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Virology 346 (2006) 7 – 14
VIROLOGY
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# Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon

Marin Chiba [a], Jonathan C. Reed [a], Alexey I. Prokhnevsky [a], Elisabeth J. Chapman [a], Munir Mawassi [b], Eugene V. Koonin [c], James C. Carrington [a], Valerian V. Dolja [a,*]

[a] *Department of Botany and Plant Pathology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331, USA*
[b] *Agriculture Research Organization, Department of Virology, Bet Dagan 50250, Israel*
[c] *National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD 20894, USA*

Received 22 July 2005; returned to author for revision 15 August 2005; accepted 21 September 2005
Available online 21 November 2005

**Abstract**

Launching the *Beet yellows virus* (BYV) minireplicon by agrobacterial delivery resulted in an unexpectedly low number of infected cells per inoculated leaf. This effect was due to a strong RNA silencing response in the agroinfiltrated leaves. Strikingly, ectopic co-expression of p21, a BYV RNA silencing suppressor, increased minireplicon infectivity by three orders of magnitude. Mutational analysis demonstrated that this effect correlates with suppressor activity of p21. Five diverse, heterologous viral suppressors were also active in this system, providing a useful approach for a dramatic, up to 10,000-fold, increase of the efficiency of agroinfection. The minireplicon agroinfection assay was also used to identify a new suppressor, a homolog of BYV p21, derived from *Grapevine leafroll-associated virus-2*. In addition, we report preliminary data on the suppressor activity of the p10 protein of *Grapevine virus A* and show that this protein belongs to a family of Zn-ribbon-containing proteins encoded by filamentous plant RNA viruses from three genera. The members of this family are predicted to have RNA silencing suppressor activity.


*Keywords:* RNA silencing; Viral suppressors; Agroinfection

## Introduction

Natural transmission of plant viruses occurs with the aid of vector organisms, via vegetative or sexual propagation, or by mechanical inoculation (Hull, 2001). Except for the latter, these pathways are difficult to reconcile with reverse genetics. Mechanical inoculation with viral nucleic acid normally results in few infection foci in epidermal cells. However, mechanical inoculation is problematic for many viruses that multiply preferentially or exclusively in the phloem. To overcome this problem, *Agrobacterium* can be used for efficient delivery of viral genomes to cells. Infiltration of leaf tissue with agrobacterial suspensions results in efficient delivery of viral cDNA copies to large numbers of epidermal, mesophyl, or phloem cells (English et al., 1997). Application of this technology permitted reverse genetic studies for several phloem-limited viruses (Grimsley et al., 1987; Leiser et al., 1992).

*Beet yellows virus* (BYV) is the prototype member of the genus *Closterovirus* (Dolja, 2003). The 15.5 kb, positive-strand RNA genome of BYV possesses nine open reading frames (ORFs; Fig. 1). The ORFs 1a and 1b encode the leader proteinase and RNA replicase components that are essential for genome amplification (Peng and Dolja, 2000; Peremyslov et al., 1998). In addition, the 21-kDa product of ORF 8 (p21) functions as an enhancer of RNA accumulation. This protein is a strong RNA silencing suppressor that is able to bind short interfering RNAs (siRNAs) in vitro and in vivo (Chapman et al., 2004; Reed et al., 2003; Ye and Patel, 2005). The products of ORFs 2–7 are dispensable for genome amplification (Peremyslov et al., 1998). The ORF 2 encodes a BYV movement protein that resides in the endoplasmic reticulum (Peremyslov et al., 2004b). The ORFs 3–7 encode virion components, each of which is also required for virus transport (Alzhanova et al., 2000, 2001; Napuli et al., 2003; Peremyslov et al., 2004a; Prokhnevsky et al., 2002). In addition to the major capsid protein, these components include a minor capsid protein, a 64-kDa protein, a homolog of 70-kDa heat shock proteins (Hsp70h), and a 20-kDa

* Corresponding author. Fax: +1 541 737 3573.
*E-mail address:* doljav@science.oregonstate.edu (V.V. Dolja).

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doi:10.1016/j.virol.2005.09.068

