Annu. Rev. Plant Physiol. Plant Mol. Biol. 2000. 51:167–94


# (TRANS)GENE SILENCING IN PLANTS: How Many Mechanisms?

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**Key Words** transgene, virus, methylation, transcription, RNA degradation

■ **Abstract** Epigenetic silencing of transgenes and endogenous genes can occur at the transcriptional level (TGS) or at the posttranscriptional level (PTGS). Because they can be induced by transgenes and viruses, TGS and PTGS probably reflect alternative (although not exclusive) responses to two important stress factors that the plant's genome has to face: the stable integration of additional DNA into chromosomes and the extrachromosomal replication of a viral genome. TGS, which results from the impairment of transcription initiation through methylation and/or chromatin condensation, could derive from the mechanisms by which transposed copies of mobile elements and T-DNA insertions are tamed. PTGS, which results from the degradation of mRNA when aberrant sense, antisense, or double-stranded forms of RNA are produced, could derive from the process of recovery by which cells eliminate pathogens (RNA viruses) or their undesirable products (RNA encoded by DNA viruses). Mechanisms involving DNA-DNA, DNA-RNA, or RNA-RNA interactions are discussed to explain the various pathways for triggering (trans)gene silencing in plants.

CONTENTS

## INTRODUCTION

Plants are subject to various endogenous and environmental stimuli that may lead to changes in genome structure and/or genome expression. Because plants are not able to move and cannot escape from their environment, they have developed defenses to limit the potentially deleterious effects resulting from such stimuli.

The movement of transposable elements (TEs) is activated by many stresses (32). Plants with a small genome, like Arabidopsis, carry a limited number of copies of TEs, whereas plants with a large genome, like maize, consist to more than 83% of TEs (9, 80). In both cases, the great majority of these elements are silent, which indicates that plants have developed efficient defenses that limit the expression and mobility of TEs (53, 56).

Many pathogens infect plants by using the cellular machinery for their own purposes. Plants have developed race-specific defenses against particular pathogens, which lead to localized cell death and necrosis around the site of infection; these defenses prevent further spread of the pathogen in the plant (66). However, more than two thirds of the reported defenses against virus infection do not involve a hypersensitive response (HR), but rather are associated with other mechanisms (30). In one mechanism, which is observed with RNA viruses, plants trigger the sequence-specific degradation of the viral RNA. Alternatively, the virus persists in a noninfectious form which is observed with DNA viruses (1, 13, 77, 78).

The genome structure of plants can also be altered by genetic transformation. Organisms such as *Agrobacterium tumefaciens* integrate part of their genome into the genome of susceptible species. Recently, genetic transformation techniques have begun to modify significantly the organization of the genome. Indeed, introducing transgenes into plants can both modify the number of copies of a given sequence and affect gene expression. Because the expression of a transgene cannot always be predicted, interest in studying the consequences of genetic transformations at the genome level has increased considerably over the past ten years (reviewed in 17, 20, 27, 29, 55, 56, 61, 83, 92). Transgenes can become silent after a (more or less) long phase of expression, and can sometimes silence the expression (at least partially) of homologous elements located at ectopic positions in the genome. In some cases, the silencing of transgenes also triggers resistance against homologous viruses; in other cases, infection by viruses triggers silencing of homologous transgenes (5, 6).

The silencing of transgenes probably results from the activation of defense mechanisms, indicating that plants possess systems for controlling genome structure and gene expression (56). The transgene itself or its product(s) are probably perceived as endogenous stimuli that activate this machinery. The study of transgene silencing provides an appropriate way to understand the different mechanisms controlling plant genome structure and expression. This review summarizes current knowledge on silencing events mediated by stably integrated transgenes and DNA and RNA viruses.

## METHODS AND RULES FOR THE CLASSIFICATION OF SILENCING EVENTS

One factor that makes it difficult to determine the precise number of mechanisms involved in silencing is the diversity of analytical methods used by different research groups. It is important to define the largest number of parameters and criteria that allow one mechanism to be discriminated from others and then to analyze each silencing event according to these parameters and criteria.

### Sources and Targets of Silencing

In analyzing silencing events, it is important to distinguish the source leading to silencing from the target that is being silenced. Four scenarios are described in the literature.

1. An element can be exclusively the source of silencing without being subjected to the silencing process it triggers in *trans*. Examples of transgenes or viruses that silence homologous genes but are not affected themselves have been reported (58, 78, 79) and are described in detail below.
2. An element can be a source of silencing but affect only itself, in which case, it is said to occur in *cis*. Examples of transgenes that are silenced when inserted into a particular structure or into a particular location of the genome, and that do not affect the expression of any other element have been reported (4, 49, 51, 64, 100) and are detailed below.
3. An element can be a source of silencing for itself and for homologous ectopic elements, i.e. silencing occurs in both *cis* and *trans*. Examples of transgenes or viruses that are simultaneously sources and targets of silencing, and that trigger silencing of homologous ectopic elements have been reported (3, 14, 15, 25, 34, 38, 50, 60, 68, 71, 73, 82, 86, 88, 91) and are detailed below.
4. An element can be exclusively a target for silencing by trans-acting elements, i.e. it is not a source leading to silencing. Many examples exist of transgenes, endogenous genes, and viruses in which expression is silenced only when the element is brought into the presence of other homologous silenced elements.

