J Gen Plant Pathol (2008) 74:326–330
DOI 10.1007/s10327-008-0099-z

VIRAL AND VIROID DISEASES

Degree of RNA silencing and the ability of a viral suppressor vary depending on the cell species in a protoplast system

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Received: 27 December 2007 / Accepted: 30 January 2008 / Published online: 8 May 2008

Abstract RNA silencing is a sequence-specific defense mechanism against viruses. As a counterdefense, viruses evolved silencing suppressors to interfere with host silencing. In analyses using protoplasts prepared from cultured cells (BY-2) and mesophyll cells of *Nicotiana tabacum* and *N. benthamiana*, viral suppressors differentially functioned in different cell types. This phenomenon has not been discussed in earlier papers on protoplast systems and RNA silencing. In investigations of the cellular activities of viral suppressors and their role in the RNA-silencing pathway, assays with host protoplasts offer many advantages and can complement other in planta assays such as *Agrobacterium*-mediated transient expression.

Keywords Protoplast · Silencing suppressor · BY-2 · *Nicotiana tabacum* · *Nicotiana benthamiana*

Plants have an antiviral defense system that targets viral RNAs for degradation in a sequence-specific manner. This phenomenon, called posttranscriptional gene silencing (PTGS), is triggered by double-stranded RNAs (dsRNAs) that originated from either viral replicative intermediates or hairpin structures on viral genomes (Baulcombe 2004; Pantaleo et al. 2007). A dsRNA-specific-enzyme, Dicer, digests long dsRNA into 21–26 nt short interfering RNAs (siRNAs), the hallmark of PTGS. One strand of such siRNA is then incorporated into RNA-induced silencing complex (RISC) and serves as a guide for sequence-specific cleavage or translational repression of a target RNA.

As a counter defense, plant viruses have evolved the ability to produce silencing suppressors that interfere with the host PTGS (Vance and Vaucheret 2001). The suppressor proteins are structurally diverse, but many use a common strategy of binding to dsRNAs to interfere with the PTGS pathway (Mérai et al. 2006; Lakatos et al. 2006). Among the known suppressors, potyvirus HC-Pro, *Cucumber mosaic virus* 2b, and tombusvirus P19 have been studied most extensively (Roth et al. 2004). They are all reported to have an ability to bind to dsRNAs (Ye et al. 2003; Mérai et al. 2006; Goto et al. 2007). Recently, 2b was also found to bind Argonaute 1 (AGO1), which is one of the components of RISC (Zhang et al. 2006).

The activity of viral suppressors has been analyzed using transgenic plants, viral vectors, and *Agrobacterium*-mediated transient expression assays (Roth et al. 2004). However, such in planta systems all suffer from intrinsic problems. For example, in the *Agrobacterium* system, we can never rule out the effect of the bacterial infection. In transgenic plants, controlling the degree of expression of the host PTGS and the given suppressor gene is very difficult. In addition, PTGS does not uniformly operate in all organs and tissues in a plant. Kubota et al. (2003) demonstrated that GFP silencing was differently suppressed by *Tomato mosaic virus* (ToMV) infection depending on the position of the leaves on the transgenic plant. Thus, we might find that the tested suppressor has different abilities, depending on the leaves that were tested. Recently, a protoplast-based system for RNA silencing has been demonstrated (Vanitharani et al. 2003; Qi et al. 2004; An et al. 2003) to have several advantages over in planta systems, First, we can control the amounts of a silencing inducer and a tested suppressor when the protoplast is co-

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transfected. Second, we can scrutinize each step in the PTGS pathway in a time-course experiment. Third, we can test compounds that may affect silencing and the ability of a suppressor. Fourth, considering that the ability of a suppressor is sometimes assessed differently depending on the plant organs (tissues) and even the plant species (Kubota et al. 2003; Senda et al. 2004), it is obviously better to use protoplasts from the plant of interest.

