Gene expression in plants can be suppressed in a sequence-specific manner by infection with virus vectors carrying fragments of host genes. Recent developments have revealed that the mechanism of this gene silencing is based on an RNA-mediated defence against viruses. It has also emerged that a related mechanism is involved in the post-transcriptional silencing that accounts for between line variation in transgene expression and cosuppression of transgenes and endogenous genes. The technology of virus-induced gene silencing is being refined and adapted as a high throughput procedure for functional genomics in plants.

**Introduction**

A limiting step in genetics and molecular biology is the ability to relate genes to phenotypes and vice versa. In the shadow of genome projects this limitation is more conspicuous than ever. Here, I describe a novel approach to bridging this gap between genetics and molecular biology. This approach is based on recent discoveries concerning plant viruses and gene silencing and is still under development. The early results, however, provide encouragement that virus-based technology will be a useful complement to existing functional genomics tools.

The most straightforward iteration of this approach is referred to as virus-induced gene silencing (VIGS) and employs virus vectors carrying elements from the exons of plant host genes. Analyses in Nicotiana species have shown that the symptoms on these plants are phenocopies of mutations in the corresponding host genes. For example, with tobacco mosaic virus (TMV) [1] and potato virus X (PVX) vectors [2•] carrying a fragment of phytoene desaturase (PDS) mRNA, the upper leaves of the infected plant develop a spectacular bleached effect (Figure 1). The cause of these symptoms is a decline in the level of the endogenous PDS mRNA leading to low levels of phytoene desaturase, a block in carotenoid production and, consequently, the absence of protection against photobleaching. In other examples VIGS has been targeted against a chelatase gene required for chlorophyll synthesis [3•] and against various transgenes [2•,3•,4••,5].

The obvious attraction of VIGS for analysis of gene function is speed and ease of adaptation to high throughput systems. If the partial sequence of a given gene is known, a virus vector construct can be assembled within two days. This construct may be infectious directly as *in vitro* transcripts of cDNA clones [6] or as DNA, if the virus has a DNA genome. Alternatively the cDNA of an RNA virus can be fused to a promoter that is active in plant cells. The cDNA of these constructs is infectious as directly inoculated DNA [7] or by employing an *A. tumefaciens* Ti plasmid vector to transfer the DNA into plant cells [8].
After inoculation of the vector there is a delay of only 7–21 days before VIGS-related symptoms develop in the upper leaves of the infected plant. The relationship from gene to phenotype, therefore, can be established within a few weeks. In genomics jargon this strategy is referred to as reverse genetics. VIGS can also be used in a forward genetics strategy although the resources required are substantial — it would be necessary to construct cDNA libraries in virus vector constructs and to systematically analyse large numbers of infected plants.

In the later sections of this article I assess the potential, the limitations and the practicalities of VIGS both for forward and for reverse genetics. Because the development of VIGS technology is influenced by knowledge, however, I first review the current views about the underlying mechanism.

The mechanism of VIGS

It is likely that VIGS is caused by an RNA-mediated defense (RMD) mechanism in plants against viruses [9,10]. According to this idea there is an as yet uncharacterised surveillance system in plants that can specifically recognise viral nucleic acids and give sequence specificity to RMD. Normally, with wild-type isolates of plant viruses, it is thought that this mechanism is activated as the virus begins to accumulate in the plant (Figure 2). Eventually, as a result of RMD, virus accumulation slows down and eventually stops.

In the situations when a genetically modified virus has similarity to a gene in the host plant, the RMD would target both the viral RNA and the corresponding endogenous mRNA. VIGS would result from the targeting of the endogenous mRNA.

There are two lines of supporting evidence for this model. First there is direct evidence that both RNA nepoviruses [11••] and DNA caulimoviruses can induce RMD [12,13••]. Initially the plants infected with these viruses exhibit symptoms and the viruses accumulate to a high level. However, the upper leaves of these plants emerging after systemic infection have recovered. They are symptom free and have only low levels of viral RNA. This recovery can be attributed to RMD because, in the nepovirus infected plants, there is RNA sequence-dependent resistance against viruses inoculated to these upper leaves [11••]. In the caulimovirus infected plants the recovery is probably due to RMD as there is post-transcriptional suppression of viral RNA in the symptom-free leaves.

The second supporting evidence for RMD is indirect and is based on analyses of plants exhibiting post-transcriptional gene silencing (PTGS) of transgenes. PTGS involves sequence-specific turnover of RNA and is one of the reasons for between-line variation in transgene expression levels [14]. Lines in which a transgene is expressed at a low level include those in which PTGS is active and targeted against the transgene RNA. High level expressors are those in which PTGS is either weak or inactive.