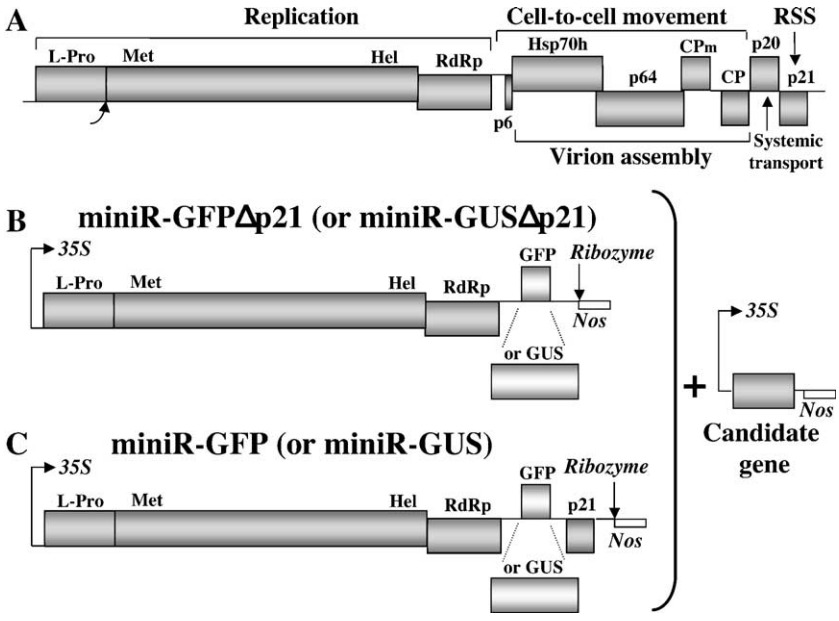


Fig. 1. Maps of BYV genome (top) and binary vectors used in this study. Functions of BYV genes are shown above and below the diagram. L-Pro, leader proteinase; Met, Hel, and RdRp, methyltransferase, RNA helicase, and RNA polymerase domains, respectively; p6, 6-kDa movement protein; Hsp70h, Hsp70 homolog; p64, 64-kDa protein, CPm, minor capsid protein; CP, major capsid protein; p20, 20-kDa protein; p21, 21-kDa RNA silencing suppressor (RSS); GFP, green fluorescent protein; GUS, β-glucuronidase; 35S, 35S RNA polymerase promoter of *Cauliflover mosaic virus*; Nos, Nos transcription terminator.

long-distance transport factor. Although BYV is not strictly limited to the phloem, mechanical transmission is inefficient (Russel, 1963). In contrast, agroinfection provides a reliable means for achieving systemic infection with wild type or GFP-tagged BYV, presumably thanks to direct delivery of virus replicons to phloem cells (Prokhnevsky et al., 2002).

Because BYV can reproduce in leaf epidermal and mesophyll cells (Medina et al., 1999; Peremyslov et al., 1999), we anticipated that delivery of viral replicons by agroinfiltration would result in massive infection of these cells. Such infection would be nearly synchronous and independent of the ability of BYV to move from cell-to-cell, providing an excellent model for measuring RNA amplification or isolating virions produced by movement-defective mutants (Liu and Lomonossoff, 2002; Voinnet et al., 2000). Surprisingly, inspection of leaves upon launching a BYV-GFP minireplicon encoding only proteins essential for replication revealed very few infected cells. Investigation of this phenomenon indicated that the limited infectivity was due, at least in part, to RNA silencing. Using this experimental system, we tested the effects of six known suppressors and two previously uncharacterized candidate suppressors on replicon infectivity. We found that, depending on a particular suppressor, the replicon infectivity can be elevated up to 10,000-fold, facilitating utilization of agroinfection for research and biotechnology.

## Results

### *Agroinfection with BYV minireplicon is enhanced by co-expression of p21*

To determine if *Agrobacterium*-mediated delivery of a viral cDNA is an efficient method to launch mini-BYV replication in leaf tissue, we compared GFP expression driven by a non-replicating GFP mRNA to that directed by a BYV minireplicon encoding a GFP reporter (miniR-GFPΔp21; Fig. 1). The miniR-GFPΔp21 contains BYV genes that are essential for RNA replication (L-Pro, Met, Hel, and RdRp in Fig. 1) (Hagiwara et al., 1999; Peng and Dolja, 2000; Peremyslov et al., 1998). Transient expression of GFP mRNA resulted in nearly uniform fluorescence of cells in the infiltrated area (Fig. 2A). In contrast, only one fluorescent epidermal cell per leaf on average was observed in tissue infiltrated with miniR-GFPΔp21 with most of the leaf areas showing no detectable GFP expression (Figs. 2B and 3A). The lack of detectable reporter expression from minireplicon in the majority of cells indicated partial or complete deficiency in virus ability to replicate. This effect could be due to low efficiency of the agrobacterial system or a strong antiviral response.

To test the possibility that agroinoculation triggers RNA silencing directed against the mini-replicon, we modified miniR-GFPΔp21 to include the BYV silencing suppressor, p21 (miniR-GFP in Fig. 1). The infectivity of miniR-GFP, defined as a number of GFP-positive, infected cells per leaf, was ~25-fold higher than that of miniR-GFPΔp21 (Figs. 2C and 3A), indicating that RNA silencing was at least in part responsible for the low infectivity of the original minireplicon.