Although a protoplast system will not be suitable to study systemic signaling of RNA silencing, it certainly complements the in planta systems many researchers commonly use. We therefore developed our own protoplast system to study the roles of viral suppressors in the host PTGS pathway. In a preliminary experiment using cultured cells of *N. tabacum* (BY-2 cells), the viral suppressor that we used did not effectively function in BY-2 cells, but it seemed to function well in mesophyll cells of *N. benthamiana* (Nb cells). This observation led us to do detailed analyses because the earlier reports did not handle this unique difference, which may significantly influence our understanding of the mechanisms of RNA silencing and the roles of viral suppressors.

Protoplasts were prepared either from leaves of *N. benthamiana* and *N. tabacum* cv. BY-4 (Yamaguchi et al. 2005) or from BY-2 cells (Watanabe et al. 1987; Suzuki et al. 1995). DNA and RNA transcripts were introduced into protoplasts in the presence of polyethylene glycol (PEG) as described earlier (Yamaguchi et al. 2005). We used two reporter genes [for green fluorescence protein (*GFP*) and firefly luciferase (*Fluc*)] to construct the transient expression assay for RNA silencing. The *GFP* gene and its internal control, the *DsRed* gene were cloned in pE2113 (Mitsuhara et al. 1996) to create pE-GFP and pE-DsRed, respectively. For the *Fluc* gene and its internal control, the Renilla luciferase (*Rluc*) gene, plasmids, pBI221 (Clontech, Palo Alto, CA, USA) and pE2113 were used to create pBI-Fluc and pE-Rluc, respectively. As a silencing inducer, dsRNA was prepared by in vitro transcription using a PCR-amplified fragment containing the T7 promoter sequence at both the 5′ and 3′ ends. RNA silencing in protoplasts was induced by co-transfecting with the reporter genes and the inducer, dsRNAs. When GFP was used as a reporter, 3 μg of pE-GFP, 6 μg of pE-DsRed, and 0.3 μg of dsGFP were co-transfected. At 36 h after transfection, transient expressions of GFP were observed with a fluorescence microscope (Leica DMI 6000B) and a filter block containing a 480/40 nm excitation filter, a 505 nm dichroic mirror and a 527/30 nm barrier filter for GFP fluorescence. DsRed fluorescence was viewed with a filter block containing a 515–560 nm excitation filter, a 580 nm dichroic mirror and a 590 nm barrier filter. To measure the expression levels of the reporters, we chose the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) using Fluc and Rluc because this system is far more sensitive than the measurement of GFP fluorescence used in earlier papers. We first tested the effects of dsRNA concentration on Fluc expression in protoplasts at 20 h after transfection. The expression levels of luciferase were measured with a fluorometer (Wallac 1420 ARVO MX, PerkinElmer, Waltham, MA, USA). Viral suppressors (2b of CMV-Y and tombusvirus P19) were cloned in pE2113 to create a recombinant plasmid. For RNA preparation, we used the CMV subgenomic RNA, RNA4A for the 2b gene so that the 2b and P19 genes should be transcribed from the common background. RNA transcripts of 2b and P19 were prepared from the PCR-amplified RNA4A fragment and from the RNA4A fragment whose 2b gene was replaced with the P19 gene, respectively. The PCR products contained the T7 promoter sequence at the 5′ end. Three micrograms of respective suppressor plasmids or RNA transcripts were co-transfected into the protoplasts.

We first compared two types of protoplasts in the dose of dsRNA to induce silencing against GFP and also in the function of a suppressor to interfere with the GFP silencing. With microscopy, we found that the addition of dsGFP effectively induced GFP silencing in Nb cells, but induction was weak in BY-2 cells (Fig. 1). A similar result was obtained consistently when mesophyll cells and cultured cells of *Arabidopsis* were compared; protoplasts from the cultured cells required approximately tenfold higher amount of dsRNA than did the mesophyll protoplasts for sufficient induction of silencing (An et al. 2003). These observations suggest that cultured cells are more tolerant of induction to RNA silencing than mesophyll cells. Surprisingly, viral suppressor 2b failed to inhibit GFP silencing in BY-2 cells, while the 2b transcript successfully restored GFP fluorescence in Nb cells, indicating that the activity of the suppressor varies depending on the plant species (Fig. 1).