## Molecular Parameters

Many molecular criteria can help to classify silencing events. Ideally, analysis of silencing would include all the molecular characteristics listed below. Unfortunately, as shown in Tables 1 and 2, none of the silencing events reported in the literature has been analyzed with all these criteria. Consequently, it is a matter of speculation to determine if separate silencing events rely on the same type of mechanism.

***TGS versus PTGS*** Silencing may result from a block of transcription (TGS; 60), or from the degradation of RNA (PTGS; 15). Northern blot assays performed on cytoplasmic RNA combined with run-on transcription or RNase protection assays performed with isolated nuclei enable TGS to be distinguished from PTGS. For TGS, the absence of transcription in the nucleus and the failure of RNA to accumulate in the cytosol provide the result, while for PTGS transcription occurs but RNA fails to accumulate. Not all silencing events have been analyzed at the nuclear level (by run-on or RNase protection assays); thus the question of which type of silencing occurs remains unresolved in many cases (for example, see 14, 35). Other types of analyses may help to solve this ambiguity; for example, TGS correlates with methylation in the promoter, whereas PTGS correlates with methylation in the coding sequence; TGS is both mitotically and meiotically heritable, whereas PTGS is meiotically reversible. However, such short-cuts may be dangerous because they could prevent the discovery of counter-examples, such as TGS events not associated with methylation as in yeast and Drosophila.

***Copy Number*** In only a few cases have transgene loci been recloned and sequenced (37, 58, 67, 74). Two analyses indicated that a single transgene copy can be subjected to silencing in *cis* (37, 74), whereas two other cases demonstrated clearly the requirement of a particular inverted repeat structure to trigger silencing in *trans* (58, 67). It is thus unresolved whether a single transgene copy can trigger silencing in *trans*. The studies of *trans*-silencing loci that suggested the presence of a single transgene copy were all based on southern blot analysis (19, 22, 71, 76, 99). This is an imperfect method to score for small rearrangements of the transgene such as partial duplications of the inserted DNA that are thought to play a role in triggering silencing (4, 25, 34, 42, 51, 58, 81, 84).

***Transcription*** Silencing loci may be transcribed at a high level, low level, or not at all (i.e. below detectable levels). Whether the level of transcription is important for triggering silencing is an important question, one that may be addressed by introducing transgenes driven either by promoters of different strengths or without a promoter, and then comparing their effect (75, 88). However, due to position effect, this approach does not provide definite proof. Transcription of transgenes driven by the 35S promoter may be blocked by the 35S-specific silencing locus of the tobacco line 271 (86, 91), which allows the requirement for transcription

**TABLE 1** Characteristics of representative TGS events triggered by transgenes or viruses

| Examples | Trans-silenced homologous targets | | Molecular characteristics of silencing locus | | | | | Genetic modifiers | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Viruses | | Mutations | |
| | Endogenous genes or transgenes | Viruses | Minimal copy number | Transcription (run-on) | Transcription required | Methylation of promoter region | Systemic acquired silencing | CMV | TEV, PVY | *sgs* | *ddm, som, hog, sil* |
| Surrounding heterochromatin | | | | | | | | | | | |
| p35S-A1 | — | — | 1 (74) | — | — | — | — | — | — | — | — |
| Repetitive sequences (RPS) | | | | | | | | | | | |
| RPS/p35S-GUS | — | — | 1 (85) | — | — | N (85) | — | — | — | — | — |
| Transgene-genomic junctions | | | | | | | | | | | |
| p35S-A1 | Y (60) | — | 1 (74) | N (60) | — | Y (60) | — | — | — | — | — |
| p35S-HPT | — | — | 1 (37) | — | — | — | — | — | — | — | — |
| (Trans)gene repeats | | | | | | | | | | | |
| p35S-HPT | — | — | >1 (62) | — | — | Y (63) | — | — | — | N (21) | Y (63) |
| p35S-GUS | N (u) | — | >1 (u) | N (u) | — | Y (u) | — | — | — | N (u) | Y (u) |
| pNos-NPT | — | — | >1 (14) | — | — | Y (31) | — | — | — | — | Y (31) |
| pNos-OCS | Y (54) | — | >1 (54) | — | — | Y (54) | — | — | — | — | — |
| p35S-RiN | Y (91) | — | >1 (91) | N (93) | — | Y (91) | N (u) | N (u) | N (u) | — | — |
| PAI1-PAI4 | Y (39) | — | >1 (8) | Y (8) | N (52) | Y (52) | — | — | — | — | Y (39) |
| Aberrant promoter transcripts | | | | | | | | | | | |
| p35S-pNos | Y (58) | — | >1 (58) | Y* (58) | Y (58) | — | — | — | — | — | — |
| DNA viruses | | | | | | | | | | | |
| CaMV | Y (1) | — | — | Y (1) | — | — | — | — | — | — | — |

Y: yes; N: no; —: not determined; Y*: determined by northern; (number): reference; (u): unpublished data from our lab.

