Potato leafroll virus amplicons in the study of RNA silencing in plants

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Introduction RNA silencing is a type of post-transcriptional regulation of gene expression that is based on the sequence-specific degradation of RNA molecules. Processes related to RNA silencing have been found in a broad variety of eukaryotic organisms including plants, animals and fungi. Thus, cells of 'silenced' eukaryotes are able to degrade RNA species in the cytoplasm that share sequence homology with the triggering RNA molecules. Common to RNA silencing pathways is the generation of dsRNA that corresponds to the target RNA and that acts as a trigger or an intermediate in the degradation process. The dsRNA is cleaved into short interfering (si) RNAs 21 to 25 nucleotides in length that correspond in sequence to both sense and antisense strands of the target RNAs, and these are thought to mediate the specificity for target RNA degradation.

In plants, RNA silencing has been studied most extensively as a post-transcriptional gene silencing (PTGS) mechanism by using transgenic plants. In many studies, plants have been transformed with virus genes in order to determine if resistance is induced against infection with the original virus. However, viruses are both inducers and targets of RNA silencing, and infection of plants with a broad range of viruses results in host defensive RNA silencing responses even in the absence of homologous nuclear sequences. This has led to the idea that RNA silencing in plants may have evolved as a general antiviral defence mechanism. Some viruses encode specific proteins that suppress PTGS, which suggests a co-evolution of defence and counter defence mechanisms between the plant hosts and viruses. [For recent reviews on PTGS see Carrington (2000)[1], Vance and Vaucheret (2001)[2] and Voinnet (2001)[3], SCRI Ann. Rep. 2000-2001, 120-123].

We have been investigating the utility of amplicons (virus-based transgenes) as an exciting and innovative tool for the study of RNA silencing. Amplicons were first described by Angell and Baulcombe (1997)[4] for *Potato virus X*, and extended to other viruses such as *Potato leafroll virus* (PLRV) by Franco-Lara *et al.* (1999)[5] and Barker *et al.* (2001)[6]. An amplicon is a transgene that comprises a full-length (biologically active) copy of a virus genome. In plants that contain an amplicon, transgene-derived mRNA transcripts initiate infection and replication of the amplicon in much the same way as the RNA genome of an invading virus. An important difference is that this process can potentially take place in every cell of the plant. However, such replication also induces very effective silencing of the amplicon. Therefore this system, based on replicating viral RNA, may well prove to be more suitable for obtaining an accurate picture of RNA silencing caused by virus infection than PTGS systems using transgenes. This report describes our recent work to understand the mechanisms involved in amplicon-mediated silencing and our attempts to develop new and more useful amplicons (Fig. 1).

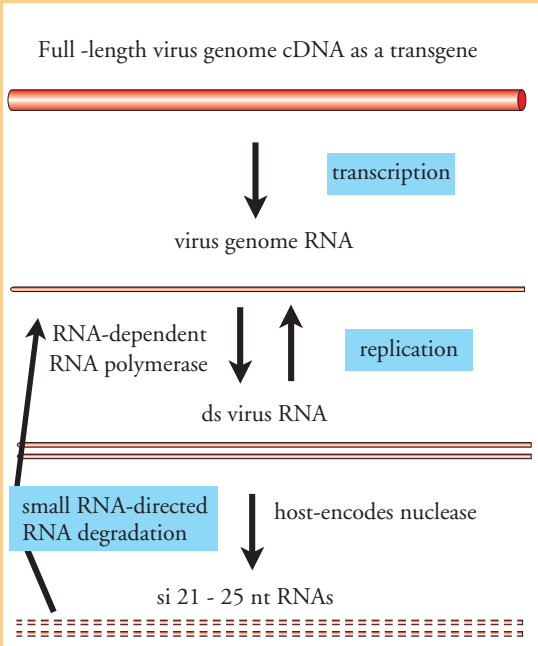

Figure 1 Diagram of amplicon mode of action.

Transformation of *Nicotiana* species with a PLRV amplicon The first amplicon we developed was based on PLRV, (see *SCRI Ann. Rep.* 1999/2000, 142-145). Like other luteoviruses, PLRV cannot be transmitted to virus-free plants by mechanical inoculation. In nature, PLRV is transmitted by aphid vectors that introduce particles into

the vascular tissue of plants where PLRV multiplies and in which it remains largely restricted. PLRV is normally restricted to phloem tissue because the virus lacks movement functions that can operate in epidermal and mesophyll cells and/or because PLRV cannot suppress putative host defence responses in non-vascular tissues. In tobacco plants transformed with the PLRV amplicon, immunoprints of tissue pieces showed that approx. 5% of phloem companion cells were infected, and also that a few mesophyll cells and epidermal cells contained virus. We concluded in 1999 that some mechanism was restricting PLRV multiplication in most cells of these plants whilst permitting replication in a few cells.

