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Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome

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Viral infection in both plant and invertebrate hosts requires a virus-encoded function to block the RNA silencing antiviral defense. Here, we report the identification and characterization of three distinct suppressors of RNA silencing encoded by the ≈20-kb plus-strand RNA genome of Citrus tristeza virus (CTV). When introduced by genetic crosses into plants carrying a silencing transgene, both p20 and p23, but not coat protein (CP), restored expression of the transgene. Although none of the CTV proteins prevented DNA methylation of the transgene, export of the silencing signal (capable of mediating intercellular silencing spread) was detected only from the F_1 plants expressing p23 and not from the CP- or p20-expressing F_1 plants, demonstrating suppression of intercellular silencing by CP and p20 but not by p23. Thus, intracellular and intercellular silencing are each targeted by a CTV protein, whereas the third, p20, inhibits silencing at both levels. Notably, CP suppresses intercellular silencing without interfering with intracellular silencing. The novel property of CP suggests a mechanism distinct to p20 and all of the other viral suppressors known to interfere with intercellular silencing and that this class of viral suppressors may not be consistently identified by *Agrobacterium* coinfiltration because it also induces RNA silencing against the infiltrated suppressor transgene. Our analyses reveal a sophisticated viral counter-defense strategy that targets the silencing antiviral pathway at multiple steps and may be essential for protecting CTV with such a large RNA genome from antiviral silencing in the perennial tree host.

The discoveries of viral suppressors of RNA silencing play an important role in establishing RNA silencing as a natural antiviral response in both plant and invertebrate hosts (1). Many plant viral proteins have been identified as suppressors of RNA silencing since the initial reports in late 1998 (2–4). RNA silencing suppressors from different virus taxons are structurally diverse. However, they are typically required for long-distance virus spread and influence virulence and accumulation levels in infected plants (5–7). Progress is being made toward understanding the molecular mechanisms involved in viral suppression of RNA silencing (8–11). In RNA silencing assays, plant viral suppressors differ by their ability to suppress intracellular and/or intercellular silencing (12–16). Intercellular silencing is mediated by the non-cell-autonomous silencing signal that can spread to destroy homologous RNAs in neighboring or distant tissues that do not contain the initial trigger, such as a silencing transgene or a replicating virus (17–19). The differential silencing suppression is best illustrated by the potyviral helper component-proteinase (HC-Pro) and the Cucumber mosaic virus (CMV) 2b protein. When introduced by genetic crosses into the *Nicotiana tabacum* line 6b5 carrying a silencing β-glucuronidase (GUS) transgene, intracellular silencing was suppressed by either viral protein, whereas inhibition of intercellular silencing was detected in the progeny plants expressing CMV 2b but not in the progeny plants expressing HC-Pro (20, 21). It is not clear whether viral suppression of intracellular and intercellular silencing plays specific roles in facilitating the cell-to-cell and long-distance virus spread. However, it is of interest to note that virus synergy observed in mixed virus infections, such as potyvirus synergism with PVX and CMV, often involves two unrelated viruses that encode distinct suppressors targeting intracellular and intercellular silencing, respectively (22).

In this study, we investigated the strategy of Citrus tristeza virus (CTV) in counter-defense against the RNA silencing antiviral defense. As the most destructive virus of the citrus industry worldwide, CTV represents the first pathogen of a perennial tree to be examined for the activity in silencing suppression. CTV, a member of the genus *Closterovirus* of the Closteroviridae, has a plus-strand RNA genome of ≈20 kb (23), about twice as large as the genome size of most plant viruses from which a silencing suppressor has been identified. The 5′ half of the genome encodes the viral replicase of 400 kDa that is translated from the genomic RNA with ribosomal frameshifting. The 3′ half of the genome encodes 10 genes that are not required for replication in protoplasts and are each expressed through a nested set of 3′-coterminal subgenomic mRNAs (24, 25). This large genome size, as well as the genome organization and expression strategy, is very similar to that of the members of the animal Nidovirales, of which the human severe acute respiratory syndrome Coronavirus is a member (24, 26). Our results show that the large CTV genome encodes at least three suppressors of RNA silencing for protection against the host RNA silencing defense. Importantly, p23 and coat protein (CP) target the intracellular and intercellular silencing, respectively, whereas the third, p20, inhibits silencing at both levels. We suggest that the simultaneous suppression of intracellular and intercellular silencing antiviral defense by CTV proteins may explain, in part, why CTV causes the most destructive viral disease in citrus worldwide (27).