**TABLE 2** Characteristics of representative PTGS events triggered by transgenes or viruses

| Examples | Trans-silenced homologous targets | | Molecular characteristics of silencing locus | | | | | Genetic modifiers | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Viruses | | Mutations | |
| | Endogenous genes or transgenes | Viruses | Minimal copy number | Transcription (run-on) | Transcription required | Methylation of transcribed region | Systemic acquired silencing | CMV | TEV, PVY | *sgs* | *ddm, som, hog, sil* |
| Sense transgenes | | | | | | | | | | | |
| p35S-NIA | Y (94) | — | 1 (71) | Y (94) | Y[1] (94) | Y (u) | Y (70) | Y (7) | — | Y (21) | — |
| p35S-GUS | Y (22) | Y (24) | 1 (22) | Y (22) | Y[1] (24) | Y (21) | Y (70) | Y (10) | Y (2) | Y (21) | — |
| p35S-TEV | — | Y (50) | 1 (19) | Y (50) | — | — | N* (19) | — | — | — | — |
| Δpro-CHS | Y (88) | — | >1 (88) | N (84) | — | Y (84) | — | — | — | — | — |
| Antisense transgenes | | | | | | | | | | | |
| p35S-aMET1 | Y (28) | — | — | — | — | — | — | — | — | N (u) | — |
| p35S-aGUS | Y (u) | — | — | — | — | — | N (u) | — | — | N (u) | — |
| p35S-aPVY | — | Y (99) | >1 (99) | — | — | — | — | — | — | — | — |
| p35S-aNII | Y (86) | — | >1 (91) | N (93) | — | Y (91) | N (u) | Y (u) | Y (u) | — | — |
| Sense/antisense transgenes | | | | | | | | | | | |
| p35S-ACC-aACC | Y (34) | — | — | — | — | — | — | — | — | — | — |
| p35S-GUS-aGUS | Y (99) | — | — | — | Y[2] (99) | — | — | — | — | N (u) | — |
| p35S-PVY-aPVY | — | Y (99) | 1 (99) | — | — | — | — | — | — | — | — |
| Viruses inducing recovery | | | | | | | | | | | |
| CaMV | Y (1) | — | | | | | — | — | — | — | — |
| TBRV | — | Y (77) | | | | | — | — | — | — | — |
| TRV | Y (78) | Y (78) | | | | | — | — | — | — | — |
| Viruses not inducing recovery | | | | | | | | | | | |
| TGMV | Y (46) | — | | | | | Y* (46) | — | — | — | — |
| PVX | Y (79) | Y (78) | | | | | Y* (79) | — | — | — | — |

Y: yes; N: no;—: not determined; Y[1]: construct brought into the presence of the 35S-silencing locus 271; Y[2]: transformation with a promoterless contruct; N*: primary determination could be reinterpreted; Y*: hypothesized when infecting transgenic plants; Δpro: promoterless construct; (number): reference; (u): unpublished data from our lab.

to be evaluated independently of position effect (24, 94). However, such a strong and specific promoter silencer exists only in tobacco. Transcription may also be controlled by inducible promoters. Surprisingly, no examples of silencing events triggered by such promoters have been reported in the literature. Finally, the promoter of transgenic silencing loci may be eliminated by using the Cre-*lox* system. However, site-directed deletions such as these may also trigger structural changes in the transgene locus that could modify its silencing properties.

***Production of a Systemic Silencing Signal*** Basic grafting experiments have demonstrated clearly that a sequence-specific systemic silencing signal is produced in some cases of PTGS, which allows PTGS to propagate and become amplified throughout the plant (70, 72, 95, 96). Whether this is a common aspect of all PTGS events is unknown. Also to be determined is whether TGS could rely on the production of a presumably diffusible or transported molecule (98).

***Methylation*** Methylation has often been associated with silencing. Although methylation can sometimes affect a large part of a transgene locus, TGS correlates mainly with methylation of the promoter sequence (4, 18, 49, 51, 54, 57, 60, 63, 73, 74, 84, 91, 100), whereas PTGS correlates with methylation of coding sequences (21, 25, 38, 41, 84). However, whether methylation is a cause or a consequence of silencing is not known. Furthermore, methylation has usually been scored using methylation-sensitive enzymes, and rarely by genomic sequencing in which methylation of all sites is assayed. It is thus difficult to conclude that methylation is not involved in silencing when only a limited number of methylation-sensitive enzymes have been used.

### Genetic Modifiers

Mutants affected in TGS or PTGS have been identified recently (16, 21, 31, 39, 63), allowing a genetic classification of silencing events. To date, few silenced loci have been transferred to these mutants to test whether release of silencing occurs, mainly because the mutants were obtained in Arabidopsis whereas a larger number of silencing events were identified in crop species.

