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Dissecting RNA silencing in protoplasts uncovers novel effects of viral suppressors on the silencing pathway at the cellular level

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ABSTRACT

Short interfering RNA (siRNA)-mediated RNA silencing plays an important role in cellular defence against viral infection and abnormal gene expression in multiple organisms. Many viruses have evolved silencing suppressors for counter-defence. We have developed an RNA silencing system in the protoplasts of *Nicotiana benthamiana* to investigate the functions of viral suppressors at the cellular level. We showed that RNA silencing against a green fluorescent protein (GFP) reporter gene in the protoplasts could be induced rapidly and specifically by co-transfection with the reporter gene and various silencing inducers [i.e. siRNA, double-stranded RNA (dsRNA) or plasmid encoding dsRNA]. Using this system, we uncovered novel roles of some viral suppressors. Notably, the Cucumber mosaic virus 2b protein, shown previously to function predominantly by preventing the long-distance transmission of systemic silencing signals, was a very strong silencing suppressor in the protoplasts. Some suppressors thought to interfere with upstream steps of siRNA production appeared to also act downstream. Therefore, a viral suppressor can affect multiple steps of the RNA silencing pathway. Our analyses suggest that protoplast-based transient RNA silencing is a useful experimental system to investigate the functions of viral suppressors and further dissect the mechanistic details of the RNA silencing pathway in single cells.

INTRODUCTION

Short interfering RNA (siRNA)-mediated gene silencing plays essential roles in cellular defence against viral infection and abnormally expressed genes in plants and animals (1–7). This RNA silencing pathway involves many distinct steps (8,9), which generally starts with the detection of long double-stranded RNAs (dsRNAs) that may arise from a variety of sources including viral replication intermediates, dsRNA made from viral RNAs by a cellular RNA-dependent RNA polymerase (RdRP), aberrant cellular RNAs, over-expressed transgenes and transposons. A long dsRNA is cleaved into 21–26 nt siRNA duplexes by RNase III Dicer in animals and by Dicer-like (DCL) proteins in plants. Upon unwinding using an RNA helicase, one strand is incorporated into the RNA-induced silencing complex (RISC), and the other strand is degraded. Using the siRNA as a guide, the RISC then carries out sequence-specific cleavage of a target RNA or repression of translation. Some siRNAs also act to promote further production of long dsRNAs and their cleavage into siRNAs to enhance silencing (10,11). In plants, RNA-containing signals can move from the cell of origin into neighboring cells or even distant organs to cause systemic silencing [reviewed in (12)]. Furthermore, two size-classes of siRNAs appear to have functional divisions. The 24–26 nt siRNAs seem to be involved in systemic silencing and homology-dependent DNA methylation, whereas the 21–22 nt siRNAs are involved in sequence-specific mRNA cleavage (13).

As a counter-defence strategy, many plant and animal viruses studied to date encode suppressors of RNA silencing [reviewed in (6,7,14)]. The functions of plant viral suppressors have been studied in plants by stable transgenic expression, viral vector-based expression and Agrobacterium-mediated transient expression as well as by *in vitro* protein–RNA interactions [reviewed in (6)]. These RNA silencing suppressors function at distinct steps of the RNA silencing pathway, providing important insights into the mechanisms of viral counter-defence and establishing viral suppressors as valuable tools to probe the inner workings of the silencing pathways. The specific functions of many viral suppressors remain to be fully understood. For some suppressors, inconsistent or even opposing results have been reported. As has been well discussed (6,15–17), there are limitations for each *in planta*-based experimental system: constitutive expression of a suppressor in a transgenic plant makes it difficult to separate early and late events of silencing; and transient expression by viral or bacterial infection may introduce effects of other viral components as well as elicit plant defence/stress responses. There may be additional complications. The cells in a plant

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or plant organ may not be homogeneous in RNA silencing function at a given time. The dynamic aspects of suppressor function may be masked or compromised by unknown plant reactions during the long periods of time, days to weeks, between silencing initiation and sampling. All *in planta* methods are limited to the use of DNA constructs. Therefore, one is forced to examine the entire pathway from dsRNA production to siRNA function, making it difficult to dissect individual steps.

A recent study demonstrated that RNA silencing targeted towards a reporter gene and DNA virus could be induced in tobacco BY2 cells by the delivery of synthetic siRNAs, offering the promise to complement *in planta* systems to dissect mechanistic details of the silencing pathway (18). Here, we have expanded on these observations to establish a RNA silencing system in cultured cells of *Nicotiana benthamiana*, a plant species that has been extensively used to study the phenomenon of RNA silencing as well as the function of viral suppressors at the whole plant level, previously. We used green fluorescent protein (GFP) as a reporter for RNA silencing and DsRed as an internal control. Co-expression of GFP and DsRed was achieved by co-electroporation of plasmids carrying their respective genes, as established previously in tobacco BY2 cells (18). Protein expression, as monitored by fluorescence microscopy, became readily visible within 20 h of electroporation and peaked at 36 h post electroporation. Specific silencing of GFP could be achieved by co-delivery of *in vitro* synthesized *GFP* siRNA (si*GFP*), *in vitro* synthesized double-stranded *GFP* RNA (ds*GFP*), or plasmid encoding ds*GFP* RNA, greatly expanding the possibilities to dissect specific steps of the silencing pathway by using different forms of silencing inducers. Using this system, we tested the effects of five viral suppressors, which have been shown to have diverse effects on RNA silencing, within 36 h of electroporation. Our results revealed novel functions for some suppressors. Furthermore, our analyses suggest that a viral suppressor may interfere with multiple steps of the RNA silencing pathway to cause suppression of silencing. Finally, our analyses also suggest that protoplast-based RNA silencing offers a versatile experimental system to probe the mechanisms of RNA silencing in single cells.