To determine if agroinfection efficiency could be further enhanced by overexpression of p21 in trans, a mixture of bacterial strains engineered to express miniR-GFP and p21 was introduced to leaves. Strikingly, this resulted in a vast increase in the number of GFP-positive cells (Fig. 2D). Protoplasts were isolated from individual leaves, and the number of GFP-positive cells was determined. Addition of p21 resulted in a ~1000-fold increase in the number of fluorescent cells per leaf

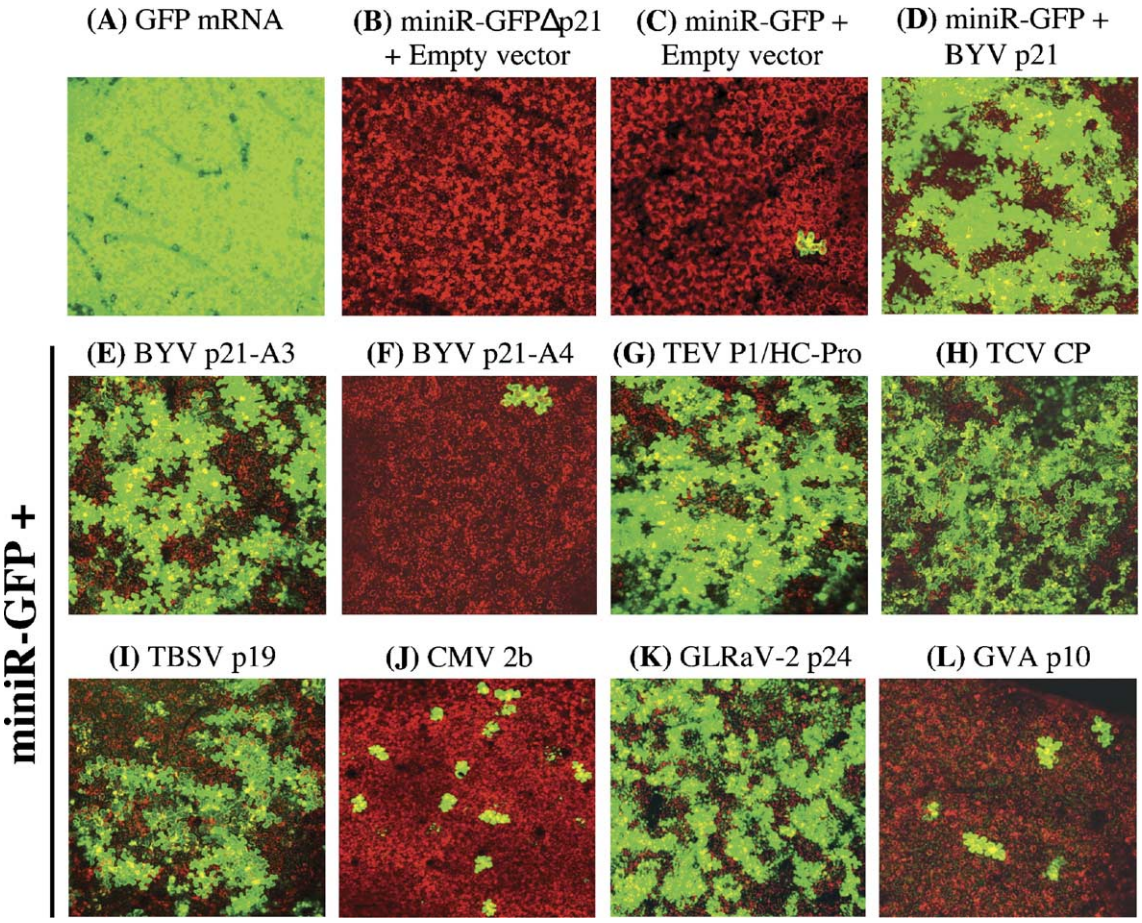


Fig. 2. Confocal laser scanning microscopy of *N. benthamiana* leaves infiltrated with agrobacterial strains expressing GFP mRNA (top left), or BYV minireplicons and viral RNA silencing suppressors as indicated. Red background is due to chlorophyll autofluorescence, whereas green is due to GFP fluorescence.

compared to the control in which miniR-GFP was used in combination with empty vector (Fig. 3A).

To confirm that the observed GFP expression was due to miniR-GFP amplification rather than translation of the GFP sequence from non-replicating transcripts, a replication-deficient miniR-GFP variant with a frameshift mutation in the replicase gene (miniR/FS-GFP) was generated and agroinfiltrated to leaves. Because this variant failed to yield any detectable fluorescent cells (data not shown), we concluded that the observed GFP expression was dependent on BYV