The release of PTGS by non-homologous viruses has also been reported recently (2, 7, 10, 44), indicating that viruses can interfere with the plant silencing machinery. Therefore, an additional criterion to classify silencing relies on the analysis of their sensitivity to infection by such viruses.

## TRANSCRIPTIONAL GENE SILENCING

TGS corresponds to a block of transcription. TGS has been shown to affect sequences that are integrated in the genome and not extrachromosomal DNA. However, it has been reported that artificially methylated sequences introduced

transiently in plant cells are not expressed, even when using methylation-target-free promoters. This indicates that methylation of the coding sequence is sufficient to block expression (35). Since it has not been determined whether these methylated sequences are transcribed or not, it is not possible to classify this type of silencing event as TGS or PTGS.

As shown in Table 1, TGS of integrated sequences can be classified into six classes according to the nature of the source of silencing. Whether TGS occurs in *cis*, simultaneously in *cis* and *trans*, or in *trans* only is discussed individually.

## TGS Mediated by Surrounding Heterochromatin

Transgenes insert randomly into the genome. Depending on the position of their insertion, they may be surrounded by euchromatin or heterochromatin. In the latter case, the transgene adopts the structure of the surrounding transcriptionally silent chromatin, thus leading to TGS. This phenomenon can affect transgenes present even as a single copy (74).

## TGS Mediated by Endogenous Repetitive Sequences

Repetitive sequences (RPS) exist in the genome of most plant species and are often methylated. The association of a methylated RPS element from petunia with a 35S-GUS transgene destabilizes its expression in transgenic tobacco and petunia plants, leading to variegation (85). This RPS element probably attracts repressive chromatin complexes, which then spread into the neighboring 35S-GUS transgene. Although de novo methylation of the RPS element has been observed, there is no evidence for methylation of the 35S-GUS transgene (85).

To determine whether or not this TGS effect relies on a *trans*-effect of endogenous RPS elements on the RPS-associated transgenes, the RPS-p35S-GUS transgene was introduced into Arabidopsis, which lacks this RPS element. Methylation occurred at the RPS element, even when present as a single copy, which suggests that a stem loop region present in this RPS element is a target for de novo methylation by the cellular machinery (P Meyer, personal communication). A protein was characterized that binds to this RPS element. It shows similarities to proteins that form repressive chromatin complexes in yeast and Drosophila (two organisms that show TGS but lack methylation), suggesting that methylation per se is not necessary to repress transcription. Rather, methylation of the RPS element probably recruits chromatin components that induce TGS of neighboring transgenes (P Meyer, personal communication).

Of interest will be the resolution of whether the *cis*-TGS effect mediated by RPS can be modified in the Arabidopsis *ddm1* mutant, which is impaired in synthesizing a chromatin remodeling factor (39, 40) or in the *ddm2* mutant, which is affected in synthesizing the MET1 DNA-methyltransferase (26, 28; E Richards, personal communication). This analysis will allow a precise determination of the respective roles of methylation and chromatin structure on TGS.

## TGS Mediated by Transgene-Genomic Junctions

Integration of a single transgene copy in a nonmethylated area of the genome generally allows transgene expression. However, expression may be unstable, leading to variegation when part of the plant genome is silenced, for example by environmental factors, or to non-Mendelian segregation when the DNA of part of the progeny is silenced. Transcriptionally silenced individuals show methylation and a condensed chromatin structure (60, 87). Molecular analysis of such unstable TGS events affecting single transgene copies indicated that either the GC content of the transgene differed significantly from that of the surrounding genomic sequences (23, 60), or the presence of backbone plasmid DNA unexpectedly transferred with the transgene (37). It was therefore hypothesized that such a local discrepancy may disorganize chromatin structure and contribute to destabilizing gene expression (48, 60, 74). Surprisingly, one of these TG-Silenced loci was able to silence the expression of an active allelic copy brought in by crossing; this copy then was able to silence another active allelic copy (60). This phenomenon is reminiscent of paramutation in plants, a phenomenon involving conversion of the epigenetic state of an endogenous allele (paramutator) which is silent and methylated to an active allele (paramutable) that suggests cross-talk between homologous chromosomes in somatic tissues.

## TGS Mediated by (Trans)Gene Repeats

Integration of multiple copies of a transgene in a particular spatial arrangement may lead to methylation and TGS (4, 14, 49, 63). In one case, TGS was shown to correlate with chromatin condensation (4, 100). The implication of repeats in this process was elegantly demonstrated by analyzing internal deletions within this transgene locus that eliminate TGS (4), thus ensuring that TGS was not mediated by *cis*-surrounding sequences or by particular transgene-genomic junctions, as outlined above. In other cases, the contribution of repeats versus that of surrounding sequences remains unclear because either no internal deletions were identified (14, 49) or internal deletions that eliminate TGS could also have modified transgene-genomic junctions (62).