Materials and Methods

***Agrobacterium* Coinfiltration Assay.** The binary plasmid 35S-GFP that directs the *in planta* expression of GFP is described in ref. 3. The CTV ORFs were cloned between the Cauliflower mosaic virus 35S promoter and terminator in the binary plasmid pCAMBIA1300 (GenBank accession no. AF234296) to generate constructs for both plant transformation and leaf infiltration. These plasmids were then transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation and selected in Luria–Bertani medium containing kanamycin at 50 μg/ml and rifampicin at 10 μg/ml. The leaf infiltration of *A. tumefaciens* strains was as described in ref. 3. For the coinfiltration experiments, equal volumes of an *Agrobacterium* culture containing 35S-GFP and an

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Abbreviations: CMV, Cucumber mosaic virus; CTV, Citrus tristeza virus; GUS, β-glucuronidase; HC-Pro, helper component-proteinase; siRNA, small interfering RNA.

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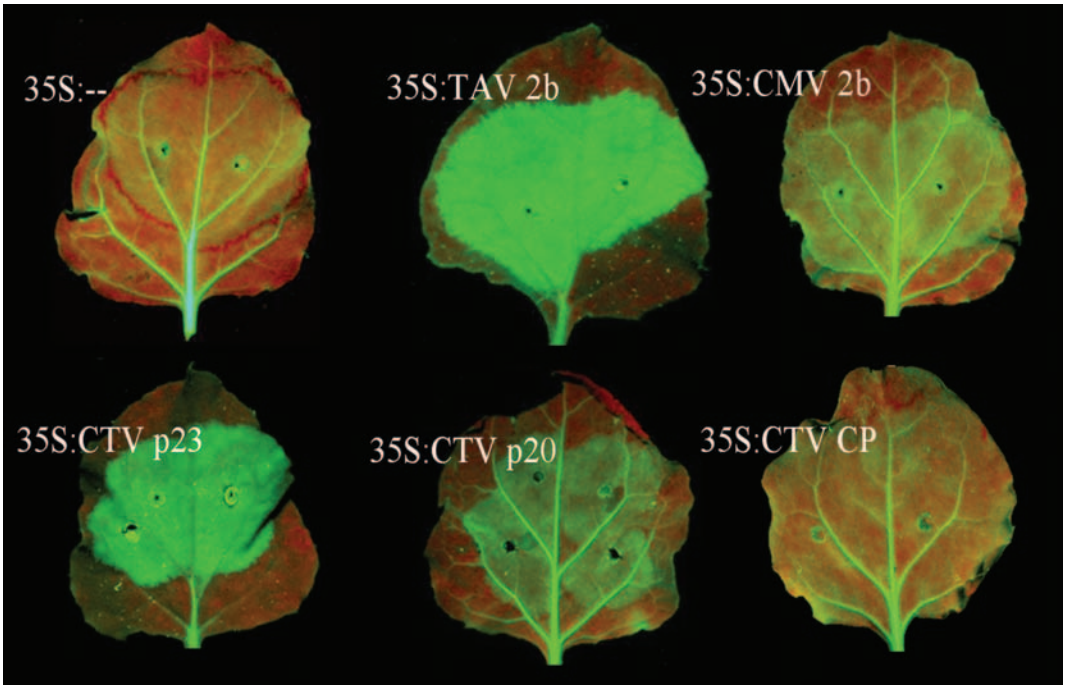

Fig. 1. Identification of p20 and p23 as suppressors of RNA silencing by the *Agrobacterium* coinfiltration assay. Leaves of the 16c GFP plants were infiltrated with an *A. tumefaciens* strain carrying 35S-GFP together with an *A. tumefaciens* strain carrying the empty binary plasmid (35S:--), 35S:TAV 2b, 35S:CMV 2b, 35S:CTV p23, 35S:CTV p20, or 35S:CTV CP. The green fluorescence images of the coinfiltrated leaves with the abaxial-side up were taken 3 days postinfiltration under a long-wave UV lamp.