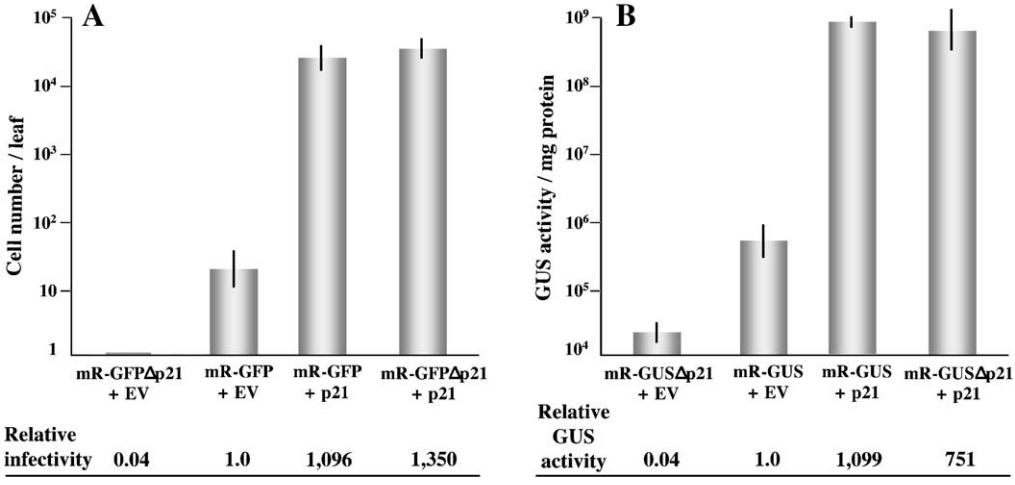


Fig. 3. Increased specific infectivity and accumulation of the BYV minireplicons due to co-expression of p21. (A) Mean of GFP-positive cells per leaf. Leaves were agroinfiltrated with the mixtures of GFP-tagged minireplicons (mR) and either empty vector (EV) or p21. The infectivity of each variant defined as a number of GFP-positive, infected cell per leaf relative to that of miniR-GFP is shown below the graph. (B) Mean GUS activity (pmol/μg protein/30 min) in leaves agroinfiltrated with mixtures of GUS-tagged minireplicons and either EV or p21. The relative GUS activity of each variant is shown at the bottom.

replicase-mediated amplification of miniR-GFP. In accord with this conclusion, abundant accumulation of miniR-GFP genomic and subgenomic RNAs in the presence of ectopically expressed p21 was revealed by hybridization analysis (Fig. 4A, lanes p21). In contrast, only low levels of subgenomic RNA were detected after co-infiltration of miniR-GFP with an empty vector, whereas genomic RNA was beyond the detection level (Fig. 4A, lanes EV). Quantitative analysis revealed that addition of p21 increased accumulation of subgenomic RNA ~830-fold, which is in close agreement with the results obtained by counting GFP-positive cells. No minireplicon-specific RNA was detected after agroinoculation with miniR/FS-GFP (Fig. 4A, lanes FS + EV).

This latter result indicated that the steady-state levels of minireplicon RNAs produced by nuclear transcription of the integrated cDNAs were beyond the sensitivity of hybridization analysis. To determine if RNA silencing was directed against the minireplicon, we analyzed for virus-derived siRNAs. Indeed, siRNAs of 21–24 nts were readily detected in plants agroinfiltrated with either miniR/FS-GFP or miniR-GFP (Fig. 4B). These results showed that transcription, but not replication of a BYV minireplicon was sufficient to trigger the silencing response and that siRNA was likely derived from positive-sense viral RNAs. The amounts of siRNAs were elevated in the presence of p21 (Fig. 4B) likely due to the increase in the levels of miniR-GFP genomic RNA in the presence of p21 (Chapman et al., 2004; Reed et al., 2003). Interestingly, the level of ~21-nt siRNAs was increased ~8-fold, whereas that of ~24-nt siRNAs was increased only ~3-fold (Fig. 4C). This may indicate either that p21 stabilizes 21-nt duplexes preferentially in infected cells (Chapman et al., 2004), or that 21-nt species is preferentially produced in response to elevated levels of replicating viral RNA.

Overexpression of p21 may have promoted susceptibility of cells that would otherwise have remained resistant, or may have increased accumulation of replicon RNAs such that GFP became visible in a greater number of cells. To distinguish between these possibilities, we used GUS-tagged minireplicons (Fig. 1) that allow sensitive quantification of virus replication using GUS activity (Peng and Dolja, 2000; Peng et al., 2002). In parallel experiments, agroinoculation with miniR-GFPΔp21 yielded only one fluorescent cell per leaf, while GUS activity of ~26,000 units was measured for miniR-GUSΔp21 (Fig. 3B). In the case of miniR-GUS, GUS activity was ~25-fold higher than that for miniR-GUSΔp21, which correlated well with a difference in infectivity of the corresponding GFP-tagged variants (Figs. 3A and B). Furthermore, combined agroinfiltration of miniR-GUS and p21 resulted in GUS activity that was ~1000-fold higher than that in control infiltrations (Fig. 3B). A correlation between the increase in GUS activity and the number of miniR-GFP-infected cells in response to either minireplicon-driven or ectopic expression of p21 (Figs. 3A and B) strongly suggests that p21 enables the minireplicon to establish infection in a greater number of inoculated cells.