Two transgenic lines hypothesized to be TG-Silenced (14, 62) and one transgenic line in which run-on assays clearly identified TGS (P Mourrain & H Vaucheret, unpublished data) were used to identify mutants and/or to test the effect of previously identified genetic modifiers. Mutants impaired in the *SGS1* or *SGS2* genes, which control PTGS (21), failed to release TGS from the two tested loci (21; P Mourrain & H Vaucheret, unpublished data), suggesting that *SGS* genes play a role specific to PTGS. Conversely, mutants impaired in the *DDM1* gene encoding a chromatin remodeling factor (40) released TGS from the three loci (31, 63; P Mourrain & H Vaucheret, unpublished data). Mutants impaired in the *DDM2* gene encoding the major DNA-methyltransferase of Arabidopsis (also termed *MET1*; 26, 28: E Richards, personal communication) or transgenic plants expressing an

antisense *MET1* RNA failed to release TGS from one line (63), had very little effect on another line (I Furner, personal communication), but released TGS from the third line (P Mourrain & H Vaucheret, unpublished data). These results suggest a range of efficiency in TGS that might be due to methylation alone or a combination of methylation and chromatin remodeling (see conclusions on TGS below). Analysis of the effect of *som* (63), *hog*, and *sil* (31) mutants on the different reporter loci, as well as characterization of the corresponding genes, should ensure a more complete analysis of the genetic determinism of TGS.

In two cases, integration of multiple copies of a transgene in a particular spatial arrangement led to methylation and TGS in both *cis* and *trans*, i.e. transgenic loci were able to silence ectopic target transgenes driven by homologous promoters (54, 57, 86, 91). The molecular mechanism of transmission of TGS from these two silencing loci to their targets remains unclear. It may involve transient DNA-DNA pairing between the silencing loci and their targets, followed by the imposition of a mitotically and meiotically heritable transcriptionally repressive state on the targets (54, 73, 94). Alternatively, it may result from the production of specific molecules by the silencing loci that impose such a mitotically and meiotically heritable transcriptionally repressive state on the targets (73, 98). The molecules required to trigger TGS may be below detectable amounts. In addition, the diffusion of putative silencing molecules would certainly be restricted to the cell, and these molecules would be unable to propagate from cell to cell, as there is no evidence for graft-transmission of *trans*-TGS from silenced rootstocks to target scions (H Vaucheret, unpublished data). *Trans*-TGS seems to require a specific arrangement of transgene copies and a specific degree of methylation of the silencing locus, because hypomethylated epigenetic variants as well as mutants with a rearranged hypomethylated locus are unable to trigger *trans*-TGS (P Mourrain & H Vaucheret, unpublished data). *Trans*-TGS does not require the presence of symmetrical methylation sites in the targeted promoters, whereas symmetrical sites are required to maintain silencing after meiotic elimination of the silencing locus (18). This latter experiment shows that methylation plays a role in maintaining *trans*-TGS rather than in its establishment.

Strong evidence for a DNA-DNA directed trans-methylation mechanism was suggested by the analysis of the effect of an endogenous inverted repeat of the *PAI1* and *PAI4* genes carried by the Ws strain of Arabidopsis on the unlinked *PAI2* and *PAI3* single copies (8). When introduced by crossing into the Col strain (carrying single nonmethylated *PAI1*, *PAI2*, and *PAI3* copies), this inverted repeat triggers methylation of unlinked endogenous *PAI2* and *PAI3* copies (52). Surprisingly, one of the *PAI* genes of the endogenous inverted repeat of the Ws strain is expressed at a high level despite being methylated (8; J Bender, personal communication), thus leaving open the possibility that it produces silencing RNA molecules. However, introduction of a transgene consisting in a promoterless *PAI1-PAI4* inverted repeat in the Col strain also triggers methylation of unlinked endogenous *PAI2* and *PAI3* copies. The absence of fortuitous expression of transgene RNA (checked by RT-PCR) led the authors to suggest a direct DNA-DNA pairing

mechanism for the transmission of methylation (52). Methylation of the multigene *PAI* family requires *DDM1* and *DDM2* genes. Indeed, when brought into the Ws strain, the *ddm1* mutation strongly reduces methylation of *PAI2* and *PAI3* (80% reduction), but has little effect on the *PAI1-PAI4* inverted repeat (20% reduction). Conversely, when brought into the Ws strain, the *ddm2* mutation reduces methylation of *PAI2, PAI3,* and the *PAI1-PAI4* inverted repeat (70% reduction), which suggests that the *PAI1-PAI4* inverted repeat is in a more open chromatin configuration than the singlet *PAI2* and *PAI3* genes, and is thus less dependent on *DDM1* for access of the DNA to methylation (J Bender, personal communication). Methylation of *PAI2* is accompanied by silencing (39). Both *ddm1* and *ddm2* cause a loss of *PAI2* methylation and silencing when brought in the Ws *pai* mutant background (which carries a deletion of the *PAI1-PAI4* inverted repeat). This indicates that the maintenance of *PAI2* silencing in the absence of the *PAI1-PAI4* inverted repeat requires the integrity of both *DDM1* and *DDM2* genes (39; J Bender, personal communication). Here again, analysis of the effect of other genetic modifiers (*som*, *hog*, *sil*, and *sgs*) is needed.

