Cross-kingdom activity of plant virus-encoded silencing suppressors

B. Reavy & S.A. MacFarlane

Plants have evolved several ways of resisting infection by pathogens including viruses. Systemic acquired resistance (SAR) is a general resistance mechanism that exists in tobacco and other plants. Typically, challenge to the lower leaves of a plant with a virus such as tobacco mosaic virus (TMV), induces partial resistance to both TMV and to other unrelated pathogens in upper, uninoculated leaves. More specific resistance to viruses results from the presence of resistance genes in the plant. For example, potato plants carrying the Ry gene are resistant to strains of *Potato virus Y* but are not resistant to other, related viruses. Recently, it has been discovered that plants may also combat virus infection by targeting the virus RNA for sequence-specific degradation by a mechanism known as post transcriptional gene silencing (PTGS). PTGS in plants was first identified as the cause of cosuppression, in which transformation of plants with an additional copy of a host gene could abolish expression of both the new transgene and the host homologue by inducing degradation of cytoplasmic mRNA. This sometimes results in a visible phenotype depending on the gene involved (Fig. 1). This system was shown also to act on viruses; firstly by the demonstration that transformation of plants with non-translatable viral sequences often resulted in either extreme resistance to the same virus, or in a 'recovery' phenotype where plants developed resistance following an initial, virus-susceptible phase. Subsequently, it was shown that PTGS can be induced in plants by virus infection without the need for plant transformation. Some plant viruses have developed suppressor genes that interrupt PTGS in order to protect themselves from inactivation by the plant PTGS system. For example, *Tobacco etch virus* (TEV) and *Cucumber mosaic virus* (CMV) contain genes (HC-Pro and 2b, respectively) that suppress PTGS. These genes appear to operate in different ways so that HC-Pro reverses silencing in all tissues including those in which it is already established, whereas the 2b gene prevents initiation of silencing in newly emerging tissues but has no effect on already established silencing.

Figure 1 Silencing of the endogenous phytoene desaturase gene in *Nicotiana clevelandii* leads to photobleaching of the leaves.

PTGS is not unique to plants and similar mechanisms, called RNA interference or RNA silencing, have been observed in a variety of organisms, including fungi, mice, nematodes, zebrafish and the fruit fly *Drosophila melanogaster*. Recently, it was shown that silencing could be induced in cultured *Drosophila* cells by treatment with double-stranded (ds) RNA, and biochemical studies of this system have revealed details of some of the enzymatic activities involved in this process. The *Drosophila* system is ideally suited to allow the identification of cellular components that interact with and regulate the activities of the biochemical components of the silencing process. Because dsRNA-mediated silencing occurs in a wide variety of organisms, we hypothesised that components of the process might be common between the different organisms. In particular, we were interested to determine if suppressors of silencing from plant viruses could also act in *Drosophila* cells. Double-stranded RNA and DNA plasmids encoding different viral suppressors and the enzyme β-galactosidase were introduced into the cells by a process known as transfection. This leads to a short term or 'transient' expression of the introduced genes, and the induction of PTGS by the dsRNA. The *Drosophila* system mimics some but not all of the events occurring during PTGS in plants, as there is no movement of proteins or RNA molecules between the insect cells and no events similar to the systemic spread of silencing in plants. Nevertheless, it is a very useful system for studying parts of the PTGS mechanism that may be common to a number of organisms.

Suppression of silencing in *Drosophila* cells by the HC-Pro protein As a first test of the activity of plant virus silencing suppressors in *Drosophila* cells, we looked at the effect of expression of the HC-Pro gene on silencing of the *lacZ* gene. The *lacZ* gene codes for the enzyme β-galactosidase and the activity of this enzyme can be detected by staining cells with a substrate that produces a blue colour in the presence of the enzyme. Introduction of this gene alone into *Drosophila* cells produces a blue colour in the cells that have taken up and expressed the DNA. Co-introduction of dsRNA derived from the *lacZ* gene is expected to silence expression of the *lacZ* gene, reducing the number of cells that stain blue. The function of plant virus silencing suppressors can be examined by adding plasmids encoding the suppressor genes to *Drosophila* cells together with the *lacZ* gene and dsRNA, and assaying the number of cells that subsequently stain blue. In these experiments, transfection of the *lacZ* gene alone into *Drosophila* cells resulted in ~80% of the cells staining blue (Fig. 2). The number of cells staining blue was reduced to only ~10% when the *lacZ* expression gene and dsRNA corresponding to the first ~500nt of the *lacZ* gene were introduced into the cells. By contrast, introduction of the *lacZ* gene and *lacZ*-specific dsRNA, together with the TEV HC-Pro gene, resulted in staining of ~50% of the *Drosophila* cells, indicating that the silencing was partially suppressed by expression of the virus suppressor protein.