Agrobacterium culture containing the CTV constructs were mixed before infiltration. The final concentration for each *Agrobacterium* culture is 0.4 at OD_{600}. The GFP fluorescence in whole plants was visualized by using a 100-W, hand-held, long-wave UV lamp (Blak-Ray B-100AP, Ultraviolet Products, Upland, CA). Plants were photographed with a Canon G2 digital camera, using preset white balance under UV light.

Plant Material and Grafting Procedure. Transgenic *Nicotiana benthamiana* line 16c expressing GFP (28), *N. tabacum* line T19 expressing GUS (29), and line 6b5 containing a silenced GUS transgene (30) were described previously. Pollen grains from line 6b5 were used in crosses with the *N. tabacum* lines p23, p20, or CP to generate progenies P23 × 6b5, P20 × 6b5, and CP × 6b5, respectively. A wedge-grafting method (17, 21) was used to generate single-grafted tobacco plants. Tobacco plants used for grafting experiments were 8 weeks old. Leaf samples for RNA extraction were taken both from the individually labeled stock plants immediately before grafting and from the scions 6 weeks after grafting.

DNA and RNA Analysis. The analysis of DNA methylation at the *Mlu*I site in the GUS coding sequence was performed as described in refs. 20 and 21, using ^{32}P-labeled DNA probes corresponding to the full coding sequence of GUS. Total plant RNA extraction and RNA gel-blotting analysis were performed as described in ref. 31. RNA gel-blot analysis of high- and low-molecular-weight RNA was on 15 μg of total RNA, unless otherwise stated. Hybridization probes were labeled with ^{32}P by using the Megaprime DNA labeling kit (Amersham Pharmacia). Ethidium bromide staining of total RNA before transfer was used to confirm equal loading except when the blots were rehybridized by using *N. tabacum Nectin* probe. Densitometric analysis of at least two independent RNA gel blots exposed to x-ray film was used to assess relative RNA levels. Small interfering RNA (siRNA) detection was as described in ref. 21. All experiments on the DNA and RNA analyses were repeated at least twice.

Results

Screening for RNA Silencing Suppressors Encoded by CTV. The initial screen to identify CTV suppressors of RNA silencing was carried out by using the *Agrobacterium* coinfiltration assay, essentially as described in refs. 19 and 21. To assay for silencing suppression, coding sequences for p33, p6, p61, p27, CP, p18, p13, p20, and p23 of CTV were cloned in a binary vector, and the resulting plasmids were transformed into *A. tumefaciens*. The *A. tumefaciens* strain carrying the 35S-GFP binary plasmid and an *A. tumefaciens* strain carrying one of the CTV constructs were mixed before infiltration into *N. benthamiana* plants expressing GFP (line 16c). As expected, silencing of the GFP transgene was induced by agro-infiltration with 35S-GFP, leading to reduction of both green fluorescence (Fig. 1 *Upper Left*) and GFP mRNA accumulation in the infiltrated leaves as well as detection of GFP-specific siRNAs (Fig. 2 *Top* and *Middle*, compare lanes 6 and 14). Also as expected (21, 32), both cucumoviral 2b proteins suppressed GFP silencing, and the Tomato aspermy virus (TAV) 2b was more effective in silencing suppression than CMV 2b (Fig. 1 *Upper Center* and *Upper Left*, and Fig. 2, lanes 5, 7, 13, and 15).

Both fluorescence and RNA analyses on GFP expression in the infiltrated leaves identified p20 and p23 as suppressors of RNA silencing among the nine CTV proteins examined (Figs. 1 and 2) (data not shown). Expression of p23 resulted in the detection of intense green fluorescence (Fig. 1 *Lower Left*) and a significant increase in the accumulation of GFP mRNA (Fig. 2 *Top*, lane 4) in the infiltrated leaves. By comparison, silencing suppression by p20 was weaker (Fig. 1 *Lower Center*, and Fig. 2, lane 3) as measured by both the intensity of green fluorescence and the accumulation levels of GFP mRNA and siRNAs in the infiltrated leaves. However, suppression of GFP RNA silencing by either CTV protein in the infiltrated leaves was transient in

