro$^+$ plants (Fig. 3 *Upper Right*) were notable in that the number of endogenous 24-nt smRNA clones was ≈2-fold lower than that of the 21- to 22-nt clones, in sharp contrast to the at least 4- to 5-fold excess of the 24-nt smRNAs present in the total smRNA population (Fig. 1*A*, lanes 3 and 4). In addition to this remarkable imbalance, expression of HC-Pro resulted in a notable decrease in the proportion of tRNA or rRNA-derived smRNA clones relative to that of endogenous or viral smRNA clones. This effect of HC-Pro was most prominent in uninfected plants (Fig. 3 *Upper*) and also occurred in infected plants (Fig. 3 *Lower*). Assuming that the production of tRNA or rRNA-derived fragments is independent of HC-Pro expression, as appears reasonable from an assessment of the background RNA abundances (e.g., the 18- to 19-nt region in Fig. 1*A*), these two results suggest that the expression of HC-Pro in uninfected plants increases the amount of unmodified 21- to 22-nt-long endogenous smRNA relative to the unmodified 24-nt smRNA population. However, the total amount of unmodified 21- to 22-nt smRNAs induced by HC-Pro expression is likely to be small; these smRNAs were not readily detectable by radiolabeling and periodate treatment of the total smRNA population (Fig. 1*A*).

HC-Pro Expression Strongly Affects Viral smRNA Modification but only Mildly Influences Endogenous miRNA Modification. Although our cloning data suggested that most of the unmodified 21- to 22-nt

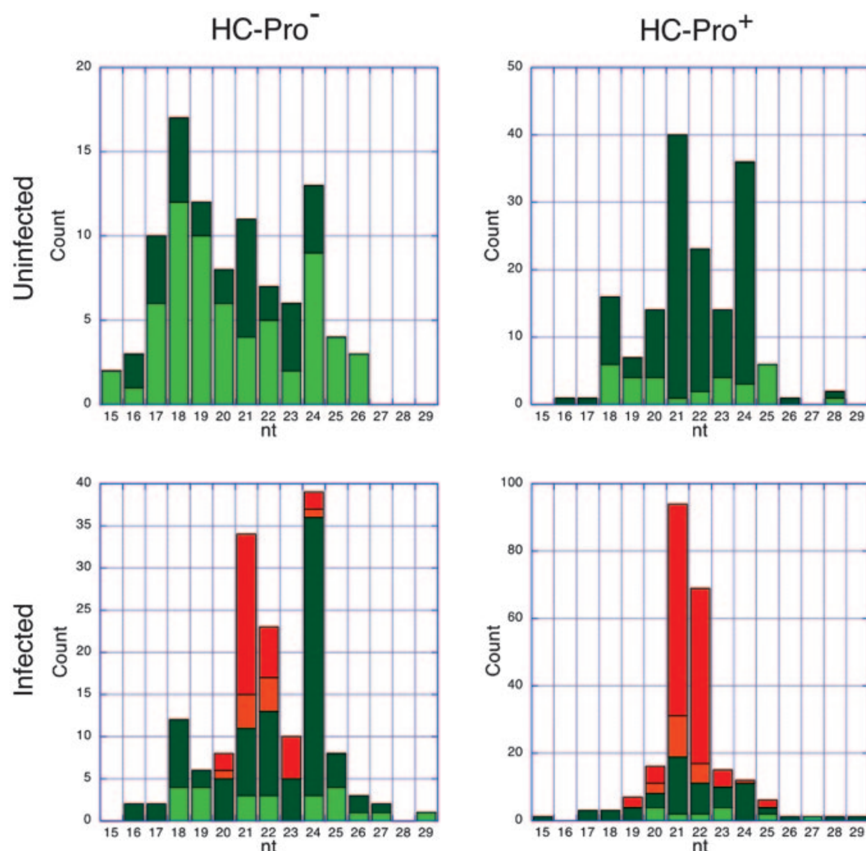

Fig. 3. Source and size distribution of cloned smRNA populations. Data are shown for uninfected (*Upper*) and infected (*Lower*) plants lacking (*Left*) or expressing (*Right*) HC-Pro. The green bars indicate smRNA clones derived either from tRNA or rRNA (light green bars) or from other endogenous sequences (dark green bars). The red and orange bars indicate Y-Sat- and CMV-derived smRNA clones, respectively. All smRNAs that were successfully cloned and sequenced are included in these histograms. smRNA clones of 21–22 nt are dominant in virally infected HC-Pro-expressing plants (*Lower Right*), consistent with the marked periodate sensitivity (Fig. 1*A*, lane 8) and ligation efficiency (Fig. 2*B Left*) exhibited by these RNAs. Note also the large excess of 21- to 22-nt smRNA seen in uninfected HC-Pro$^+$ plants (*Upper Right*) relative to that observed in Fig. 1.

smRNA in infected HC-Pro$^+$ plants was of viral origin, it was unclear whether the endogenous miRNAs, which overlap with the viral smRNAs (21–22 nt) in size, were also affected by HC-Pro expression. We therefore examined the 3′ modification of two known endogenous miRNAs together with specific Y-Sat-derived smRNAs using periodate treatment. To purify the specific miRNAs and Y-Sat smRNAs, we developed a gel-shift method involving hybridization of smRNAs with biotinylated complementary oligonucleotides (Fig. 6*A*, which is published as supporting information on the PNAS web site). This purification method also allows the determination of the relative concentration of a particular smRNA in the total smRNA population. Using this method, we purified Y-Sat smRNA species and two miRNAs (MIR166 and MIR168; Fig. 6*A*). These purified smRNAs were subjected to periodate treatment to determine the extent of their terminal modification.