## TGS Mediated by Aberrant Promoter Transcripts

The production of diffusible silencing RNA molecules that trigger TGS in *trans* was shown when a transgene made of the Nos promoter sequences (pNos) under the control of the 35S promoter was constructed for this purpose (58). Plants expressing polyadenylated pNos RNA failed to silence pNos-driven transgenes, whereas one plant producing truncated non-polyadenylated pNos RNA triggered *trans*-TGS and methylation. This plant carries two incomplete copies of the transgene arranged as an inverted repeat (IR), with pNos sequences at the center. This transgene locus produces RNA that could potentially adopt a hairpin conformation. The production of this distinctive RNA is required for *trans*-TGS of pNos-driven target transgenes since *trans*-TGS does not occur when transcription from the 35S promoter is impeded by the tobacco line 271-locus (58). This is the first evidence for *trans*-TGS mediated by an RNA, and it is not known whether other previously described *trans*-TGS events involve the production of an aberrant RNA that triggers methylation of the promoter of target transgenes and TGS. Once again, introduction of this system into Arabidopsis and confrontation with the previously identified genetic modifiers *ddm*, *som*, *hog*, *sil*, and *sgs* should provide insight into the mechanisms involved.

## TGS Mediated by DNA Viruses

One example of *trans*-TGS mediated by a nuclear DNA virus was reported recently (1). Wild-type *Brassica napus* plants recover naturally from CaMV-infection by a PTGS-like mechanism, i.e. 19S and 35S RNA encoded by CaMV are degraded while replication of CaMV DNA is occurring in the nucleus (see PTGS section). CaMV-infection of transgenic *B. napus* plants expressing a p35S-GUS transgene with a 35S or Nos terminator leads to recovery from CaMV infection and PTGS

or TGS of the p35S-GUS transgene, respectively. These results led the authors to suggest that, in the presence of homology in both promoter and transcribed regions, PTGS preferentially occurs, whereas TGS occurs only if the homology is restricted to the promoter region (1). Such *trans*-TGS mediated by DNA viruses resembles *trans*-TGS mediated by the tobacco transgenic line that expresses an aberrant RNA homologous to the Nos promoter (58). In both cases, the source of *trans*-TGS (CaMV, p35S-pNos transgene) is not subjected to TGS, and TGS involves the production of RNA either of aberrant structure (p35S-pNos transgene) or targeted for degradation by the cellular machinery (CaMV).

## Conclusions on TGS

TGS can be triggered in *cis* or in *trans*. *Cis*-acting elements may be endogenous heterochromatin surrounding the transgene locus (74), endogenous repeated and methylated elements located close to the transgene locus (85), transgene-genomic junctions that disturb chromatin organization (37, 60, 87), or particular arrangements of transgene repeats that create heterochromatin locally (4, 100). *trans*-acting elements may be allelic or ectopic homologous loci that potentially transfer their epigenetic state by direct DNA-DNA pairing or protein-mediated DNA-DNA interactions (52, 54, 60, 86), or ectopic transgenes (58) or nuclear DNA viruses (1) that produce a diffusible signal (aberrant RNA, PTGS-targeted viral RNA) that potentially imposes an epigenetic silent state by interaction with the homologous promoter of target transgenes.

In all cases, TG-Silenced transgenes show hypermethylation (4, 18, 49, 51, 54, 57, 60, 63, 73, 74, 84, 91, 100). In cases where it was tested, chromatin condensation was also observed (87, 100). Some, but not all, TG-Silenced (trans)genes are reactivated in the methylation-deficient mutant *ddm2* or in plants expressing a *MET1* antisense RNA (63; J Bender, personal communication; I Furner, personal communication; P Mourrain & H Vaucheret, unpublished data), suggesting that methylation plays a critical role in some but not all TGS events. At these loci, transgene methylation could constitute the primary determinant that allows the attraction of nuclear factors, such as MeCP2, which specifically bind to methylated cytosines and assemble local chromatin into a repressive complex (43). Since the *DDM2* gene encodes the major DNA methyltransferase activity (MET1; 26, 28: E Richards, personal communication), *ddm2* mutants could release TGS only from loci in which the formation of repressive chromatin complexes depends essentially on the presence of methylation. Conversely, at other loci, repressive complexes could be formed independently of methylation, and methylation could be an indirect consequence of this chromatin state. The *DDM1* gene encodes a protein of the SWI2/SNF2 family that plays a role in various functions including transcriptional co-activation, transcriptional co-repression, chromatin assembly, and DNA repair (40). Both the repressive chromatin state and hypermethylation associated with TGS are expected to be lost in *ddm1* mutants, allowing the release of TGS from any locus.