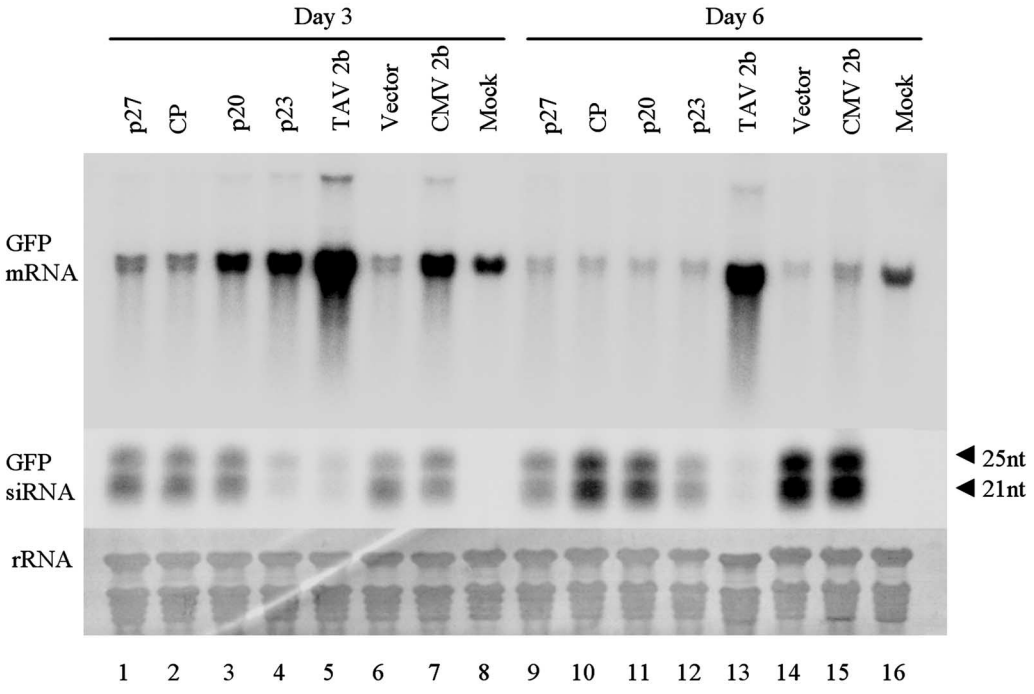

Fig. 2. The accumulation of GFP mRNA and siRNAs in the infiltrated leaves of 16c plants. RNA was extracted from leaves of the 16c GFP plants 3 and 6 days postinfiltration with an *A. tumefaciens* strain carrying 35S-GFP together with an *A. tumefaciens* strain carrying the empty binary plasmid (vector), 35S:CTVp27, 35S:CTVCP, 35S:CTVp20, 35S:CTVp23, 35S:TAV2b, or 35S:CMV2b. RNA samples were also obtained from noninfiltrated leaves of similar developmental stages and used as controls to show the levels of GFP mRNA from the stably integrated copy of the GFP transgene. Arrows indicate the positions of synthetic siRNAs of 21 and 25 nt.

this assay and became almost undetectable by 6 days postinfiltration (Fig. 2, lanes 11 and 12). In contrast, expression of any of the remaining seven CTV proteins, including CP (Fig. 1 *Lower Right*, and Fig. 2, lane 2) and p27 (Fig. 2, lane 1), had no detectable effect on GFP RNA silencing in the infiltrated leaves either at 3 or 6 days postinfiltration (data not shown). These results indicate for the first time that a viral genome may encode more than one silencing suppressor.

Suppression of Intracellular Silencing by p20 and p23 but Not by CP of CTV. We next analyzed the silencing suppression activity of the CTV proteins in an independent silencing system based on the GUS transgene in the 6b5 tobacco line (30). RNA silencing of the GFP and GUS transgenes shares many features such as cytosine methylation of the transgene DNA in the transcribed region and production of siRNAs and graft-transmissible silencing signal; however, silencing of the GUS transgene in the 6b5 *N. tabacum* plants occurs autonomously in each generation in contrast to transgene silencing in the 16c *N. benthamiana* plants that requires induction by *Agrobacterium* infiltration. To assay for silencing suppression in the 6b5 system (20, 21), *N. tabacum* lines that expressed a candidate viral suppressor from a stably integrated transgene were first generated. After the silencing GUS transgene in line 6b5 was introduced by genetic crosses, the potential effect of the candidate suppressor on the intracellular and intercellular silencing of the GUS transgene was analyzed in the F_1 progeny plants. Thus, expression of the candidate protein was constitutive and persistent, unlike the *Agrobacterium* coinfiltration assay in which ectopic expression of the candidate protein ceases after 2–3 days unless it could suppress intracellular silencing (33). In addition to p20 and p23, we also created lines expressing the CTV CP because we occasionally observed a partial suppression of systemic silencing in 16c plants coinfiltrated with the CP construct (data not shown) despite the fact that CP did not suppress silencing in the infiltrated leaves (Figs. 1 and 2).