MATERIALS AND METHODS

DNA constructs and preparation

Plasmid pRTL2 contains an enhanced 35S promoter of Cauliflower mosaic virus (CaMV) and was described by Restrepo *et al*. (19). pRTL2:smGFP and pRTL2:dsGFP, which contain the coding region of the soluble-modified *GFP* (sm*GFP*) gene and an inverted repeat of sm*GFP* respectively, were described in the study of Johansen and Carrington (16). pRTL2:0027 carries the Tobacco etch virus (TEV) P1/HC-Pro coding region and was described by Carrington *et al*. (20). The above plasmids were kindly provided by Dr James Carrington (Oregon State University, Corvallis, OR). pRTL2:TBSV P19 and pRTL2:TCV CP, which carry coding regions for the P19 protein of Tomato bushy stunt virus (TBSV) and the coat protein of Turnip crinkle virus (TCV) respectively, were described by Qu *et al*. (21) and were kindly provided by Dr Feng Qu and Dr Jack Morris (University of Nebraska-Lincoln, Lincoln, NE). The sm*GFP* gene was PCR-amplified using pRTL2:smGFP as template and cloned into pSP72 vector (Promega, Madison, WI), giving rise to pSP:smGFP that was used as template to generate sense and antisense sm*GFP* RNAs. The 2b coding region of Cucumber mosaic virus (CMV) was PCR-amplified from construct Fny 201 (22) and inserted into NcoI and BamHI sites of pRTL2 to obtain pRTL2:2b. Fny 201 was kindly provided by Dr Marilyn Roossinck (The Samuel Roberts Noble Foundation, Ardmore, OK). pRTL2:AL2 contains Tomato golden mosaic virus (TGMV) AL2 coding region at NcoI and BamHI sites and was kindly provided by Dr David Bisaro (The Ohio State University, Columbus, OH). pRTL2:mDsRed, which contains a copy of *DsRed* gene, was described by Qi *et al*. (23). pTGA443, which carries insert *CP[-657]-GUS* that has β-glucuronidase (*GUS*) gene under the control of a minimal sequence of TGMV coat protein (CP) promoter necessary and sufficient for AL2 activation (24), was a gift from Dr David Bisaro. All plasmid DNAs were prepared by using Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA), concentrated by ethanol precipitation and dissolved in double-distilled water. DNA concentration was determined by UV spectrometry.

RNA preparation

Sense and antisense sm*GFP* RNAs were produced by *in vitro* transcription (MEGAscript; Ambion, Austin, TX) using EcoRI or HindIII-digested pSP:smGFP as template and with SP6 and T7 RNA polymerases, respectively. RNA transcripts were purified by using MEGAClear kit (Ambion). Equal amounts of sense and antisense RNAs were mixed in an annealing buffer (100 mM potassium acetate, 4 mM $MgCl_2$ and 60 mM HEPES–KOH, pH 7.4), boiled for 5 min and incubated overnight at 37°C to produce ds*GFP*. The ds*GFP* was purified by ethanol precipitation and dissolved in distilled water. To generate siGFP, ds*GFP* RNA was incubated with recombinant Dicer enzyme (Gene Therapy Systems, San Diego, CA) following manufacturer's instructions. The reactions were stopped by adding equal volumes of RNA loading buffer (95% formamide, 0.025% xylene cyonal, 0.025% bromophenol blue, 18 mM EDTA and 0.025% SDS), boiled for 5 min, and loaded onto 15% polyacrylamide/8 M urea gel. The gel was stained by ethidium bromide and visualized under UV light. The band of 20–25 nt was cut, and soaked in the annealing buffer at room temperature overnight. After phenol extraction, the aqueous phase was transferred to a new tube, and RNA was precipitated by ethanol and dissolved in distilled water.

Protoplast isolation and electroporation

Cultured cells of *N.benthamiana* were maintained as described previously (24,25). Protoplast isolation and electroporation were performed essentially as described by Qi and Ding (25). Cultured cells were collected by centrifugation at 70 *g* for 5 min, resuspended in 1.5% cellulase 'Onozuka' RS (Yakult Pharmaceutical Ind. Co., Ltd, Tokyo, Japan) and 0.2% macerozyme (Calbiochem-Novabiochem, La Jolla, CA) in solution I [0.5 M mannitol and 3.6 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 5.5]. After incubation at room temperature for 3–4 h when over 90% of the cells became

round-shaped, the protoplasts were filtered through 40 μm nylon mesh, washed twice with solution I, and resuspended in solution II (solution I plus 0.1 mM $CaCl_2$) to a density of 2×10^6 protoplasts/ml. The protoplasts were kept on ice for 1 h before electroporation. Plasmids and RNA transcripts were mixed with 1×10^6 protoplasts on ice in a cuvette with a 0.4 cm of gap (Bio-Rad Laboratories, Hercules, CA) and electroporated with an Electroporator™ Plus (BTX, San Diego, CA) at 0.2 kV. After electroporation, protoplasts were transferred to a new eppendorf tube containing 1 ml of solution II and incubated on ice for 30 min. The protoplasts were collected by centrifugation at 70 *g* for 5 min, and then cultured in 1 ml of culture solution (30 g/l of sucrose, 256 mg/l of KH_2PO_4, 100 mg/l of myo-inositol, 1 mg/l of thiamine, 0.2 mg/l and 0.45 M of 2,4-D mannitol) in Costar® 6-well cell cultured cluster (Corning Incorporated, Corning, NY). For each electroporation, 1×10^6 protoplasts (in 400–500 μl of medium) were transfected with 5 μg of pRTL2:smGFP in the presence of 30 μg Herring sperm DNA. RNA silencing was induced by co-transfecting protoplasts with pRTL2:dsGFP, si*GFP* RNA or ds*GFP* RNA (see figures for amounts used). For viral suppressor assays, 10 μg of respective suppressor plasmids (pRTL2:suppressor) were co-transfected. At 36 h post transfection, protoplasts were collected for fluorescence measurement or RNA extraction.

Fluorescence measurement and fluorescence microscopy

Total fluorescence of 0.2×10^6 cells for each experiment was measured by Cytofluor™ 2350 Fluorescence Measurement System with the associated software (Millipore, Billerica, MA). Excitation (EX) and emission (EM) parameters were set as follows: EX 485 nm and EM 530 nm for GFP; EX 530 nm and EM 645 nm for DsRed. The fluorescence values of GFP and DsRed from pRTL2:smGFP and pRTL2:mDsRed transfected cells were arbitrarily set to 1. Fluorescence measurements from all other treatments were shown as relative values.

Transfected protoplasts were examined under an E600 fluorescence microscope (Nikon, Tokyo, Japan). GFP fluorescence was visualized with a filter set consisting of an excitation filter of 450–490 nm, a dichroic mirror of 510 nm, and a barrier filter of 520–560 nm. DsRed fluorescence was visualized with a filter set consisting of an excitation filter of 540–580 nm, a dichroic mirror of 595 nm, and a barrier filter of 600–660 nm. Images were captured with a SPOT 2 Slider charge-coupled device camera and the associated software (Diagnostics Instruments, Sterling Heights, MI).