The link with RMD is from the finding that PTGS is suppressed by virus-encoded proteins that are also suppressors of anti-viral defense in the host [15••–18••]. Presumably the suppressed anti-viral defense is the putative RMD and the suppressed PTGS was activated when the transgene or its RNA was detected by the RMD surveillance system. The suppressors of PTGS would be part of a counter defense system that allows viruses to accumulate after activation of RMD [19•]. With some viruses the final steady state level of virus accumulation would be determined by the effectiveness of the suppressors. In other instances the virus may use evasion instead of suppression as a strategy to counteract RMD.

Analysis of PTGS has revealed that there is a signal of gene silencing that can move systemically in plants [20••,21••,22•,23•]. This signal can be rationalised in terms of RMD as it would allow the plant to prevent spread of the virus. If the signal moves at the same time or ahead of the virus it would prime RMD in the cells at, or just beyond the infection front. Consequently the RMD would be activated more rapidly than in the initially infected cells and the spread of the virus would be delayed.

A final point about PTGS concerns DNA methylation. There is an association of DNA methylation and PTGS in several experimental systems [4••,24–27] and evidence that the methylation may be targeted to transgenes by RNA [4••,28]. These observations are difficult to reconcile with the proposed similarity of PTGS and RMD as most plant viruses have RNA genomes. Perhaps the DNA methylation data are an indicator that the underlying mechanism of PTGS is involved not only in anti-viral defense. It could be, for example, that antiviral defense is
related to the mechanisms used to protect against retroposons and other forms of mobile DNA in plants [29].

Development of VIGS technology

This relationship of VIGS, PTGS and RMD has important implications for further development of VIGS technology. It means, for example, that VIGS would be weak if the virus vector encodes a suppressor of PTGS and that the most effective VIGS vectors will be those that use evasion rather than suppression as a strategy to overcome RMD. Vectors based on potyviruses, therefore, would be poor VIGS vectors because they produce strong suppressors of PTGS [15++,16++,18++]. Cucumber mosaic virus would be unsuitable for the same reason [16++,17++]. Conversely, PVX and tomato blackring virus (TBRV) would be suitable vectors for VIGS. Neither of these viruses is able to reverse PTGS in transgenic plants [16++,17++] and it can be assumed that they do not produce suppressors or that their suppressors are weak.

TBRV is a seed and pollen transmitted virus as a result of its ability to infect the progenitor cells of the pollen and eggs in the meristems [30]. This ability to penetrate the meristem means that TBRV would be a particularly effective vector for VIGS. Most plant viruses, including PVX, are excluded from the meristematic zone of infected plants [31] and would not be able to silence genes required for meristem identity and early in leaf development. Unfortunately, as yet, there are no full length cDNAs of TBRV or related viruses that could be developed as VIGS vectors with the ability to target these important genes.

There are several other ways in which understanding of PTGS could facilitate development of VIGS technology. For example, it may be possible to enhance effectiveness of VIGS vectors by engineering increased potential to produce double stranded RNA. Double stranded RNA has been implicated as an initiator of PTGS in plant systems [32++,33++,34++] and of a similar gene silencing phenomenon in nematodes [35,36++].

It may also be possible to enhance VIGS by modification of the host plant. For example, it might be expected that VIGS would be stronger in the background of host gene mutations that enhance gene silencing (egs) [37]. The products of these genes could be repressors of RMD. It might also be expected that VIGS would be stronger in plants that over-express genes required for RMD than in a wild-type host. The genes that are required for gene silencing can be recognised by analysis of the suppressed gene silencing (sgs) [38++] phenotype of mutant alleles.

Transgenic VIGS

In the applications of VIGS, as described above, the activator of gene silencing is the viral RNA in the infected plant cells. The silencing effect is systemic because the virus vector is able to spread through the infected plant. In transgenic VIGS the aim is to produce an RNA that can be recognised by the RMD surveillance system. As in VIGS, this RNA would include an element from a host sequence so that the RMD silences the corresponding gene. This RNA would not need to be infectious, however, because it would be delivered into cells by expression of a transgene.