As shown in Fig. 6 *B* and *C*, the purified Y-Sat smRNAs from the HC-Pro$^+$ plant had high sensitivity to periodate ($\approx$50% cleavage; Fig. 6 *B*, lanes 3 and 4, and *C*, lanes 7 and 8), but the same Y-Sat smRNAs from the HC-Pro$^-$ plant were completely resistant (Fig. 6 *B*, lanes 5 and 6, and *C*, lanes 9 and 10). These data are consistent with our previous periodate treatment, RNA ligation, and cloning results, which suggest that viral smRNAs are responsible for the observed periodate sensitivity of the total 21- to 22-nt smRNA population in the HC-Pro$^+$ plant. This HC-Pro-induced change in viral smRNA modification was not observed with the 24-nt smRNAs, whose 3′ modification appeared to be only slightly affected by HC-Pro (Fig. 1*B*).

Similar to the endogenous 24-nt smRNAs, the two most frequently cloned endogenous miRNAs, MIR168 and MIR166, showed high levels of resistance to periodate. This resistance was only weakly affected by HC-Pro expression (Fig. 6 *B*, lanes 7–14, and *C*, lanes 3–6). It is unclear why two bands appeared in the purified MIR168 samples, but this observation has been made previously with other miRNA samples (15, 28, 29). The small increase in periodate sensitivity observed with HC-Pro expression is consistent with our cloning results that showed increased representation of endogenous 21- to 22-nt smRNA clones in the uninfected HC-Pro-expressing plants relative to the tRNA and rRNA backgrounds.

Discussion

Using a range of different approaches, we have shown that a substantial fraction (typically in excess of 90%) of all 21- to 24-nt-long RNAs in normal or virus-infected tobacco plants are modified at their 3′ terminus. The ability of these smRNAs to serve as substrates for T4 RNA ligase suggests that a substantial fraction of this 3′-terminal modification occurs at the 2′-O-hydroxyl group. Based on the recent finding of Yu *et al.* (10), it is reasonable to assume that in most of the tobacco smRNAs, this modification is a terminal 2′-*O*-methyl group.

Our results show that modified smRNAs could not be extended by PAP. This finding suggests a potential biological significance of the 3′ modification in plants: It might have evolved to prevent the smRNAs from serving as primers for RNA-dependent RNA polymerases (9), preventing the primer-dependent synthesis of secondary dsRNA and thereby restricting the function of the

smRNAs to their primary targets. In support of this, viral satellite siRNA-directed DNA methylation and RNA cleavage are highly restricted to the viral satellite sequence in a fusion transgene and its transcript (30). Also, siRNA-directed heterochromatin silencing in *Arabidopsis* is restricted to transposons and repetitive sequences from which the siRNAs originate and does not spread to the neighboring gene-rich areas (31).

The differential regulation of viral and endogenous smRNA modification by HC-Pro suggests that these different smRNAs are generated in different biogenesis/methylation pathways. Consistent with this, recent studies have indicated that plant miRNAs and most endogenous 24-nt siRNAs are processed in the nucleus by Dicer-like (DCL) 1 and DCL3, respectively (7, 8). However, viral siRNAs appear to be processed by different Dicer proteins (e.g., DCL2) (7), which may function mainly in the cytoplasm, where most plant viral RNA replicates. HC-Pro is known to be localized in the cytoplasm (32) and may therefore preferentially interfere with the methylation of the cytoplasmically processed viral smRNAs.

The effect of HC-Pro expression on viral smRNA modification is unlikely to be directly associated with its silencing suppressor activity, because it does not significantly affect the 3′ modification of endogenous smRNAs. Nevertheless, the significant effect of HC-Pro on viral smRNA modification does suggest a direct interaction of this silencing suppressor with the methylation factor HEN1, presumably in the cytoplasm. It is possible that HEN1 is an integral component of a cytoplasmic silencing complex and that its interaction with HC-Pro disrupts the function of the complex, thereby resulting in suppression of silencing.

The specific effect of HC-Pro (and perhaps some other silencing suppressors) on viral smRNA modification may serve as a self-defense strategy for certain groups of viruses. Unmodified viral smRNAs, induced by the silencing suppressor, may act as primers for RNA-dependent RNA polymerases to produce secondary viral dsRNA and hence siRNAs. This possibility is consistent with our results that show that increased levels of Y-Sat smRNAs occur in $HC\text{-}Pro^{+}$ plants. The amplification of viral smRNAs by RNA-dependent RNA polymerases could make the infected cells resistant to repeated viral infections, which would minimize damage to the host and thereby increase the viability of the virus itself (33). Similarly, by not preventing the modification of endogenous smRNA, HC-Pro would ensure that no permanent damage occurs to endogenous smRNA function, thereby minimizing the effect of viral infection on host development.

As demonstrated explicitly with purified 24-nt smRNAs, smRNAs with terminal modifications can be significantly underrepresented in the cloned populations (Fig. 3). It is therefore possible that some rare modified smRNAs have been overlooked in plants. A modified cloning procedure where a particular size range of smRNAs is first periodate-treated and then repurified to select only those smRNAs that are periodate-resistant would allow the specific enrichment of modified smRNAs and would be useful in identifying rare smRNAs in plants.

In conclusion, our study has revealed that 3′ modification is a universal feature for all silencing-associated smRNAs in plants. The extent of this modification is modulated by HC-Pro, an important viral suppressor of RNA silencing. Further studies on possible spatial, temporal, and environmental regulation of this 3′ modification are likely to provide insight into the biological role of this unanticipated aspect of RNA silencing.

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