RNA gel blot analysis

Total RNA was isolated from the protoplasts by using RNeasy Plant Mini Kit (Qiagen) and quantified by UV spectrometry. An aliquot of 3 μg of total RNA from each sample were loaded on 0.8% agarose–formaldehyde gels. After electrophoresis, the RNAs were transferred onto Hybond-XL nylon membrane (Amersham Biosciences Corp., Piscataway, NJ) and UV cross-linked. Sense and antisense *GFP* RNA probes were prepared as described above except in the presence of [α-^{32}P]UTP (Perkin Elmer, Boston, MA). DNA probes specific for each RNA silencing suppressor were prepared by using the Rediprime II random prime labeling system (Amersham Biosciences Corp.) and suppressor gene fragments excised by NcoI and XbaI digestion from pRTL2:suppressor constructs. The membranes were hybridized in ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion) at 65°C (RNA probes) or 42°C (DNA probes) overnight, washed twice in 2× SSC/0.1% SDS at 65°C or 42°C for 15 min, and twice in 0.2× SSC/0.1% SDS at 65°C or 42°C for 15 min.

For siRNA detection, the small RNA species were isolated from the protoplasts by using *mir*Vana miRNA isolation kit (Ambion). An aliquot of 10 μg of small RNA were loaded on 15% polyacrylamide gels containing 0.5× TBE and 8 M urea and transferred onto nylon membrane. The hybridization was performed in ULTRAhyb®-Oligo Hybridization Buffer (Ambion) at 37°C overnight. The membranes were washed twice in 2× SSC/0.1% SDS at 37°C for 15 min and twice in 0.1× SSC/0.1% SDS at 42°C for 15 min.

For all experiments, the washed membranes were exposed to Storage Phosphor Screen (Kodak, Rochester, NY). The Phosphor Screen was scanned by Molecular Imager FX using Quantity One-4.1.1 software (Bio-Rad, Hercules, CA).

Assay of β-glucuronidase (*GUS*) expression

N.benthamiana protoplasts were transfected with plasmid pTGA443 (expressing GUS), together with plasmid pRTL2 (empty vector) or pRTL2:AL2 (expressing AL2). After incubation in the dark for 36 h, 0.2×10^6 cells were harvested and GUS activity assay was performed as described by Grotewold *et al*. (26). The GUS activity from protoplasts co-transfected by plasmids pTGA443 and pRTL2 was arbitrarily set to 1. The GUS activity from protoplasts co-transfected with pTGA443 and pRTL2:AL2 was expressed as a relative value. Data presented are from two independent experiments.

RESULTS

Multiple inducers could each trigger rapid transient RNA silencing in protoplasts

To study RNA silencing in the protoplasts of *N.benthamiana* cultured cells, we first established co-expression of *GFP* and *DsRed* as reporter genes in the same cells. We co-electroporated plasmids pRTL2:smGFP (16) and pRTL2:mDsRed (23) in equal parts into the protoplasts. Driven by the constitutive 35S promoter of CaMV, the sm*GFP* and m*DsRed* open reading frames expressed soluble-modified GFP and monomeric DsRed, respectively. Fluorescence microscopy revealed co-expression of these proteins in the same cells within 20 h of post-electroporation. By 36 h post-electroporation, the protein expression reached nearly maximal levels in ~50% of the protoplasts (Figure 1A). In subsequent experiments, the *GFP* reporter was used to test the effects of silencing, and the *DsRed* reporter served as an internal control. For consistency, samples were taken at 36 h post-electroporation for analyses. Furthermore, fluorescence images of GFP- and DsRed-expressing cells in all treatments were taken with the same illumination intensity and exposure time.

After establishing the co-expression system, we tested the function of three types of silencing inducers to trigger specific silencing against GFP expression. These included (i) si*GFP* RNA generated *in vitro* by Dicer cleavage of ds*GFP* RNAs,