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N. tabacum lines expressing these CTV transgenes, all verified by Northern blot hybridizations (Fig. 3*B*), were crossed with the line 6b5, essentially as described in ref. 21, to give F_1 progenies referred as P20 × 6b5, P23 × 6b5, and CP × 6b5 plants. The resulting F_1 plants were used for the analyses of silencing

F3

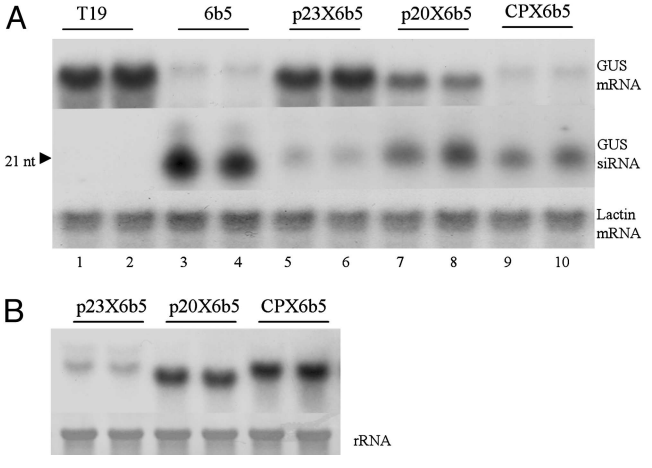

Fig. 3. Suppression of intracellular silencing by p20 and p23 but not by CP of CTV. Shown is Northern blotting detection of expression of the GUS (*A*) and CTV (*B*) transgenes in tobacco plants. Total high- and low-molecular-weight RNAs were individually extracted from tobacco plants of various genotypes as indicated and analyzed for the accumulation of GUS mRNA (*Top*) and siRNA (*Middle*) as described in ref. 21. The CTV probes corresponded to the full-length coding sequences of respective CTV genes. Equal loading was further demonstrated by probing for the lactin mRNA (GenBank accession no U60489.1) or by staining for the 28S rRNA. Two of the 10 individual plants examined for each genotype were shown. The position of a 21-nt synthetic siRNA is indicated by an arrowhead.

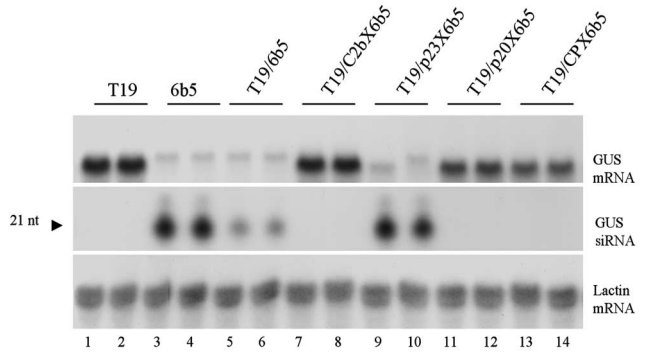

Fig. 4. Suppression of intercellular silencing by p20 and CP but not by p23 of CTV. Six weeks after the T19 scions were grafted on the rootstocks made from 6b5, p23 × 6b5, p20 × 6b5, CP × 6b5, or CMV2b × 6b5, total high- and low-molecular-weight RNAs were individually extracted from the new growth of those T19 scions (lanes 5–14) and analyzed for the accumulation of GUS mRNA (*Top*) and siRNA (*Middle*) as described in Fig. 1. Two of the 10 individual plants examined for each genotype are shown. The position of a 21-nt synthetic siRNA is indicated by an arrowhead.