## POSTTRANSCRIPTIONAL GENE SILENCING

Three papers published in 1990 (68, 82, 89) demonstrated that introduction of transcribed sense transgenes could down-regulate the expression of homologous endogenous genes, a phenomenon called co-suppression (68). Co-suppression results in the degradation of endogenous gene and transgene RNA after transcription (15, 36, 38, 88, 90, 94). Because posttranscriptional RNA degradation can affect a wide range of transgenes expressing plant, bacterial, or viral sequences, it was more generally renamed PTGS. This section explores whether related silencing phenomena occurring with sense transgenes, antisense transgenes, and viruses rely on the same mechanism as the originally described co-suppression.

As in TGS (Table 1), PTGS may be classified according to the nature of the silencing source (Table 2), which can be a sense transgene, an antisense transgene, simultaneously expressed sense/antisense transgenes, or viruses. Many PTGS events have been reported in the literature, but only a few representative examples of PTGS events targeting endogenous sequences, foreign sequences, or viral sequences are presented for each class (when available). PTGS, like TGS, can occur in *cis* (only the RNA transcribed from the silencing source is degraded), simultaneously in *cis* and *trans* (RNA transcribed from the silencing source and all homologous RNA are degraded), or in *trans* (only RNA that is homologous to RNA transcribed from the silencing source is degraded, but not the RNA transcribed from the source).

### PTGS Mediated by Sense Transgenes

***Strongly Transcribed Sense Transgenes*** Comprehensive analysis of PTGS events with strongly transcribed sense transgenes allows the characteristics of this phenomenon to be defined precisely. Once initiated against the RNA of a given transgene, PTGS leads to the degradation of homologous RNA from either endogenous genes (co-suppression; 36, 68, 88), transgenes (*trans*-inactivation; 22, 25, 38), or RNA viruses (RNA-mediated virus resistance; 19, 25, 50, 81). In RNA-mediated virus resistance, plants can be either immune, i.e. virus resistance is established prior to the infection (19, 25, 81), or can recover from infection in newly emerging leaves (19, 50).

A single transgene copy appears to be sufficient to trigger this type of PTGS (19, 22, 71, 76). Transgene transcription seems to be required, since the frequency of silencing correlates with the strength of the promoter used to drive the transgene (75), and since transcriptional silencing of 35S-driven transgenes mediated by the tobacco locus 271 (86, 91) impedes co-suppression of homologous endogenous genes (94) as well as resistance against homologous RNA viruses (24).

The production of aberrant RNA by PTG-Silenced transgenes is evoked in many models that try to explain the mechanism of PTGS (6, 17, 20, 50, 56, 59, 92, 98). Because PTGS depends on active transcription of the transgene itself, it is unlikely that aberrant RNA is directly produced by readthrough transcription from neighboring transgenes beyond their terminators, or from transcription from neighboring

endogenous promoters. However, such unintended transcription events could interfere with regular transcription of transgenes, leading to the production of aberrant RNA instead of regular mRNA, or could produce antisense RNA that could interact with regular mRNA to form aberrant (partially) double-stranded RNA. Alternatively, transgenes could produce directly single-stranded aberrant RNA because they are methylated. Indeed, in some cases, PTGS correlates with methylation of the transgene coding sequence (21, 25, 38, 41, 81, 84). In addition, de novo methylation of the transgene appeared to precede the onset of PTGS-mediated virus resistance (41). Since *de novo* methylation can be triggered in sequence-specific transgenes by introduction of homologous viroid RNA (97), an RNA signal is suggested to trigger transgene methylation and subsequently trigger PTGS (41, 98). Despite these data, it is still not clear whether methylation plays an active role in the triggering and/or the maintenance of PTGS, or whether it is an indirect consequence of PTGS. Analysis of the effect of methylation mutants like *ddm* on PTG-Silenced transgenes should help clarify this issue.

Grafting experiments revealed that PTG-Silenced plants produce a sequence-specific systemic silencing signal that propagates long distance from cell to cell and triggers PTGS in non-silenced graft-connected tissues of the plant (70, 72, 95). Because of its sequence-specificity and its mobility, this signal is assumed to be (part of) a transgene product, probably the putative aberrant RNA hypothesized above, that could migrate alone or within a ribonucleoprotein complex.

In one case of RNA-mediated virus resistance, PTGS was found not to be graft transmissible (19). However, transmission was scored by infection with a virus (TEV) that is itself a source and a target of silencing. In addition, the propagation and/or maintenance of PTGS is counteracted by viruses like TEV, PVY, or CMV, even when they do not exhibit any homology with the PTG-Silenced transgene (2, 7, 10, 44). The HC-Pro protein of potyviruses (TEV, PVY) and the 2b protein of cucumoviruses (CMV) are the genetic determinants of this PTGS-inhibitory effect (2, 10, 44). These proteins could either interact directly with proteins of the cellular machinery involved in PTGS, and/or they could impede the propagation of the systemic silencing signal. Viruses such as TEV, PVY, and CMV do not enter the meristems and are not transmitted through the seeds. Note that PTGS is also absent from meristems (7, 96), a result consistent with the absence of transmission of PTGS through meiosis (15, 16, 22, 36, 71). These observations therefore reinforce the similarities between the movement of the silencing signal and the movement of viruses.