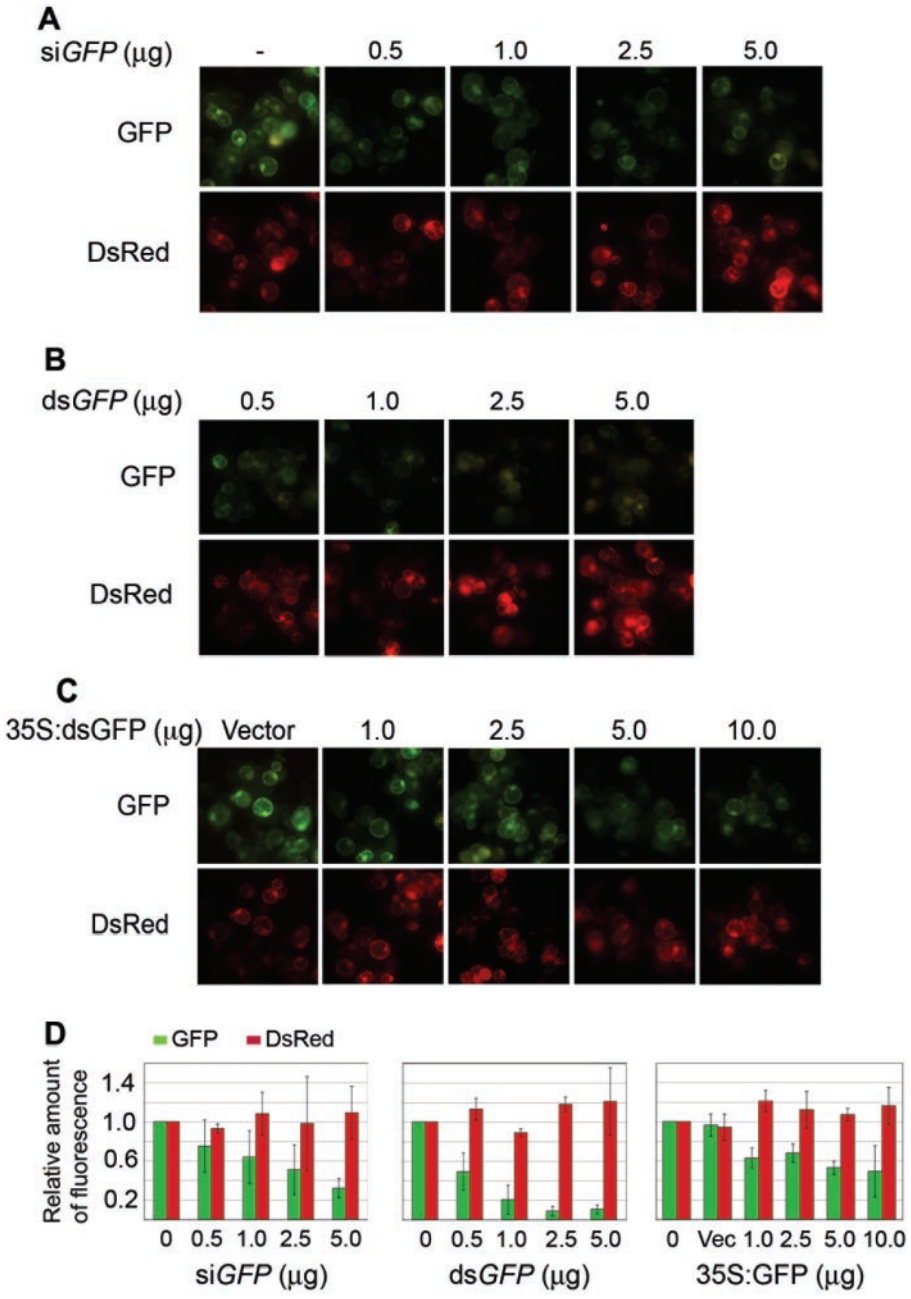

Figure 1. Co-delivery of multiple silencing inducers each triggers RNA silencing targeted to reporter GFP expression in protoplasts of *N.benthamiana* cultured cells. Increasing amounts of co-delivered si*GFP* RNA (**A**), ds*GFP* RNA (**B**) and 35S:dsGFP DNA (**C**) enhance silencing. Expression of DsRed in the same cells is not altered, demonstrating specificity of silencing against GFP. (**D**) Quantitative analysis of fluorescence intensities of GFP (green bars) and DsRed (red bars). The intensities in untreated cells are arbitrarily set to a value of 1, and those in treated cells are presented as relative values to the controls.

(ii) *in vitro* synthesized ds*GFP* RNA and (iii) plasmid pRTL2:dsGFP (16). This plasmid carries a cassette containing 35S promoter-driven full-length GFP coding sequences in the sense and antisense orientations spaced by an intron. RNA splicing of a transcript derived from this gene would generate a ds*GFP* RNA that functions as a potent silencing inducer (16).

When si*GFP* RNA was co-electroporated at increasing amounts (ranging from 0.5 to 5.0 μg) with plasmids expressing the GFP and DsRed reporters, GFP fluorescence decreased accordingly during the 36 h experimental period (Figure 1A). When ds*GFP* RNA or plasmid pRTL2:dsGFP was co-electroporated at increasing amounts with the reporter plasmids, GFP fluorescence also decreased (Figure 1B and C). Co-electroporation of an empty vector with the reporter plasmids had no effect on GFP fluorescence (Figure 1C). The DsRed fluorescence, in contrast, remained constant in all treatments (Figure 1A–C). Results from these control experiments demonstrated that the reduced GFP expression was a specific response to RNA silencing triggers. Four

independent experiments produced essentially the same results. Thus, specific and efficient RNA silencing could be induced rapidly in the protoplasts.

As an independent approach to measure GFP and DsRed expression, the fluorescence levels of both proteins in all experiments were quantitatively analyzed using fluorometry. Fluorescence intensities from equal amounts of protoplasts from each experiment were measured and normalized relative to the intensity from control experiment without silencing inducers. As shown in Figure 1D, with increasing amounts of the silencing inducers used, the GFP fluorescence intensity decreased. Direct introduction of ds*GFP* RNA caused stronger silencing of GFP expression (80–90% reduction) than the introduction of either si*GFP* RNA or 35S:dsGFP DNA. The fluorescence intensity of DsRed was not altered. These quantitative measurement data are fully consistent with microscopic observations.

To confirm that the reduced fluorescence intensity of GFP in the presence of silencing inducers was attributed to RNA silencing targeted to the *GFP* mRNA, we performed RNA gel blot analyses. As shown in Figure 2, the accumulation of *GFP* mRNA was drastically reduced in the presence of each of the silencing inducers. Thus, we concluded that the various RNA silencing inducers elicited efficient and specific RNA silencing in the protoplasts.

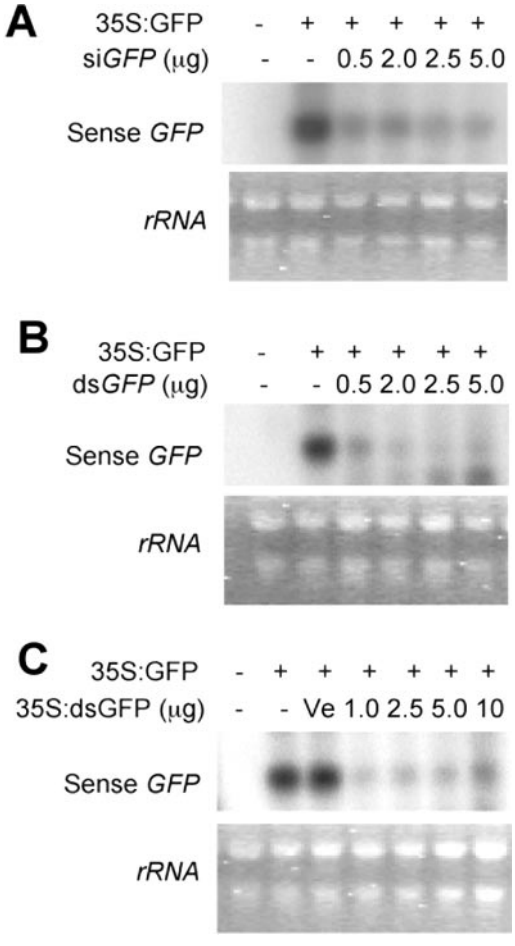

Figure 2. Reduced accumulation of *GFP* mRNA in the presence of increasing amounts of silencing inducers: si*GFP* RNA (**A**), ds*GFP* RNA (**B**) and 35S:dsGFP DNA (**C**).