Interestingly, co-expression of p21 with miniR-GFPΔp21 resulted in a specific infectivity similar to that of miniR-GFP plus p21 (Fig. 3A). Similar results were obtained with the corresponding GUS-tagged minireplicons (Fig. 3B) indicating

Table 1
Modulation of the specific infectivity of miniR-GFP by co-expressed mutant variants of p21

| p21 variant | Mutation | Suppressor activity[a] | Stability[b] | Relative infectivity |
|---|---|---|---|---|
| Empty vector | NA | NA | NA | 1.0 |
| Wild type | NA | + | + | 922 |
| A1 | $DCE_{7-9}AAA$[c] | – | +/– | 0.4 |
| A2 | $RSE_{16-18}AAA$ | + | + | 856 |
| A3 | $QSE_{33-35}AAA$ | + | + | 861 |
| A4 | $EHR_{57-59}AAA$ | – | + | 0.7 |
| 8A-21 | $R_{120}A$ | – | + | 0.3 |
| A9 | $RST_{133-135}AAA$ | + | + | 267 |

[a] Silencing suppressor activity of the p21 variants was determined as described (Johansen and Carrington, 2001). NA, not applicable.
[b] Protein stability was determined by immunoblot analysis.
[c] Identity in a single-letter code and positions of the amino acid residues changed to alanines are shown.

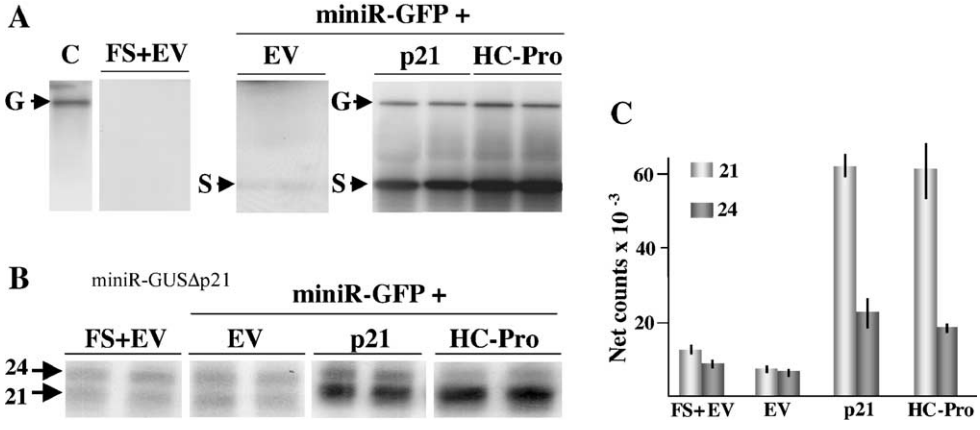


Fig. 4. Hybridization analysis of minireplicon-specific RNAs. (A) High-molecular weight RNAs. C, control that represents in vitro transcript of miniR-GFP cDNA; FS, miniR/FS-GFP; EV, empty vector; p21, BYV RNA silencing suppressor; HC-Pro, TEV RNA silencing suppressor P1/HC-Pro; G, genomic RNA; S, subgenomic RNA that encodes GFP. Two replicates of each variant are shown. (B) Radioautograms of siRNAs. Arrows mark positions of 24- and 21-nucleotide markers. (C) Comparative analysis of radioactivity corresponding to 21- and 24-nucleotide siRNAs for each experimental variant. Means and standard deviations of four replicates are shown.

Table 2
Effects of the heterologous silencing suppressors on infectivity of BYV miniR-GFP

| Suppressor | Relative infectivity |
|---|---|
| BYV p21 | 981[a] |
| TEV P1/HC-Pro | 9660[a] |
| TBSV p19 | 620[a] |
| TCV CP | 6040[a] |
| CMV 2b | 15[b] |
| PVX p25 | 27[b] |
| GLRaV-2 p24 | 6530[a] |
| GVA p10 | 12[b] |

[a] Determined as a ratio of the mean of GFP-positive protoplasts per leaf for each variant to that in the control (miniR-GFP plus empty vector).
[b] Determined by counting GFP-positive cells as a ratio of the mean for each variant to that of the control (miniR-GFP plus empty vector).

that ectopic expression of p21 is sufficient to obtain high levels of minireplicon infectivity.