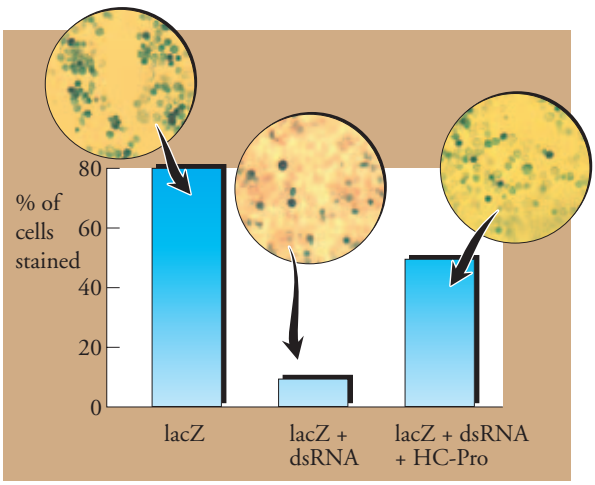

Figure 2 Suppression of gene silencing in *Drosophila* cells by TEV HC-Pro. Cells were transfected with the *lacZ* gene alone (+ lacZ) or with the *lacZ* gene and dsRNA to induce silencing (+ lacZ + dsRNA) or with the *lacZ* gene, dsRNA and the HC-Pro gene to assess suppression of silencing (+ lacZ + dsRNA + HC-Pro). Cells expressing the *lacZ* gene stain blue. The number of cells stained with each treatment is shown in the chart.

This assay is dependent upon simultaneous introduction of three molecules into the cells and the transient expression of the genes involved. Thus, complete suppression of silencing is very difficult to achieve. The efficiency of the silencing suppression assay was increased by production of a stable cell line (DS2-HC-Pro) expressing the HC-Pro protein constitutively. An unrelated cell line (DS2-VCL) expressing a recombinant antibody was used as a control in order to eliminate the possibility that the process of stable transformation of the cells could in some way interfere with the gene silencing mechanism. Silencing was strongly induced in the DS2-VCL control cells when the *lacZ* gene and dsRNA were introduced together, and only 5% of the cells stained for β-galactosidase activity compared to 42% when the *lacZ* gene was introduced alone (Fig. 3). However, ~33% of cells expressed β-galactosidase when the *lacZ* gene and dsRNA were introduced into the DS2-HC-Pro cells, compared to 42% when the *lacZ* gene was introduced alone. This indicates that silencing was significantly suppressed in the cell line expressing the TEV HC-Pro silencing suppressor protein.

Protection of *lacZ* RNA in cells producing the HC-Pro protein A feature of suppression of silencing in

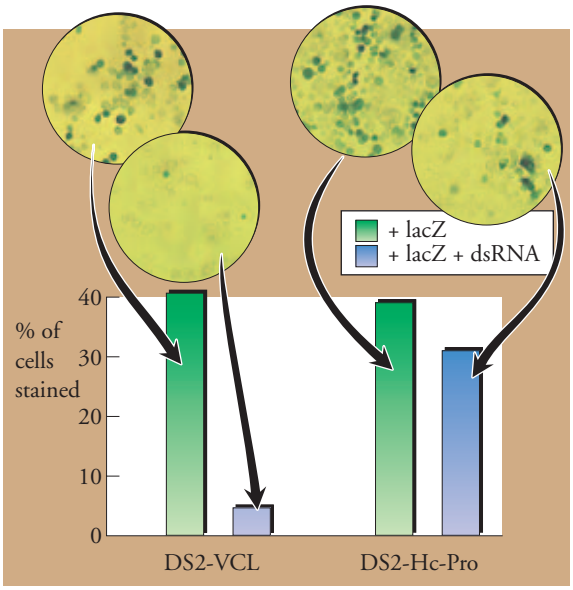

Figure 3 Suppression of gene silencing in a stably-transformed cell line expressing TEV HC-Pro. Control cells (DS2-VCL) on the left, or cells expressing TEV HC-Pro (DS2-HC-Pro) on the right, were transfected with either the *lacZ* gene alone (+ lacZ) or with the *lacZ* gene and dsRNA (+ lacz + dsRNA). The number of cells from each line that stained with either treatment is shown in the chart.

plant cells is that the RNA species targeted by the silencing process is protected from degradation. The RNA present in *Drosophila* cells after transfection with different combinations of the *lacZ* and HC-Pro genes with dsRNA, was examined by northern blotting to determine if the HC-Pro protein was effective in preventing *lacZ* mRNA degradation. No RNA with the expected size of the *lacZ* transcript RNA could be detected in extracts of *Drosophila* cells transfected with the *lacZ* gene and dsRNA (Fig. 4). In contrast, intact *lacZ* RNA was present in *Drosophila* cells when the *lacZ* gene and dsRNA were introduced along with the HC-Pro gene. Similarly, the *lacZ* RNA was intact in DS2-HC-Pro cells after transfection with the *lacZ* gene and dsRNA.