In more recent work we have explored this phenomenon further by transforming a better PLRV host, *Nicotiana benthamiana*, with the same amplicon. A small proportion of mesophyll cells (about 2%) in these PLRV amplicon plants contained detectable amounts of PLRV. Surprisingly, we also found that approximately 2% of mesophyll cells in PLRV-infected WT *N. benthamiana* that had been inoculated by aphids, also contained virus. The titres of PLRV, estimated by ELISA, and the extent of mesophyll cell infection were about the same in amplicon plants and in PLRV-infected WT *N. benthamiana*.

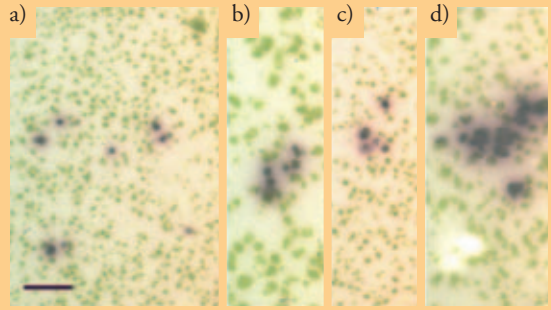

Figure 2 Immunoprints of leaf mesophyll tissue stained for PLRV. Purple spots are PLRV infected cells stained with indoxyl precipitate. (a) PLRV-infected WT *N. benthamiana* showing infected cells, some dispersed and some in pairs; (b) PLRV-infected WT *N. benthamiana* showing a typical cluster of six cells; (c) CW1 N. benthamiana leaf showing a typical cluster of five cells; (d) CW1 *N. benthamiana* leaf showing a typical cluster of approx. 20 cells. Magnification bar in (a) represents 0.5 mm

Because stained mesophyll cells occurred in WT *N. benthamiana* plants inoculated with PLRV, we assume that such cells were infected following cell-to-cell movement of PLRV from cells within vascular tissue. In PLRV amplicon *N. benthamiana* plants, mesophyll cells could be infected *via* a similar route, or as a result of RNA transcript from the PLRV amplicon initiating infection in particular cells. However, because similar numbers of infected mesophyll cells were detected in amplicon transformed and WT plants, it seems likely that the majority of stained mesophyll cells in amplicon plants became infected *via* the same route, as do the cells in WT plants inoculated with PLRV. Immunoprinting (a technique designed to reveal PLRV-infected cells by light microscopy based on a staining method using an anti-PLRV antibody) of leaves from amplicon and PLRV-infected WT *N. benthamiana* showed that a large proportion of stained mesophyll cells were located in clusters (groups of between 2 and 20 cells) (Fig. 2).

Effects of inoculation with PVY on PLRV accumulation in amplicon plants PLRV amplicon *N. benthamiana* plants were inoculated with *Potato virus Y* (PVY) and, 12 days after inoculation, systemically infected leaves were tested by ELISA and immunoprinting. The PLRV titre in these leaves was about 5-fold greater and the number of infected mesophyll cells was about 4-fold greater than in uninoculated control amplicon plants (approximately 1 in 24 mesophyll cells were infected in PVY-infected amplicon plants, and in some areas of the tissue up to 1 in 10 mesophyll cells were stained).

After PVY inoculation of tobacco plants transformed with the PLRV amplicon, the amounts of PLRV in leaf tissue samples were compared with those in leaves of non-inoculated plants; the mean titres were 240 ng/g leaf in non-inoculated plants, and 1600 ng/g leaf in PVY-infected plants. From immunoprints of the non-inoculated amplicon tobacco plants, 1 in 3000 mesophyll cells were found to be stained, whereas in PVY-infected amplicon tobacco plants, 1 in 625 mesophyll cells contained PLRV and clusters of up to four infected cells were found.

Thus potyvirus infection resulted in a 5- to 6-fold enhancement in the accumulation of PLRV in the amplicon transformed plants of tobacco and *N. benthamiana*. The effect of potyvirus infections on suppressing post transcriptional gene silencing (PTGS) is well known, and so our findings raised the possibility that PLRV accumulation in amplicon plants was limited by PTGS (Fig. 1).