suppression because silencing of the GUS transgene at the 6b5 locus also occurs when it is in the hemizygous state (17, 20). Northern blot hybridizations revealed that the GUS transgene remained silenced in CP × 6b5 plants, as indicated by the absence of the GUS mRNA accumulation in these plants that was found in the 6b5 plants (Fig. 3*A*, compare lanes 3 and 4 with lanes 9 and 10). This finding shows that expression of the GUS transgene was not restored when CP was coexpressed with the silencing transgene in the same tissue. In contrast, abundant accumulation of the full-length GUS mRNA was detected in both P23 × 6b5 and P20 × 6b5 plants (Fig. 3*A*, lanes 5–8). In particular, the levels of GUS mRNA in P23 × 6b5 plants (lanes 5 and 6) were similar to those detected in the GUS-expressing line T19 (lanes 1 and 2) and higher than those detected in P20 × 6b5 plants (lanes 7 and 8). Thus, both p23 and p20, but not CP, functioned as suppressors of intracellular silencing in *N. tabacum* plants, and p23 was a stronger silencing suppressor than p20. These findings are consistent with the results from the coinfiltration assay carried out in *N. benthamiana* plants (Figs. 1 and 2).

Suppression of Intercellular Silencing by p20 and CP but Not by p23 of CTV. GUS RNA silencing in 6b5 plants includes components of both intracellular and intercellular silencing (17). Thus, we next investigated whether expression of any of the CTV proteins influenced intercellular silencing by assaying for GUS silencing spread in P23 × 6b5, P20 × 6b5, and CP × 6b5 plants, as described in ref. 21. In these grafting experiments, the GUS-expressing T19 plants were grafted as scions onto rootstocks of P23 × 6b5, P20 × 6b5, or CP × 6b5 plants. Northern blot hybridizations were carried out to determine whether the GUS transgene became silenced in the new growth of the grafted T19 scions 6 weeks after grafting. As expected from previous studies (17, 21), the GUS transgene in the T19 scions grafted onto the control 6b5 rootstocks became silenced (Fig. 4, lanes 5 and 6), demonstrating export of the GUS-specific silencing signal from the 6b5 plants into the T19 scions. Similar GUS RNA silencing was also detected in the T19 scions grafted onto the P23 × 6b5 rootstocks, as illustrated by the detection of GUS-specific siRNAs and by the greatly reduced accumulation of GUS mRNA (Fig. 4, lanes 9 and 10). Thus, although p23 restored expression of the GUS transgene in P23 × 6b5 plants, p23 interfered with neither production nor export of the silencing signal from the 6b5 locus so that the GUS-specific silencing signal was exported normally into the T19 scions to direct GUS RNA silencing. This finding shows that although p23 was a suppressor of intracellular silencing, it was inactive against intercellular silencing.

In each of the ten T19 scions grafted onto either P20 × 6b5 or CP × 6b5 rootstocks, however, there was abundant accumulation of GUS mRNA, and GUS siRNAs were not detectable (Fig. 4, lanes 11–14). This pattern of accumulation of GUS mRNA and siRNAs was similar to that detected in the T19 scions grafted on the 6b5 rootstocks expressing CMV 2b (Fig. 4, lanes 7 and 8), which is known to suppress intercellular silencing (21). Thus, the GUS transgene was not silenced in these T19 scions grafted on P20 × 6b5 and CP × 6b5 rootstocks, indicating a lack of export of the GUS-specific silencing signal from these rootstocks. These results show that the intercellular spread of GUS RNA silencing was inhibited in both P20 × 6b5 and CP × 6b5 plants and therefore identify both p20 and CP as suppressors of intercellular silencing. Therefore, p20 suppresses both intracellular and intercellular silencing, whereas CP is only effective against intercellular silencing.