### *Enhancement of agroinfection requires suppressor activity of p21*

To determine if the silencing suppressor activity of p21 was indeed responsible for increase in replicon infectivity, we analyzed six p21 mutants (Table 1). Each mutant contained substitutions resulting in either one or three consecutive amino acids changed to alanine. Transient assays in which silencing of GFP mRNA was induced by co-expression of GFP inverted repeat construct (Johansen and Carrington, 2001) demonstrated that mutants designated A2, A3, and A9 retained their suppressor activity, whereas mutants A1, A4, and 8A-21 lost suppressor activity (Table 1). The agroinfection assays revealed that the silencing-competent mutants enhanced minireplicon infectivity 267–861-fold (Fig. 2E; Table 1). In contrast, the suppressor-defective mutants A1, A4, and 8A-21 had no effect on agroinfection efficiency (Fig. 2F; Table 1). Among the six p21 mutants tested here, only A1 accumulated to a level markedly below that of the wild type p21 (Table 1). Correlation between the data obtained in inverted repeat-induced silencing and infectivity assays implies that the suppressor activity of p21 is required to increase the infectivity of BYV minireplicon. Moreover, these data provide additional support to the notion that the low infectivity of BYV minireplicon is due to RNA silencing response of the agroinfiltrated plants.

### *Heterologous silencing suppressors enhance infectivity of the BYV replicon*

To determine if the increase in agroinfection efficiency requires homologous interactions between viral RNA and p21, we used five known silencing suppressors derived from diverse plant viruses: P1/HC-Pro of *Tobacco etch virus*, p19 of *Tomato bushy stunt virus*, capsid protein of *Turnip crinkle virus* (TCV CP), 2b protein of *Cucumber mosaic virus*, and p25 of *Potato virus X* (PVX) (Chapman et al., 2004; Lecellier and Voinnet, 2004; Mlotshwa et al., 2002; Silhavy and Burgyan, 2004). Ectopic co-expression of each suppressor with the miniR-GFP replicon increased replicon infectivity, albeit to different extents (Figs. 2G–L; Table 2). Isolation and quantification of fluorescent protoplasts derived from agroinfiltrated leaves revealed a ~10,000-fold increase in minireplicon infectivity in the presence of P1/HC-Pro (Fig. 2G). Correspondingly, P1/HC-Pro expression dramatically increased levels of miniR-GFP genomic and subgenomic RNAs (Fig. 4A). Accumulation of siRNA species was also elevated in the presence of P1/HC-Pro (Fig. 4B). Similar to the pattern seen for p21, the levels of ~21-nt siRNAs were increased to a larger extent than those of ~24-nt siRNAs (Fig. 4C). The effect of TCV CP on minireplicon infectivity was also very strong, while that of p19 was somewhat below a ~1000-fold increase (Figs. 4H and I; Table 2). Ectopic expression of 2b and p25 also resulted in significant increases in miniR-GFP infectivity of 15- and 27-fold, respectively (Fig. 2J; Table 2).

Because minireplicon infectivity assays were responsive to a wide range of known suppressors, these assays were used to test candidate silencing suppressors from two grapevine viruses. A 24-kDa protein (p24) of *Grapevine leafroll-associated virus-2* (GLRaV-2), genus *Closterovirus*, was predicted to be a silencing suppressor based on amino acid sequence similarity to BYV p21 (Reed et al., 2003). A 10-kDa protein (p10) of *Grapevine virus A* (GVA), genus *Vitivirus*, was suggested to be a pathogenicity determinant (Galiakparov et al., 2003), a feature frequently associated with viral suppressor proteins (Voinnet et al., 1999). Ectopic expression of GLRaV-2 p24 increased miniR-GFP infectivity ~6500-fold (Fig. 2K; Table 2), suggesting that p24 may function as a silencing suppressor. This conjecture was confirmed using the GFP-inverted repeat silencing assay (data not shown). Taken together with previous data (Reed et al., 2003), four known

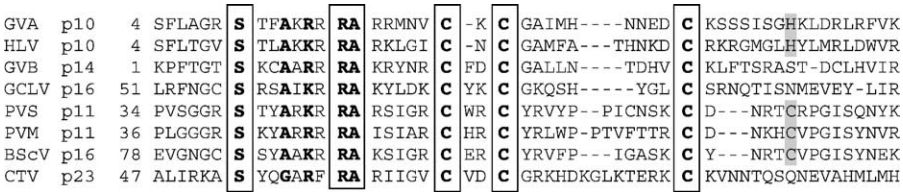


Fig. 5. A conserved amino acid sequence motif in known and putative silencing suppressors encoded by diverse filamentous viruses. Genus *Vitivirus*: GVA, *Grapevine virus A*; HLV, *Heracleum latent virus*; GVB, *Grapevine virus B*. Genus *Carlavirus*: GCLV, *Garlic common latent virus*; PVS, *Potato virus S*; PVM, *Potato virus M*; BScV, *Blueberry scorch virus*. Genus *Closterovirus*: CTV, *Citrus tristeza virus*. Similar residues are shown in boldface; invariant residues are boxed. Virus names are followed by the protein designation, which indicates its molecular mass (kDa). The numbers before each sequence show the amino acid position from the N-terminus.

members of the p21-like family of closterovirus proteins have now been confirmed as silencing suppressors.