To confirm this observation further, we switched to another assay system using a luciferase gene for rapid quantitation of reporter gene expression. The activity of Fluc was measured and normalized relative to that of the internal control, the *Rluc* gene. As shown in Fig. 2, with increasing amounts of dsRNA against Fluc, the relative Fluc activity markedly decreased in both BY-2 and Nb cells, suggesting that silencing can be artificially induced in BY-2 and Nb cells. On the basis of the amount of dsRNA necessary for induction of Fluc silencing, silencing represented by Fluc activity was 2–20-fold more sensitive in Nb cells than in BY-2 cells, suggesting that plant cells differentially operate RNA silencing.

We then compared the activity of a viral suppressor in BY-2 and Nb cells. In BY-2 cells, the presence of 2b resulted in a slight increase of the Fluc activity, but P19 had little effect on silencing in BY-2 cells (Fig. 3a). On the

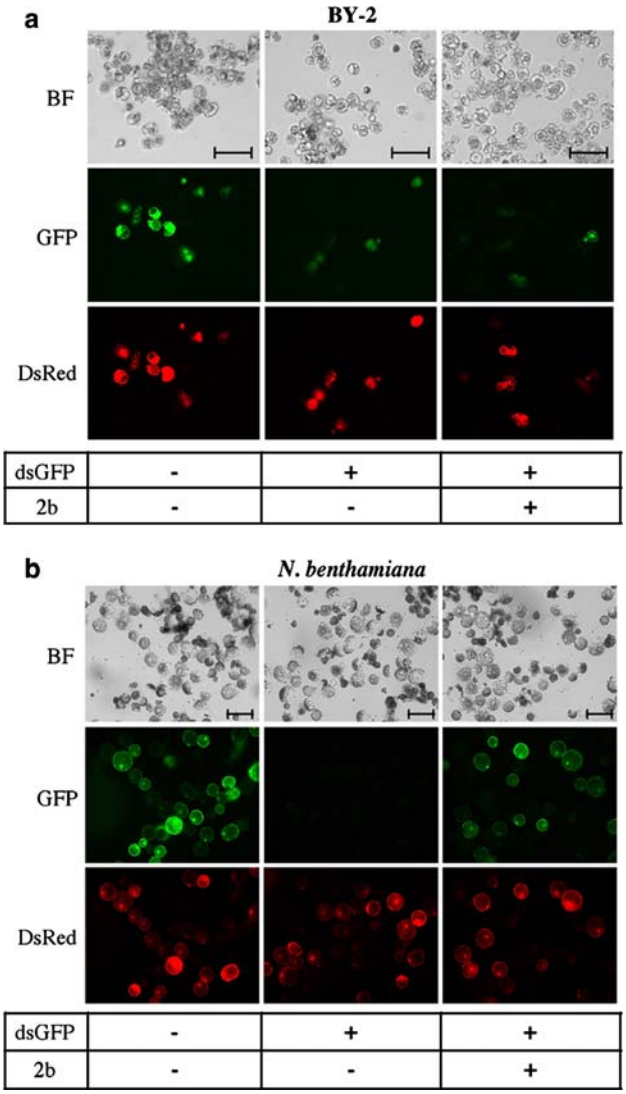


Fig. 1 GFP silencing in protoplasts from BY-2 (**a**) and *Nicotiana benthamiana* (**b**). The prepared protoplasts were co-transfected with pE-GFP (3 μg), pE-DsRed (6 μg), dsGFP (0.3 μg) and viral suppressor 2b (3 μg of RNA transcript). After a 36-h incubation, the protoplasts were observed with fluorescence microscopy. Presence (+) and absence (−) of dsGFP and 2b are indicated below the pictures. *BF* blight field. *Bars* in (**a**) and (**b**), 100 μm