None of the CTV Suppressors Interfered with the Restoration of Transgene DNA Methylation. RNA silencing of the GUS transgene in 6b5 plants is associated with cytosine methylation of the transgene DNA in the coding sequence, which was not detectably affected by HC-Pro that suppressed intracellular, but not intercellular, silencing in 6b5 plants (20). In contrast, significant reduction of GUS DNA methylation was observed in 6b5 plants expressing CMV 2b that inhibited both intracellular and intercellular silencing in 6b5 plants (20–22). Thus, we next examined the cytosine methylation status of the GUS transgene DNA in P23 × 6b5, P20 × 6b5, and CP × 6b5 plants, each of which carried a CTV protein that suppressed intracellular silencing (p23), intercellular silencing (CP), or both (p20). The GUS transgene contains three *Mlu*I sites, two of which toward the 3′ region of the GUS coding sequence are heavily methylated in the silencing 6b5 plants (Fig. 5*A*) but not in the GUS-expressing T19 plants (21, 29). As expected, the genomic DNA extracted from T19 plants was completely digested by *Mlu*I along with *Eco*RI, which cut within the NOS terminator, to generate two expected bands of 0.7 and 0.85 kb, whereas protection of the two adjacent *Mlu*I sites due to cytosine methylation in 6b5 plants resulted in a new 1.55-kb band as well as a few larger bands (Fig. 5*B*). Also as expected from previous studies (20, 21), the *Mlu*I sites remained methylated in the 6b5 plants expressing HC-Pro but remained mostly unmethylated in the 6b5 plants expressing CMV 2b (Fig. 5*B*, lanes 3 and 4). However, the diagnostic 0.7- and 0.85-kb bands were not detected in similar Southern blot analysis of genomic DNA extracted from 6b5 plants expressing any of the three CTV suppressors (Fig. 3*B*, lanes 9, 11, and 13). Thus, suppression of either intracellular or intercellular silencing by the CTV proteins did not interfere with the restoration of transgene DNA methylation in the nucleus, indicating that viral suppression of silencing occurs either independently or downstream of transgene methylation. Consistent with a previous report (34), GUS DNA in the new growth of the T19 scions did not become methylated at the *Mlu*I sites no matter whether there was GUS RNA silencing in the scion 6 weeks after grafting (Fig. 5, lanes 5–7, 10, 12, and 14).

Discussion

Whereas previous studies have identified one silencing suppressor from each of the plant and animal viruses examined (1, 13), our results show that CTV encodes three suppressors and that each exhibits distinct features in silencing suppression. p23 appears similar to HC-Pro because, although both are potent suppressors of intracellular silencing, neither prevents intercellular silencing and DNA methylation of the target transgene as examined in the same 6b5 silencing system (20). The suppressor activity of p20 was first suggested in the study that identified the