Distinct functions of viral suppressors to interfere with silencing in single cells

Having established the protoplast RNA silencing protocols, we tested the functions of five viral suppressors on silencing suppression. These suppressors included the TEV P1/HC-Pro (27–29), CMV 2b (29), TBSV P19 (30), TCV CP (21,31) and TGMV AL2 (David Bisaro, personal communication), a homolog of African cassava mosaic virus (ACMV) AC2 (30). Previous studies showed that these suppressors seemed to interfere with the RNA silencing pathway in different manners. Plasmids carrying the open reading frames encoding each of these suppressors were co-electroporated with the GFP reporter plasmid into the protoplasts, with or without co-delivery of si*GFP* RNA (5 μg), ds*GFP* RNA (5 μg) or 35S:dsGFP DNA (10 μg). Each of these suppressors was expressed under the control of the CaMV 35S promoter. Expression of all suppressors was confirmed by northern blots (Figure 3A–E).

As shown in Figure 4A, except for AL2, all suppressors enhanced the expression of GFP in the absence or presence of si*GFP* RNA, ds*GFP* RNA and 35S:dsGFP DNA. Co-electroporation of plasmid vector alone had no effect on GFP expression, indicating that the enhanced GFP expression was attributed to the biological activity of the viral suppressors. The results were quantified and presented in Figure 4B. According to fluorescence visualization and measurement, 2b and P19 were more potent than P1/HC-Pro and CP to suppress silencing.

RNA gel blots showed that the presence of P1/HC-Pro, 2b, P19 and CP each increased dramatically the accumulation of *GFP* mRNA in the absence of silencing inducers, whereas presence of an empty vector or AL2 had little effect, when compared with the accumulation with 35S:smGFP construct delivered alone (Figure 5A). In the presence of si*GFP* RNA (Figure 5B), ds*GFP* RNA (Figure 5C) or 35S:dsGFP DNA (Figure 5D), these suppressors also enhanced *GFP* mRNA accumulation when compared with the controls (35S:smGFP alone or 35S:smGFP+vector), but to a lesser extent than in the absence of these silencing inducers. Presence of AL2 did not enhance *GFP* mRNA accumulation when compared with the controls.

These data indicate that the enhanced GFP fluorescence in the presence of P1/HC-Pro, 2b, P19 and CP was attributed to enhanced stability of *GFP* mRNA. AL2 had little effect on silencing suppression in such assays. Significantly, although GFP fluorescence was much stronger in the presence of 2b than in the presence of P1/HC-Pro and CP, GFP mRNA levels were not higher. In fact, the *GFP* mRNA level in the presence of 2b was consistently lower than that in the presence of other suppressors (see Figure 5A, C and D), except in the case of co-delivery of si*GFP* RNA as the silencing inducer (Figure 5B). *GFP* mRNA showed similar levels in the presence of P19 and CP (Figure 5A–D). However, the GFP fluorescence was much higher in the presence of P19 than in the presence of CP (Figure 4B).

The correlation between biological activities and expression profiles for P1/HC-Pro, 2b, P19 and CP indicated that these suppressors were fully functional in the protoplasts. However, the lack of suppressor activity of AL2 raised the question of whether the expressed protein was biologically active. To answer this question, we tested the function of AL2 in

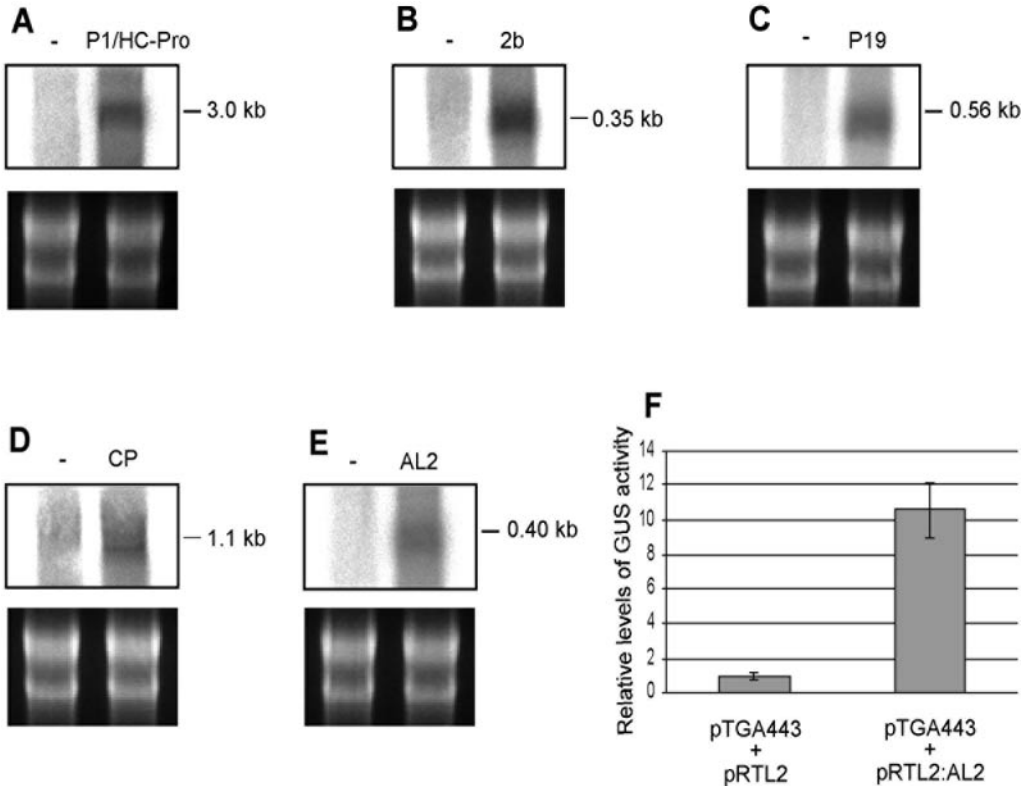

Figure 3. Expression of viral suppressors in *N.benthamiana* protoplasts. (**A**–**E**) Northern blots showing expression of the suppressor mRNAs in protoplasts co-transfected by plasmid expressing GFP and empty vector pRTL2 (lanes marked with the '−' sign) or by plasmid expressing GFP and plasmids expressing viral suppressors. (**F**) Assay of TGMV AL2 function by activation of TGMV coat protein promoter-driven *GUS* reporter expression. The level of *GUS* expression in protoplasts co-transfected by plasmids pTGA443 and pRTL2 was arbitrarily set to 1. The level of *GUS* expression in protoplasts co-transfected with pTGA443 and pRTL2:AL2 was expressed as a relative value. Data presented are the mean ± SE.