Effects of transgenically expressed *Tobacco etch virus* P1/HC-Pro on amplicon plants The experiment described above used PVY infection to test the effects of silencing suppression on amplicon transformed plants. The HC-Pro protein encoded by potyviruses, such as PVY, is known to suppress silencing. In more

direct tests, we used plants transformed so as to produce high levels of the HC-Pro protein of *Tobacco etch virus* (TEV), another potyvirus. In progeny plants from crosses between amplicon tobacco plants and tobacco transformed to express TEV HC-Pro, the mean PLRV titres were about 10-fold greater than in progeny plants from control crosses containing only the PLRV amplicon. Immunoprints made from leaves (mesophyll and sections of mid-vein) of plants of these progenies showed that there were many more PLRV-infected cells in the mesophyll and phloem tissue of amplicon plants that also expressed TEV HC-Pro than in control plants. Thus, in the control plants only 1 in 25000 mesophyll cells were infected, whereas 1 in 55 mesophyll cells were infected in amplicon plants that also expressed TEV HC-Pro. Many stained cells were in small clusters of up to six. Thus expression of TEV HC-Pro resulted in an increase in the amounts of PLRV produced in both mesophyll and phloem tissues. This result is consistent with previous experiments in which HC-Pro was produced as a result of potyvirus infection and suggests that expression of HC-Pro diminishes, at least in part, the resistance to PLRV accumulation in the mesophyll.

Inoculation of *N. benthamiana* amplicon plants with TMV(ΔCP)PLRV-CP It could be predicted that if PTGS occurs in amplicon plants, it should target all RNAs containing PLRV nucleotide sequences. Therefore, as an alternative system for examining PTGS-like effects, we used the chimeric virus TMV(ΔCP)PLRV-CP in which the CP gene of TMV RNA had been replaced by the CP gene of PLRV (Fig. 3). This virus accumulates in inoculated leaves of *N. benthamiana* but does not move systemically, probably because the CP is essential for long-distance movement of TMV and because PLRV CP is unable to provide this function.

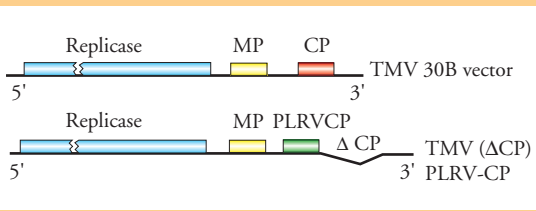

Figure 3 Schematic of TMV(ΔCP)PLRV-CP genome.

When TMV(ΔCP)PLRV-CP was inoculated to WT *N. benthamiana* plants, immunoprints made to locate PLRV antigen in leaves showed that large areas (up to 15 mm across) were infected at 4 days post inoculation (p.i.), and at 8 days p.i. approximately 80% of the leaf area was infected (Fig. 4b). By microscopy, it was possible to see that the majority of individual cells in these visibly stained areas were infected. These results suggested that virus had moved from cell to cell in these leaves. The result was different when TMV(ΔCP)PLRV-CP was inoculated to PLRV-infected WT *N. benthamiana* or amplicon plants. In these plants, by 4 days p.i. antigen-containing cells were in clusters of up to approximately 200 cells. At 8 days p.i. the areas of staining were much larger and