An advantage of transgenic VIGS over ‘conventional’ PTGS using sense or antisense transgenes should be consistency. The transgenic VIGS constructs would be designed to produce an RNA that is detected by the RMD surveillance system and it would be expected that all, or at least most, of the lines would exhibit silencing of the target gene. In PTGS it is likely that the transgene RNA is only recognised by the RMD surveillance system as a result of sequence rearrangements, DNA methylation of chromatin structures that are associated with the transgene after its introduction into the plant genome. As a result, the degree of PTGS varies greatly between lines.

Transgenic VIGS is less amenable than infectious VIGS to high throughput applications because of the transformation step. In plants that can be transformed with high efficiency, however, it is quite feasible to produce the tens of thousands of transformed lines that would be necessary to survey the gene function in a typical plant. Once produced, the seed from these lines would be a permanent resource that could be used in many different functional screens.

The version of transgenic VIGS corresponding most closely to infectious VIGS employs amplicons. Amplicons are transgenes comprising a promoter and terminator directing transcription of a modified viral vector RNA in the plant cell [39]. The modifications may include deletion or mutation of viral genes required for spread of the virus in the infected plant and of any other functions that are secondary to replication. Expression of the amplicon in the plant cell could activate RMD and, if there are host sequences in the amplicon construct, PTGS of the corresponding host gene.

There are several instances of amplicon transgenes although they are not always referred to as such. A PVX amplicon produced consistent silencing as expected [39]. Similarly, a petunia chalcone synthase gene could be targeted by a gemini virus amplicon with a chalcone synthase insert [40] and a brome mosaic virus amplicon conferred virus resistance that was probably due to RMD [41]. The phenotype of amplicons derived from cucumber mosaic virus [42] and a severe strain of TMV, however, did not appear to involve gene silencing [43]. The differences between these amplicons may be due to the suppressors of RMD encoded in the corresponding viral genomes — genomes producing little or no silencing may produce strong suppressors of RMD, whereas good silencers may be those that do not encode suppressors or from which the suppressor genes have been deleted.

Some alternative approaches to PTGS in transgenic plants may also be considered as transgenic ‘pseudoVIGS’. The success of these approaches depends on the ability of non-viral transgenes to produce the virus-like RNAs that activate
RMD. The gene silencing from simultaneous expression of sense and antisense RNAs [33•] or from transgene constructs with inverted repeats in the transcribed regions [32•] could both be considered in this ‘pseudoVIGS’ category.

**VIGS versus other techniques of functional genomics**

Conventional approaches to forward genetics generally involve insertional mutagenesis either by T-DNA [44] or transposable elements [45,46]. These are powerful approaches that have led to many advances in gene discovery; however, there are several complications with insertional mutagenesis of genes required for basic cell function or early development. In these instances, because the insertional mutation often results in complete inactivation of the target gene, the mutant phenotype will be embryonal and difficult to interpret in terms of the cellular or biochemical role of the gene product. Conversely, if the mutant gene is one of a family it is likely that there may be no phenotype of an insertional mutant because the functional homologues may compensate for the loss of function at the mutant locus.

VIGS and related PTGS-based approaches may circumvent these problems. For example, with multigene families, the gene silencing mechanism will target not only the precise homologue of the gene in the VIGS vector; it will also target partial homologues that vary by up to 10 or 20%. The chlorotic and dwarf phenotypes that result from PTGS of tobacco ribulose bisphosphate carboxylase (rubisco) is a good illustration of how gene silencing can target a multigene family [47,48]. It is unlikely that a phenotype would result from insertional mutagenesis of rubisco because it is encoded in a multigene family. The precise mismatch between different tobacco rubisco genes is not known but, by comparison with other solanaceous plants, it is likely to be up to 14% [49].

When VIGS is targeted against lethal genes the phenotype is death, as in insertional mutagenesis, but the dying process may be more informative than the embryo lethal phenotype of insertional mutants. Because the VIGS is applied to mature plants and because there may be only partial suppression of the target gene, the dying process may be informative about the role of the corresponding gene products. It might be expected, for example, that death due to a block in lipid biosynthesis would be clearly different from death due to suppression of DNA synthesis or proteins required for respiration.

**Conclusions**

VIGS vectors have been developed from the genomes of TMV, PVX and TGMV for applications in Nicotiana species. To develop these vectors for use in other plant species it will be possible to build on recent discoveries that VIGS and PTGS are related to antiviral defense in plants and that viruses produce suppressors of this antiviral defense. When these vectors are available for Arabidopsis, maize and other species it will be possible to combine insertional mutagenesis and VIGS to develop a powerful combined approach to functional surveys of plant genomes.

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**References and recommended reading**

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