activating the TGMV coat protein promoter (32), using an established protocol in *N.benthamiana* protoplasts (24). We transfected *N.benthamiana* protoplasts with plasmid pTGA443, which carries insert *CP[-657]-GUS* that has β-glucuronidase (*GUS*) gene under the control of a minimal sequence of the promoter necessary and sufficient for AL2-mediated activation (24), in the presence or absence of AL2. Co-expression of AL2 boosted GUS expression by more than 10-fold (Figure 3F), as reported by Sunter and Bisaro (24). Therefore, we concluded that AL2 expressed in the protoplasts in our silencing experiments was biologically active; however, it had little silencing suppression activity.

Different effects of viral suppressors on the accumulation of siRNA

To learn about how the viral suppressors function in single cells, we examined their effects on the production of siRNA. In these experiments, we used 35S:dsGFP expression to induce silencing, which may permit synchronized expression of dsGFP and suppressors in our assays. At 36 h after electroporation, total RNA was extracted from protoplasts, which were confirmed microscopically to exhibit silencing suppression functions of the suppressors, and subjected to RNA gel blot analysis. As shown in Figure 6A, our current protocol did not detect siRNA accumulation in untreated protoplasts (lane 1) as well as when GFP was expressed in the absence of 35S:dsGFP (lane 2) or in the presence of an empty vector (lane 3). The siRNA was not visible either when GFP was co-expressed with P1/HC-Pro, P19, 2b or CP, in the absence of 35S:dsGFP (lanes 4–7).

When protoplasts were co-transfected with 35S:smGFP and 35S:dsGFP, there was prominent accumulation of siRNA (lane 8). Presence of an empty vector, P1/HC-Pro, P19 or 2b did not cause obvious alterations in the accumulation levels of the siRNA (lanes 9, 10, 11 and 12). Presence of CP, however, did lead to significantly reduced accumulation of siRNA (lane 13).

To determine whether the different effects of CP versus the other suppressors on siRNA accumulation were attributed to their different effects on ds*GFP* RNA cleavage, we analyzed the accumulation of antisense *GFP* RNA, which serves as a marker for ds*GFP* RNA. As shown in Figure 6B, the expression of CP led to significant accumulation of antisense *GFP* RNA (lane 13). The antisense *GFP* RNA was barely visible in all other treatments. These data indicate that CP blocked cleavage of ds*GFP* RNA into si*GFP* RNA, whereas the other suppressors had little or no such effect.

DISCUSSION

We have demonstrated that an RNA silencing machinery operates in protoplasts of *N.benthamiana* cultured cells, as it does

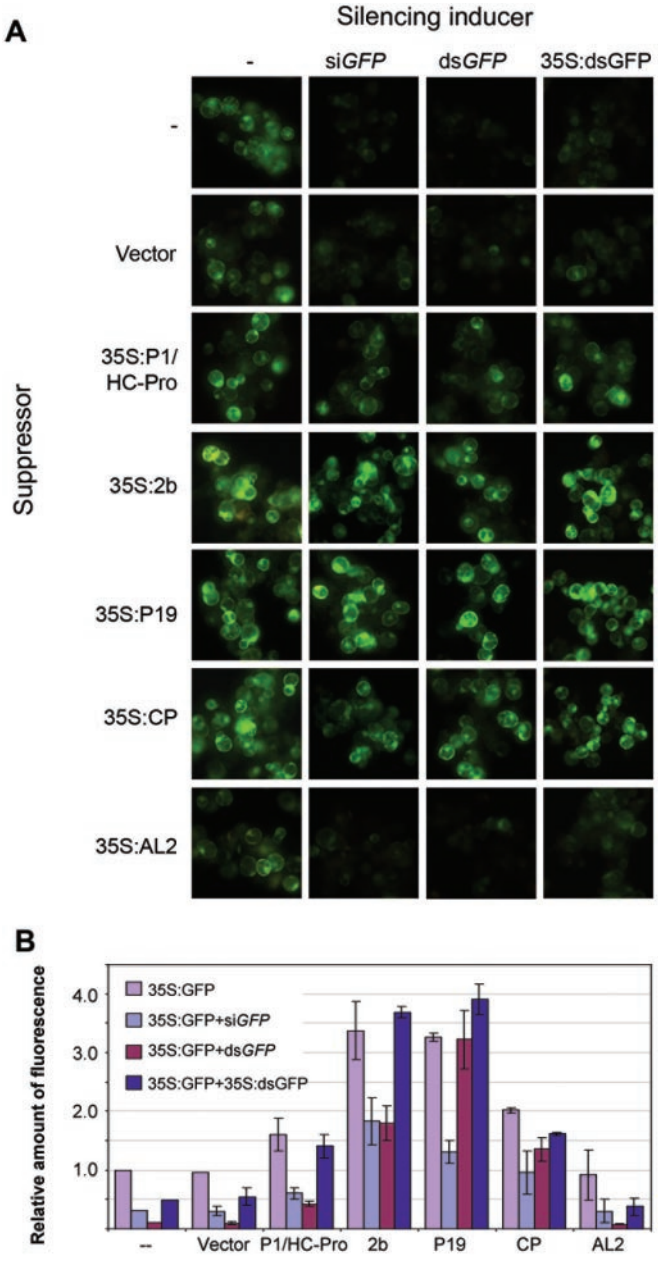

Figure 4. Effects of viral suppressors on RNA silencing against GFP expression in protoplasts. (**A**) Fluorescence images of GFP expression in the absence or presence of different silencing inducer and suppressor combinations. To express GFP, 5 μg of plasmid carrying the reporter gene was used in protoplast transfection. To induce GFP-specific silencing, 5 μg of si*GFP* RNA, 5 μg of ds*GFP* RNA or 10 μg of 35S:dsGFP DNA was used in co-transfection. To assay the effects of viral suppressors, 10 μg of plasmid encoding each of the suppressors was used in co-transfection. (**B**) Quantitative analysis of fluorescence intensities of GFP in the absence or presence of silencing inducer and suppressor combinations.