Ectopic expression of GVA p10 also increased miniR-GFP infectivity by 12-fold (Fig. 2L; Table 2), suggesting that this protein may be yet another suppressor of RNA silencing. However, an alternative interpretation, such as non-specific binding and protection of viral RNA, cannot be ruled out with the available data. Searching the non-redundant protein sequence database (National Center for Biotechnology Information, NIH, Bethesda) using the BLAST program showed that p10 contains a conserved domain present in proteins encoded by filamentous RNA viruses of the genera *Vitivirus* and *Carlavirus*, and in p23 of *Citrus tristeza virus* (CTV), a *Closterovirus* (Fig. 5). Given that CTV p23 (Lu et al., 2004) has silencing suppressor activity, it seems possible that p10 and other proteins of this family are also suppressors. In addition to an Arg- and Lys-rich motif, the conserved domain shown in Fig. 5 also contains a putative Zn-ribbon motif that includes three invariant cysteines and an additional Cys or His residue that is conserved in most, but not all, proteins of this family.

## Discussion

Agrobacterium-mediated delivery of the GFP mRNA can result in massive GFP expression in the infiltrated leaf tissue (English et al., 1997; Johansen and Carrington, 2001). In contrast, using the same approach with a GFP-tagged mini-replicon of BYV, we detected very few GFP-positive cells. Here, we demonstrate that this diminished ability to establish infection was due, at least partly, to a strong RNA silencing response in the BYV minireplicon-infiltrated leaves. This conclusion is supported by a dramatic increase in replicon infectivity upon ectopic co-expression of a homologous RNA silencing suppressor p21, and by the mutation analysis that demonstrated dependence of this effect on suppressor activity of p21. Our experiments also suggested that even non-replicating viral RNA can act as an inducer of a strong silencing response. These results are in agreement with recent work that demonstrated that siRNAs generated in virus-infected plant cells are derived primarily from structural elements within the positive strand of the viral genome (Molnar et al., 2005).

We also show that the diverse heterologous silencing suppressors are able to enhance BYV replicon infectivity albeit to a different extent. Previous research indicated that these suppressors may be mechanistically distinct. Indeed, TCV CP was proposed to act via inhibition of a Dicer-like function for siRNA biogenesis (Qu et al., 2003), whereas p19 and p21 act downstream by sequestering siRNA duplexes (Chapman et al., 2004; Lakatos et al., 2004) or other forms of silencing-associated RNAs (Ye and Patel, 2005). The 2b and p25 proteins exhibit distinct functional profiles and may act via targeting signaling components of the RNA silencing pathway (Guo and Ding, 2002; Voinnet et al., 2000).

The agroinfection assay allowed identification of GLRaV-2 p24 as a strong silencing suppressor that is also capable of preventing induction of silencing by double-stranded inverted repeat. P24 is related to BYV p21, *Beet yellow stunt virus* p22, and CTV p20 (Reed et al., 2003). Interestingly, in addition to these orthologous suppressors common to the viruses of the genus *Closterovirus*, CTV encodes an additional suppressor, p23, that is not found in other closteroviruses (Lu et al., 2004). Here, we show that p23 belongs to a family of proteins characterized by a putative Zn-ribbon (Fig. 5). Our data suggest that another member of this family, GVA p10, also possesses silencing suppressor activity, although this remains to be confirmed using one of the conventional suppression assays. The distal part of the Zn-ribbon is degraded such that the fourth ligand is not always identifiable (i.e., metal-binding might be lost in some of these viruses or else non-conserved Cys or His might be employed). Such degradation of Zn-ribbons is not uncommon in evolution (Makarova et al., 2001). A likely evolutionary scenario for p10-like proteins involves acquisition of a host gene encoding a Zn-ribbon protein by a common ancestor of one of these genera followed by horizontal transfer of the gene between viruses. The p10-like proteins comprise the only known suppressor family that spans at least three viral genera and two families, and has a putative host ancestor.

It should be emphasized that agroinoculation of replicons introduces artificial phases of DNA integration and transcription followed by nuclear export, and, possibly, incidental splicing of RNA, into the virus life cycle. It is plausible that this can accelerate and exacerbate the RNA silencing response, thereby reducing the capacity of replicons to establish infection. Interestingly, in a recent work with *Tobacco mosaic virus* (TMV) gene expression vectors (Marillonnet et al., 2005), it has been found that agroinfiltration with TMV replicons resulted in very few infected cells. By introducing multiple silent mutations and artificial introns designed to interfere with aberrant processing of TMV transcripts and improve their nuclear export, the infectivity of replicon was elevated ~1000-fold. This may suggest that the processes of nuclear maturation and export, on one hand, and cytoplasmic RNA silencing, on the other hand, are equally important for the resulting efficiency of agroinfection. However, the potential mechanistic connection between these processes is yet to be addressed experimentally.