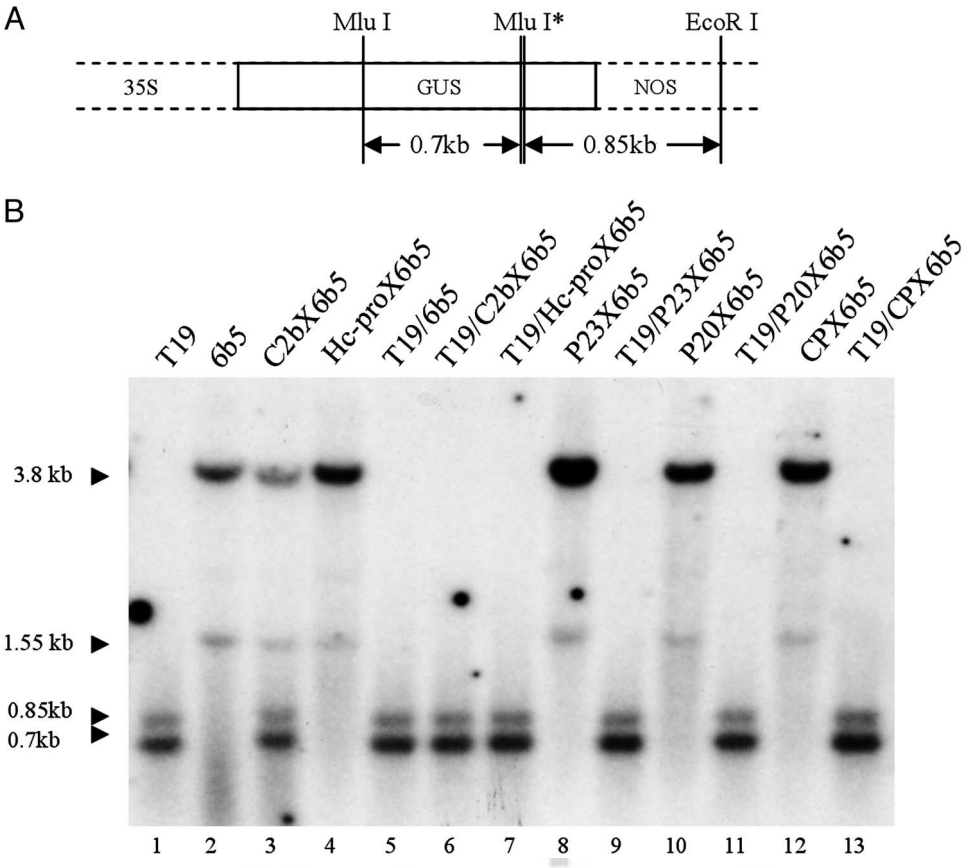

Fig. 5. None of the CTV suppressors interfered with the restoration of transgene DNA methylation. (*A*) Restriction map of the GUS transgene in transgenic tobacco plants. Lengths of predicted restriction fragments are shown below. The two adjacent *Mlu*I sites that are highly methylated (20, 21, 29) are marked by an asterisk. (*B*) Total genomic DNA was extracted from tobacco plants of various genotypes as indicated before grafting (lanes 1–4, 8, 9, 11, and 13) and from the T19 scions 6 weeks after grafting on rootstocks made from various genotypes (lanes 5–7, 10, 12, and 14). Southern blot analysis of these tobacco genomic DNA samples after double digestion with *Eco*RI and *Mlu*I was performed as described in ref. 21. The positions of the 0.7-, 0.85-, and 3.8-kb bands are indicated by arrowheads.

p20 homolog of Beet yellows closterovirus as a silencing suppressor (10, 35). p20 suppression shares features with silencing suppression by CMV 2b because both are potent suppressors of intercellular silencing but incomplete in suppressing intracellular silencing (21). Unlike CMV 2b, however, p20 suppression of intercellular silencing is not associated with reduced DNA methylation of the target GUS transgene. CP is clearly novel because its suppression of intercellular silencing spread is not associated with suppression of intracellular silencing, unlike p20 and all of the other viral suppressors known to interfere with intercellular silencing, such as CMV 2b and p25 of potato virus X (11–16). In these examples, excluding a possible role of intracellular silencing suppression in the efficient blockage of intercellular silencing requires additional experimental support (20, 21).

The novel features of silencing suppression by CP of CTV also suggest caution when interpreting data from the popular *Agrobacterium* coinfiltration assay. For example, consistent CP suppression of intercellular silencing detected in the 6b5 silencing system was not observed in the coinfiltration assay. Presumably, lack of CP suppression of intracellular silencing would not allow CP to be expressed at sufficient levels in the infiltrated leaves (33), whereas in CP × 6b5 plants CP was expressed from a preselected, highly expressing transgene locus. Thus, this novel class of intercellular silencing suppressors that are inactive against intracellular silencing is best characterized in a silencing suppression assay in which expression of the suppressor itself is not targeted by RNA silencing. AQ: O

This article describes the identification of viral suppressors of RNA silencing encoded by a natural pathogen of perennial trees (13), suggesting a role for RNA silencing in defense against viruses in trees. Although a precise role for any of the identified CTV suppressors in host infection remains to be established, it is interesting to note that all are encoded by the most abundantly transcribed subgenomic mRNAs of CTV (24, 25). Thus, it is likely that abundant expression of three distinct suppressors would potently suppress the siRNA-mediated antiviral pathway (11–16) and possibly also interfere with the host development pathway controlled by micro-RNAs (9, 10, 36–38), at multiple points in a manner similar to virus synergy in mixed infections with viruses carrying distinct suppressors (22, 39). Indeed, this may in part explain why CTV causes the most destructive viral disease in citrus worldwide (27). AQ: P

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C—Au: Is '. . . export of the silencing signal (capable of mediating intercellular silencing spread) was detected . . .' satisfactory?

D—Au: Is '. . . a sophisticated viral counter-defense strategy that targets the silencing antiviral pathway at multiple steps and may be essential for protecting CTV . . .' satisfactory?

E—Au: Please explain the relationship in 'helper component-proteinase' with regard to its hyphen.

F—Au: Please explain 'either' in '. . . was suppressed by either viral protein . . .'

G—Au: Please spell out 'PVX.'

H—Au: Please explain your varying use of capital and lowercase 'p' in 'p20' etc.

I—Au: Please explain the italicization of '*Nectin*.'

J—Au: 'ectopic' as meant and not 'ecotopic'?

K—Au: 'as indicated by the absence of the GUS mRNA accumulation in these plants that was found in the 6b5 plants' as meant? The sentence was difficult to understand.

L—Au: PNAS mandates unambiguous pronoun antecedents. Is 'This finding' satisfactory?

M—Au: Ref. 17 as meant by 'Palauqui *et al.*, 1997'?

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