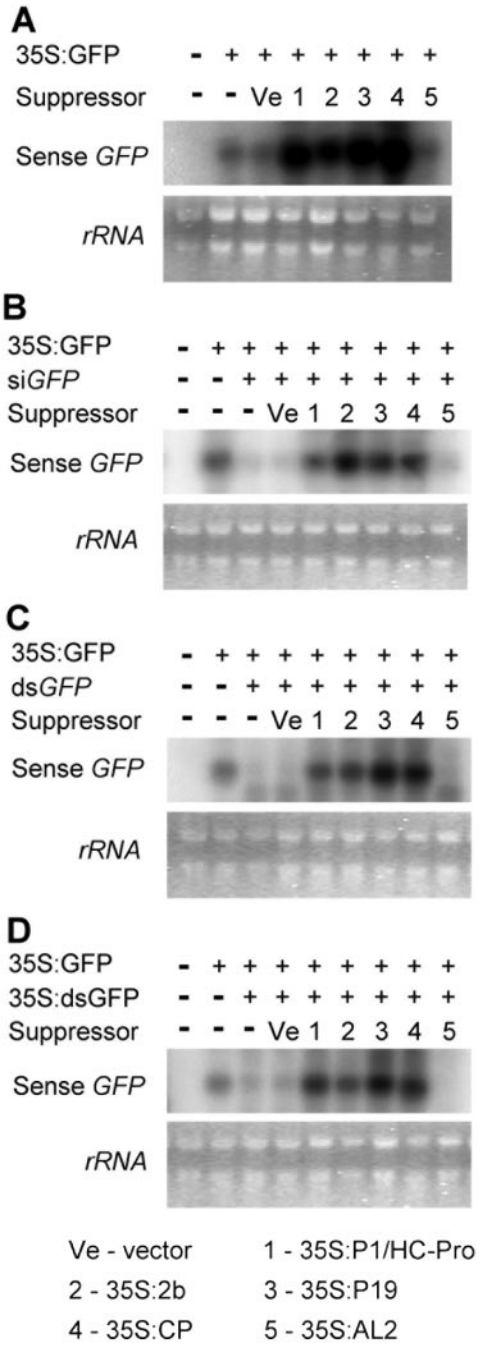

Figure 5. Viral suppressors, except AL2, increase the accumulation of *GFP* mRNA in the absence of silencing inducers (**A**) or in the presence of si*GFP* RNA (**B**), ds*GFP* RNA (**C**) and 35S:dsGFP DNA (**D**).

in a plant. First, a basal level silencing must be occurring in the absence of exogenously supplied inducers, as shown by the greatly increased expression of GFP when four of the five viral suppressors (P1/HC-Pro, 2b, P19 and CP) were each co-expressed. This observation is in accordance with previous findings from *in planta* studies (16,33). Second, and more significantly, enhanced silencing against GFP expression in the protoplasts could be triggered rapidly by multiple inducers that include siRNA, dsRNA or plasmid expressing the dsRNA. Among all inducers, direct delivery of dsRNA was the most potent in triggering the silencing. This could be explained by the following possibilities. Studies on RISC assembly in *Drosophila* showed that the generation of siRNAs by Dicer-2 is closely coupled with RISC assembly and Dicer-2 becomes a component of RISC (34). Therefore, the siRNAs derived from the dsRNAs *in vivo* may be more efficiently incorporated into RISC than the exogenously supplied siRNAs.

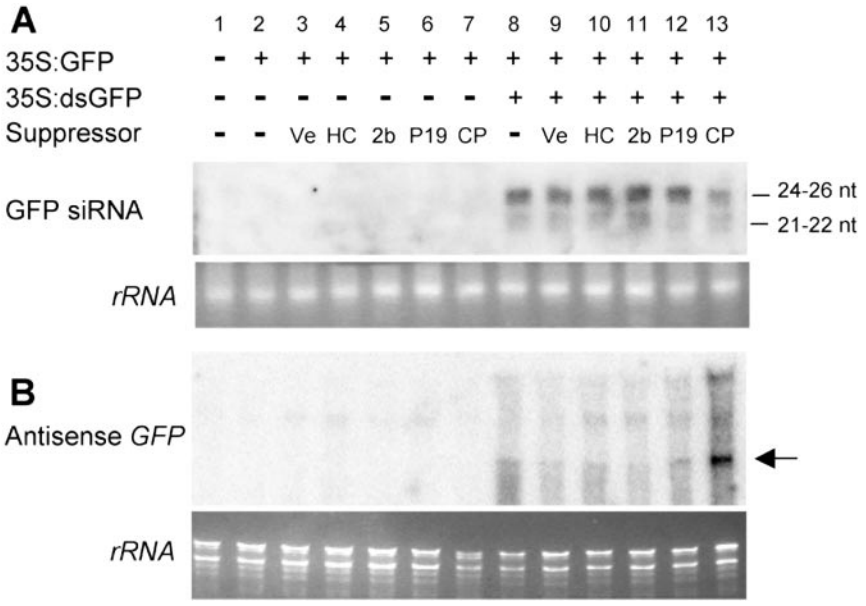

Figure 6. Different effects of viral suppressors on the accumulation of siRNA (**A**) and antisense *GFP* RNA (**B**). The arrow in (B) indicates the position of antisense *GFP*.

Furthermore, dsRNA production from a plasmid involves transcription, RNA processing and nuclear export and may therefore be less efficient in generating siRNAs than the direct use of dsRNAs.

Our analyses demonstrated that the rapid induction of RNA silencing in protoplasts offered distinct advantages to characterize the functions of viral suppressors within a short time frame, when compared with the prolonged incubation periods with *in planta* systems. Furthermore, transient expression of viral suppressors from plasmids avoids potential complications of plant responses to viral or bacterial infection. Our analyses have revealed novel information regarding the function of some suppressors.