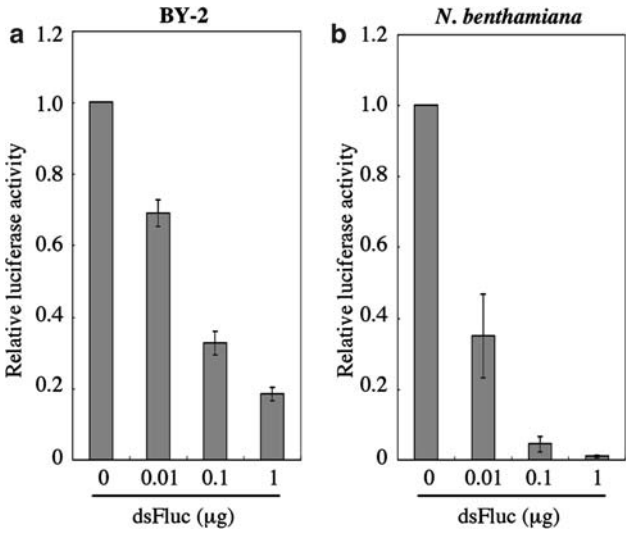
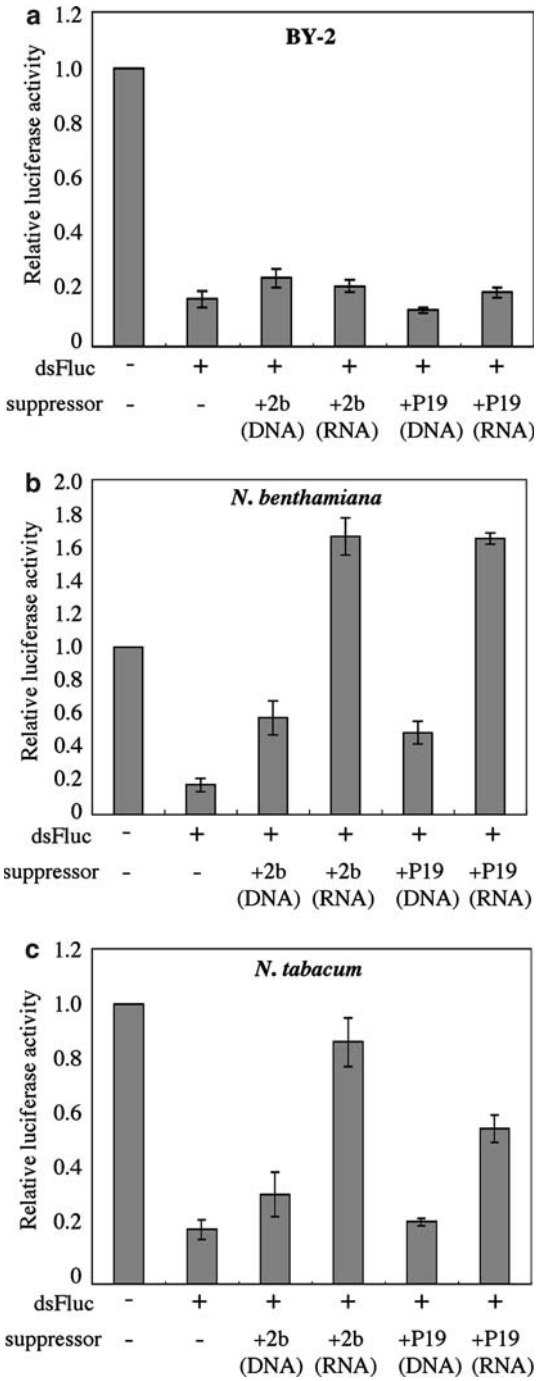