Suppressor-mediated enhancement of agroinfection efficiency is extremely useful for application of closteroviral and po tentially other plant viral gene expression vectors for both research and biotechnology purposes. In particular, efficient and synchronized infection of leaf cells after agroinfiltration provides a useful alternative to protoplast transfection for investigations of viral replication and assembly in planta (A.I.P., D.V. Alzhanova, V.V.D., unpublished results). Improved infectivity of plant viral gene expression vectors also boosts their utility for generation of vaccines and other beneficial proteins (Marillonnet et al., 2005; Pogue et al., 2002).

## Materials and methods

### *Binary vectors, agroinfiltration, and microscopic analyses*

Binary vector miniR-GUS was generated by cloning *Sna*BI–*Bst*EII fragment derived from pBYV-GUS-p21 (Hagi-

wara et al., 1999) into the appropriately digested binary vector, p35S-BYV-GFP (Prokhnevsky et al., 2002). To engineer miniR-GFP, the GUS coding sequence was replaced by the PCR-amplified EGFP coding sequence. The miniR-GUSΔp21 and miniR-GFPΔp21 plasmids were made by introducing a deletion between the *Avr*II site located downstream from reporter sequence and the *Mlu*I site located in the 3′-terminal part of the p21 sequence. A frameshift mutation in plasmid miniR/FS-GFP that inactivated BYV ORF 1a was described previously (Peremyslov et al., 1998). For transient protein expression, coding sequences were cloned into a modified mini-binary vector, pCB-302 (Reed et al., 2003). The mutant variants of p21 were generated by site directed mutagenesis using plasmid p3'BYV (Peremyslov et al., 1998), PCR-amplified, and inserted into pCB302.

*A. tumefaciens* strain C58 GV2260 was transformed by each of the binary vectors and infiltrations were done using bacterial suspensions at 1.0 OD600. GFP-positive leaf cells were visualized and counted using epifluorescent stereoscope Leica MZ FLIII (Deerfield, IL) at 7 days post infiltration. In the experiments involving PVX p25, screening was done at 4 days post-infiltration due to increased leaf senescence. Four to eight leaves for each experimental variant were used for quantification, and at least two independent experiments were done. Images in Fig. 2 were obtained using confocal laser scanning microscope Zeiss LSM 510 META.

To count GFP-positive cells in minireplicon co-infiltrations with strong silencing suppressors, protoplasts were isolated from *N. benthamiana* leaves as described (Peng et al., 2001), and numbers of fluorecent cells were determined using epifluorescence microscope and 0.1 mm deep hemacytometer.

### *Hybridization analyses, transient RNA silencing assays, immunoblotting, and GUS assays*

Hybridization analyses of miniR-GFP and miniR/FS-GFP RNAs were done as described (Peremyslov et al., 1998) using $^{32}$P-labeled, minus-sense, GFP-specific RNA as a probe. This probe was obtained by in vitro transcription using PCR-amplified GFP coding sequence followed by T7 RNA polymerase promoter. SiRNAs were analyzed as described (Reed et al., 2003) using $^{32}$P-labeled, full-length, minus-sense RNA transcripts of miniR-GFP. To prepare a probe, these transcripts were treated with 5 volumes of 100 mM NaOH at 37 °C for 5 min, followed by addition of 2 volumes of 1M Tris–HCl pH 6.8. Quantitation of radioactivity was done using a PhosphorImager. Four replicates were done for each experimental variant.

RNA silencing assays involving wild type and mutant p21 variants and GLRaV-2 p24 were done using GFP inverted repeat as inducer by visualization of GFP fluorescence and GFP mRNA hybridization analysis (Johansen and Carrington, 2001). Accumulation assays for p21 variants after agroinfiltration were done by immunoblot analysis using p21-specific antiserum (Reed et al., 2003). Accumulation of GUS activity (pMol/μg/30 min) directed by miniR-GUS or miniR-GUSΔp21 replicons was measured using fluorometric assays (Dolja et al., 1992).

## Acknowledgments

We thank Shou-Wei Ding for stimulating discussions. The cDNA clones used in this work were kindly provided by Nina Abou-Ghanem, David Baulcombe, Shou-Wei Ding, Jack Morris, Herman Scholthof, and Vicki Vance. This work was supported by grants from the National Institutes of Health (GM053190), US Department of Agriculture (NRI 2001-35319-10875), and Large Scale Biology Corp. (2000-13) to V.V.D., the National Science Foundation (MCB-0209836) and the National Institutes of Health (AI43288) to J.C.C., and BARD (IS-3784-05) to M.M. and V.V.D. This publication was made possible in part by grant number 1S10RR107903-01 from the National Institutes of Health. The authors wish to acknowledge the Confocal Microscopy Facility of the Center for Gene Research and Biotechnology and the Environmental and Health Sciences Center at Oregon State University.

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