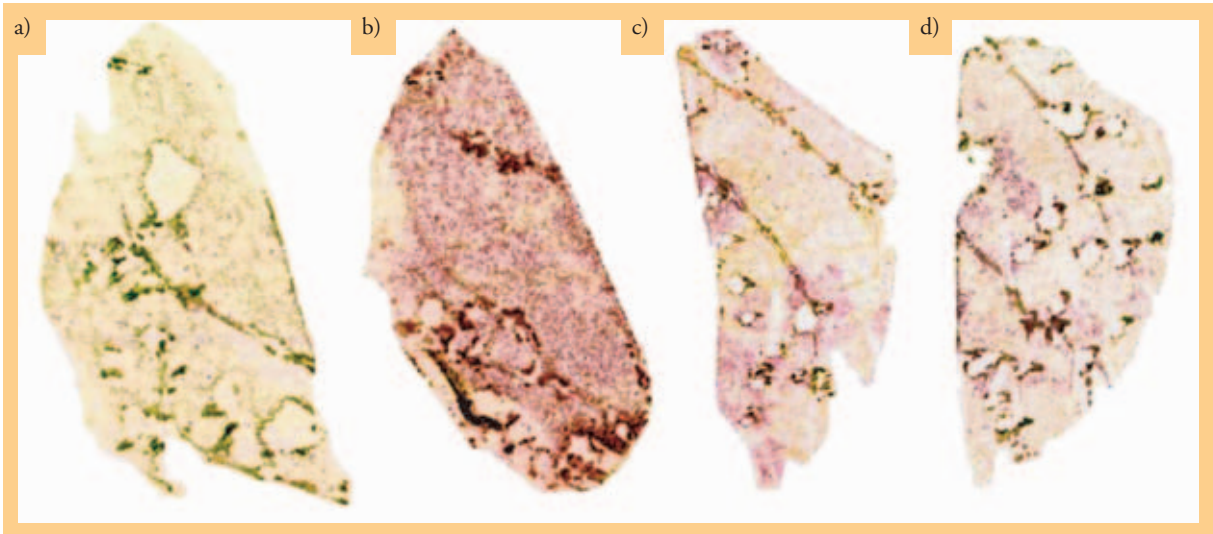

Figure 4 Immunoprints of leaves stained for PLRV. (a) WT non-inoculated *N. benthamiana;* (b) WT *N. benthamiana* 8 days p.i. with TMV(ΔCP)PLRV-CP; (c) PLRV-infected WT *N. benthamiana* 8 days p.i. with TMV(ΔCP)PLRV-CP; (d) CW1 *N. benthamiana* 8 days p.i. with TMV(ΔCP)PLRV-CP.

clusters of up to several hundred cells could be seen in the microscope, and by eye approximately 35% of the leaf area was stained (Fig. 4c & 4d). These results suggest that the delay in spread of TMV(ΔCP)PLRV-CP in inoculated leaves of PLRV-infected WT *N. benthamiana* and amplicon plants occurred because of specific resistance against the virus containing PLRV sequences as a result of PTGS induced by replicating PLRV.

Attempts to make a new PLRV amplicon expressing GFP More recently we have made an improved version of the PLRV amplicon consisting of full-length cDNA corresponding to the genome RNA of PLRV, described above, that has been modified to contain a reporter gene encoding green fluorescent protein (GFP) to monitor virus expression (Fig. 5). This plasmid carries a full-length cDNA PLRV clone in which DNA encoding GFP was inserted in the *P5* gene approximately 300 nucleotides from its 3'-end. The insertion did not prevent modified viral RNA from being replicated because virus particles and GFP-P5 fusion protein accumulated in PLRV-GFP-inoculated cells or protoplasts[7].

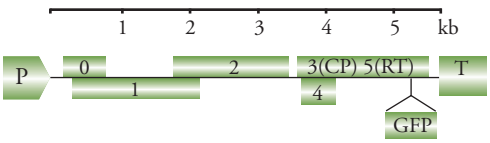

Figure 5 Schematic of PLRV-GFP genome.

Plants of *N. benthamiana* and *N. tabacum* have been transformed with this amplicon. Strong RNA silencing was induced in plants transformed with the PLRV-GFP amplicon, as evidenced by low levels of PLRV-GFP accumulation, lack of symptoms and detection of amplicon-specific si RNAs. Examination by confocal laser scanning microscopy (CLSM) showed that some cells contained GFP but this was restricted to single cells or to small clusters of usually not more than 4 cells that were distributed irregularly

Discussion The advantages of the experimental PLRV amplicon-mediated RNA silencing system we have developed are (i) that PLRV is unable to combat silencing in mesophyll and epidermis (i.e. it is unable to suppress or avoid the effects of silencing), which allows the study of silencing of virus replicating RNAs without any effect from the virus itself, and (ii) that the lack of virus movement from cell-to-cell allows the study of viral RNA silencing in the absence of possible effects from virus movement. Another advantage of amplicon-mediated RNA silencing as an experimental system over conventional silencing systems that use sense or antisense transgenes, is the reproducibility and consistency with which silencing is triggered. Results obtained in this work with the PLRV amplicon, and more recently with the PLRV-GFP amplicon, are enabling us to focus on the various factors, internal and external to the plant, that affect RNA silencing in plants and that could help to improve understanding basic virus defence and counter defence mechanisms. Results of more research on our amplicon plants could prove to be influential in many areas, including the design of improved transgenes for pathogen resistance and understanding the control of natural plant resistance genes.

References

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