Fig. 2 Fluc silencing in protoplasts from BY-2 (**a**) and *Nicotiana benthamiana* (**b**). The prepared protoplasts were co-transfected with pBI-Fluc (0.5 μg), pE-Rluc (0.5 μg) and dsFluc (0–1 μg). At 20 h after transfection, Fluc activity was divided by Rluc activity; the value for the control (0 μg of dsFluc) was set at 1.0. The values represent the mean with standard deviations (SD) from three replicates of protoplast preparation

other hand, in Nb protoplasts, both 2b and P19 markedly enhanced the expression of Fluc in the presence of dsRNA against Fluc, indicating that they are potent suppressors in protoplasts (Fig. 3b). These observations suggest that the assay system for the activity of the viral suppressor in Nb protoplasts is more effective and more sensitive than in BY-2 protoplasts. To investigate whether this difference is due to the nature of the plant species or the cell types, we conducted the protoplast experiment using mesophyll cells of *N. tabacum* cv. BY-4. We were able to induce Fluc silencing in tobacco mesophyll cells, as efficiently as in Nb cells (Fig. 3c), suggesting that tobacco actually has the potential to induce silencing efficiently in response to an inducer. It is noteworthy that the 2b and P19 transcripts could both enhance Fluc activity over the control level by suppressing the basal level of Fluc silencing induced by the introduced *Fluc* gene itself (Fig. 3b). In tobacco mesophyll protoplasts, both 2b and P19 transcripts functioned effectively just as observed in Nb cells, whereas P19 failed to suppress Fluc silencing when supplied as a plasmid, suggesting that transcription from the introduced gene in protoplasts may have affected the suppressor activity (Fig. 3c). On the basis of all these results, we concluded that suppressors did not seem to function efficiently in cultured cells. There are a couple of explanations for this lack of efficient function. First, the suppressor proteins may not be stably expressed in cultured cells perhaps, because either the suppressor RNA or the protein is rapidly degraded. Second, cultured cells may lack some host factors that are necessary for sufficient activity of the suppressors. We are currently investigating the stability of suppressors introduced in different types of cell.

Researchers primarily use *Agrobacterium*-mediated transient expression assay when they need to study the activity of a viral suppressor even though overexpression of a viral suppressor in the tissues infected with the bacteria may not necessarily reflect natural, cellular functions of the suppressor. As we reported here, the activity of a viral suppressor actually varies depending on the cell species in protoplasts. This phenomenon observed for single cells can be also true for intact plants. For example, we previously



◀ **Fig. 3** Effects of suppressors on Fluc silencing in protoplasts from BY-2 (**a**), mesophyll cells of *Nicotiana benthamiana* (**b**) and *N. tabacum* (**c**). The prepared protoplasts were co-transfected with pBI-Fluc (0.5 μg), pE-Rluc [0.5 μg for (**a**) and (**c**); 0.01 μg for (**b**)], dsFluc [0.3 μg for (**a**) and 0.03 μg for (**b**) and (**c**)] and viral suppressor (3 μg of plasmid DNA or RNA transcript). When 0.04 μg of dsFluc for 3 μg of suppressor was used in the BY-2 system, we also obtained similar results although such a low amount of inducer (0.01–0.05 μg) could not reduce Fluc activity as efficiently as in the Nb system (Fig. 2). At 20 h after transfection, Fluc activity was divided by Rluc activity; the value for the control (0 μg of dsFluc without a suppressor) was set at 1.0. The values represent the mean with standard deviations (SD) from three replicates. Presence (+) and absence (−) of dsFluc and suppressor are indicated below each graph

found that the 2b protein of CMV-Y did not function in soybean although it can efficiently suppress PTGS in many other plants including tobacco (Senda et al. 2004). It is quite conceivable that in different cell types, the expression of the genes involved in the plant silencing pathway may differ and that the proteins from orthologous genes may function differently in different plant species. The protoplast system using the plant of interest therefore offers an important advantage for measuring the natural activity of a suppressor. Use of a protoplast-based system can be an excellent tool for understanding the mechanism of gene silencing in plants.

Acknowledgment We thank Dr. David Baulcombe (Sainsbury Laboratory, John Innes Centre, Norwich, UK) for kindly providing the P19 gene.

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