Our results on CMV 2b were most surprising. Several studies showed that it is a weak suppressor of RNA silencing *in planta* when it is expressed from DNA constructs (13,21,35,36) and viral vectors (29). Its main function was proposed to be inactivation of the mobile RNA silencing signal and interference with DNA methylation in the nucleus (35). Our results show that 2b is a very potent suppressor of silencing in single cells. It did not affect siRNA accumulation. Thus, this suppressor may act at levels that directly interfere with siRNA-mediated mRNA degradation or siRNA-mediated signal amplification, in addition to the well-established role in interfering with the propagation of systemic silencing. Another intriguing observation is that although the GFP fluorescence in the presence of 2b was one of the strongest, the GFP mRNA level was relatively low. This observation raises the question of whether 2b also has a role in enhancing translation, for instance by suppressing translational repression caused by siRNA (37). The similar accumulation levels of GFP mRNA in the presence of P19 and CP, but the much higher level of GFP fluorescence in the presence of P19 also suggest the possibility of translational regulation via viral suppressor–siRNA interactions. Further studies are required to address this important issue.

Qu *et al*. (21) and Chapman *et al*. (36) showed non-accumulation of siRNA in the presence of TCV CP *in planta*, concluding that the CP blocks RNA silencing at an initiation step, specifically the cleavage of dsRNA into siRNA. Our data showing reduced accumulation of siRNA and enhanced accumulation of antisense GFP RNA in the presence of CP provided strong support of this hypothesis. However, we showed that TCV CP still suppressed the silencing induced by the direct delivery of si*GFP* RNA, suggesting that this suppressor also functions at a step downstream of silencing initiation. This could be at a step of siRNA incorporation into RISC or at a step of silencing amplification, or both.

Extensive studies on P1/HC-Pro using different *in planta* approaches have yielded different accounts of its effect on siRNA accumulation. For instance, this suppressor has been reported to eliminate siRNA production (15,38,39), suppress production of the long 24–26 nt siRNA but not the short 21–22 nt RNA (13), cause significant reduction of the short siRNA but have less effect on the long one (17), or have no effect on siRNA accumulation (16,21,36). Thus, P1/HC-Pro has been proposed to interfere with the processing of dsRNA into siRNA (17), accumulation of siRNA and DNA methylation (15), accumulation of siRNA but not DNA methylation (38), prevention of systemic silencing signal spread (13) or interference with siRNA incorporation in RISC (36). Our experiments from protoplasts showed that P1/HC-Pro suppressed silencing, but did not affect accumulation of both the short and long siRNAs within 36 h of electroporation. Consistent with this observation, there was no accumulation of antisense *GFP* RNA in the presence of P1/HC-Pro, suggesting that production of siRNA, at least at the initiation step, is not affected by P1/HC-Pro in the protoplasts. These data suggest that P1/HC-Pro functions downstream of siRNA production, consistent with the findings of Johansen and Carrington (16), Qu *et al*. (21) and Chapman *et al*. (36) from *in planta* analyses.

There are also different observations on the effect of P19 on siRNA accumulation. There is evidence that P19 drastically reduces or eliminates the accumulation of siRNAs in transgene-induced silencing and in viral-infected systemic leaves of *N.benthamiana* (13,21,40), but not in inoculated leaves or viral-infected protoplasts (40). In chalcone synthase (CHS)-based RNA silencing in *Arabidopsis*, P19 reduces the accumulation of the short siRNA but has less effect on the long siRNA (17). P19 does not affect siRNA accumulation in *N.benthamiana* with agroinfiltration (36). In the protoplasts, we showed that P19 could elicit strong silencing of GFP expression with mild effect on the accumulation of si*GFP* RNAs. Together with the observation that expression of P19 did not lead to the accumulation of antisense *GFP* RNA, these data indicate that P19 does not interfere with siRNA production. Rather, it functions downstream of siRNA production. It has been demonstrated that the P19 binds siRNAs *in vitro* (40–42) and *in vivo* (17,36,43), as a possible mechanism to sequester siRNAs to prevent its incorporation into RISC. This sequestration may lead to subsequent degradation of siRNAs.

RNA silencing could be suppressed by agrobacterium-mediated transient expression of TGMV AL2 in *N.benthamiana* leaves (David Bisaro, personal communication). ACMV AC2, a homolog of TGMV AL2, could also suppress the silencing of a sense transgene when it was expressed from a Potato virus X (PVX) vector in *N.benthamiana* plants (30). However, in our protoplast-based silencing system, AL2 did not suppress RNA silencing triggered by siRNA, dsRNA, plasmid encoding dsRNA or by overexpression of a sense gene. Our finding suggests that this suppressor does not function at the pathway that leads to RISC-mediated mRNA cleavage or translation inhibition; rather, it is consistent with the notion that AL2 functions at the DNA methylation level (David Bisaro, personal communication). This finding further demonstrates that the different behaviors of the viral suppressors in the protoplasts can be qualitatively attributed to their intrinsic biochemical modes of interactions with the RNA silencing pathway.

In summary, our analyses revealed new functions of viral suppressors in silencing suppression at the cellular level. The new findings may be explained in several ways. First, the protoplast system provided snapshot insights that may have been missed with *in planta* systems. Thus, a viral suppressor may interfere with multiple steps of the RNA silencing pathway(s), and it is crucial to employ multiple experimental systems to dissect these steps. Second, cells within a plant body and as protoplasts may have biological differences that affect quantitatively or even qualitatively, the operation of the RNA silencing pathway. Therefore, some of the differing results between protoplasts and *in planta* studies may represent unique features of each system.

The protoplast system offers many unique advantages to study RNA silencing in plants. First, the cells are more homogeneous than in a plant body, thereby allowing consistent data to be obtained. Second, synthetic siRNAs or their RNA precursors can be directly delivered into the protoplasts, greatly expanding the possibilities of experimentation with different types of inducers to dissect the various steps of the RNA silencing pathway. Third, different combinations of testing agents can be introduced together with the targets to learn about the effect of a particular agent on silencing. Fourth, detailed time-course studies may be performed to learn about the kinetic features of the RNA silencing pathway. This may be further enhanced by quantitative analyses to correlate the production level of a viral suppressor (or other factors to be discovered) with efficiency of silencing suppression. Fifth, the system may also be developed to incorporate biochemical analysis. Finally, the method may be applied to other plant species including *A.thaliana* whose mutants can be used for mechanistic studies of RNA silencing at the cellular level. The system may also be used to study the biogenesis and function of microRNAs, another class of small RNAs that play important roles in gene regulation (44–47). An obvious limitation of the protoplast system is that it does not allow studies of systemic RNA silencing. It can be predicted that a combination of *in planta*, *in vitro* and single cell studies will be necessary to develop a full understanding of the mechanisms of RNA silencing, as well as the specific role of a viral suppressor in this process.

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