Suppression of gene silencing by the *Tobacco rattle virus* 16K gene Previous studies have indicated that many plant viruses are able to overcome PTGS but often the particular genes involved in this activity have not been identified precisely. We were interested to determine if the *Drosophila* cell system could be used to ascribe silencing suppression activity to other uncharacterised plant virus proteins. *Tobacco rattle virus* (TRV) has been shown to suppress transgene silencing but the specific viral protein responsible for this activity has not been identified. TRV has two genomic RNA species that have been fully sequenced. The larger RNA (RNA1) codes for 134K and 194K proteins that form the viral replicase, a 29K cell-to-cell movement protein, and a 16K cysteine-rich protein. The smaller RNA (RNA2) codes for the coat protein (CP) and may encode other (2b and 2c) proteins involved in virus transmission by nematodes. A characteristic of tobraviruses is that RNA1 can infect plants systemically in the absence of RNA2, i.e. without CP expression and virion formation. This type of infection, referred to as NM-infection, occurs frequently in vegetatively propagated crop plants such as potato and bulbous ornamentals, and is often associated with increased symptom severity. RNA1, therefore, contains all the functions necessary for virus multiplication including, possibly, suppression of gene silencing. The 16K protein is the only protein encod-

Figure 4 Northern blot analysis of *lacZ*-gene specific RNA in control cells (lanes 3-5) or in cells expressing TEV HC-Pro (DS2-HC-Pro, lanes 1,2). Cells were transfected with the *lacZ* gene alone (lanes 1,3), or with the *lacZ* gene and dsRNA (lanes 2,4), or with both the *lacZ* and HC-Pro genes and dsRNA (lane 5).

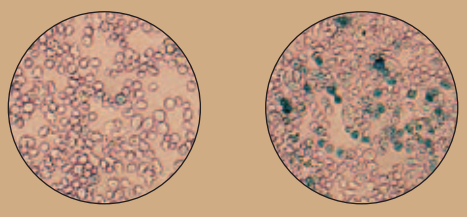

Figure 5 Suppression of gene silencing by the TRV 16K gene. *Drosophila* cells were transfected with the *lacZ* gene and dsRNA (+ lacZ + dsRNA) to induce silencing, or with the *lacZ* gene and dsRNA along with the TRV 16K (+ lacZ + dsRNA + 16K) gene to assess suppression of silencing. Cells expressing the *lacZ* gene stain blue.

ed by RNA1 without an assigned function and we decided to test this gene for silencing suppression activity in *Drosophila* cells.

As described earlier, introduction of the *lacZ* gene and dsRNA resulted in only ~6% of cells staining blue with β-galactosidase activity compared to ~80% staining when the *lacZ* gene alone was introduced. Inclusion of the 16K gene along the *lacZ* gene and dsRNA increased the number of cells staining blue seven-fold when compared to treatments lacking the 16K gene. Expression of the TRV 16K protein in *Drosophila* cells thus prevented dsRNA-mediated silencing of the *lacZ* gene confirming our hypothesis that this protein can function as a silencing suppressor. Currently, we are using this system to screen proteins from a wide variety of plant viruses for silencing suppressor activity.

Conclusions and prospects Our demonstration of suppression of PTGS in *Drosophila* cells by plant virus proteins indicates that at least part of the pathway of PTGS is conserved between plants and *Drosophila.* The TEV HC-Pro and CMV 2b proteins are thought to target different components of the silencing system, as the CMV 2b protein prevents initiation of silencing only in newly emerging tissues, whereas the potyvirus HC-Pro protein suppresses silencing in all tissues. We have been unable to observe any suppression of silencing in *Drosophila* cells by the CMV 2b protein. This could be because the 2b protein is not functional as a suppressor in this cell system, or perhaps the early stages of dsRNA-induced silencing in *Drosophila* differ from the initiation of silencing in plant tissues, possibly by-passing the step at which the 2b gene functions. The different types of silencing suppressor proteins found in plant viruses may be useful in dissecting the biochemical pathway of silencing in *Drosophila* and possibly in other organisms. In addition, it is becoming apparent that silencing may play a role in other processes as well as defence against foreign RNAs. Perturbation of the silencing process has been found to affect development and fertility in plants and germ-line development in nematodes. Intervention with silencing suppressor proteins from plant viruses may therefore have significant utility in determining the involvement of silencing in development and differentiation in both plants and animals.