The efficiency of PTGS is increased in Arabidopsis *egs* mutants that define two genetic loci (16). Conversely, PTGS is released in Arabidopsis *sgs* mutants that define three genetic loci (21; C Beclin & H Vaucheret, unpublished data). These *sgs* loci are not allelic to the *ddm1*, *ddm2*, *hog1*, and *si1l* loci (I Furner, E Richards & H Vaucheret, unpublished data). Methylation of the transgene coding sequence is lost in *sgs* mutants (21; F Feuerbach & H Vaucheret, unpublished data). Nevertheless, these mutants are unlikely to be methylation mutants since they do not show demethylation of repeated genomic sequences (21). In addition, sensitivity to RNA viruses is modified in *sgs* mutants (C Beclin & H Vaucheret, unpublished

data), indicating that *SGS* genes are likely to act at the RNA level. Characterization of the functions encoded by the *EGS* and *SGS* genes should provide insight into the mechanism(s) involved in PTGS. Mutants that are defective in quelling (a mechanism related to PTGS in *Neurospora crassa*) also define three genetic loci called *qde* (12). The cloning of the *QDE-1* gene revealed that it encodes an RNA-dependent RNA-polymerase (RdRp) for which at least four homologous genes exist in Arabidopsis (11). This enzyme is presumed to play a key role in PTGS, either through the production of aberrant RNA using mRNA or unintended transcripts as a matrix, or by the amplification of aberrant RNA up to a threshold level that would activate the cellular RNA degradation machinery (6, 20, 50, 92, 98, 99). Whether one of the Arabidopsis genes encoding a RdRp plays a role in PTGS awaits the cloning of the *SGS* genes, as well as the identification of knockouts of each of the plant RdRp genes.

***Very Weakly Transcribed or Untranscribed Sense Transgenes*** A deviation from classic PTGS came from the analysis of plants showing co-suppression of endogenous *CHS* genes by sense transgenes that are not transcribed at a high level despite the presence of a 35S promoter, or by promoterless transgenes (84, 88). All plants of this type showed complex transgene arrangements, which contain at least one inverted repeat and are methylated (84). These observations led the authors to propose that such structures could efficiently pair with homologous endogenous genes, thereby impairing the regular production of RNA (84). Alternatively, this type of structure could be as efficient as a strongly transcribed single transgene to produce the amount of aberrant RNA that is hypothesized to activate the RNA degradation machinery. In the absence of data on the actual requirement for transcription from these loci, on the production of a systemic silencing signal, and on the release of this type of PTGS by viruses or *sgs* mutations, it is not possible to determine if this type of co-suppression event relies on a different mechanism from that triggered by strongly transcribed sense transgenes.

## PTGS Mediated by Antisense Transgenes

***Transcribed Antisense Transgenes*** Before the discovery of co-suppression by sense transgenes, down-regulation of endogenous genes was usually achieved using antisense transgenes. It was therefore hypothesized that PTGS could result from the unintended production of antisense RNA by those sense transgene loci that trigger PTGS, leading to antisense-like inhibition (33). However, a precise comparison of sense and antisense inhibition reveals many differences, suggesting that few, if any, steps are common to these two processes . Although antisense inhibition is efficient against endogenous genes and foreign transgenes (28, 42, 76), patterns of silencing produced by antisense transgenes are usually different from those produced by sense transgenes (42, 68). This pattern was elegantly demonstrated by conversion of a sense transgene into an antisense one using the Cre-*lox* system (76), thereby avoiding interference of position effect. In addition, an antisense 35S-aGUS transgene that is able to silence a sense 35S-GUS transgene

when it is present in the same cell fails to produce a graft-transmissible silencing signal that would silence a sense 35S-GUS transgene present in another cell, which suggests that the PTGS systemic signal is not made strictly of antisense RNA (M Fagard & H Vaucheret, unpublished data). Moreover, antisense inhibition of the endogenous *MET1* gene or of a p35S-GUS transgene occurs efficiently in *sgs* mutants, impaired in PTGS (C Beclin, F Feuerbach & H Vaucheret, unpublished data). Finally, antisense transgenes generally fail to inhibit virus infection (99). Although other characterizations are still required to determine if there are any common steps between sense and antisense inhibition, they clearly exhibit distinct steps. The identification of mutants impaired in antisense inhibition and the analysis of PTGS in such mutants will help to identify possible common steps.

***Untranscribed Antisense Transgenes*** One instance of silencing by an antisense transgene that closely resembles PTGS mediated by sense transgenes was observed in the transgenic tobacco line 271 (86, 91, 93). Silencing of homologous endogenous genes in line 271 showed several characteristics of co-suppression mediated by transcribed sense transgenes: transcription of endogenous genes in the nucleus without accumulation of the corresponding RNA in the cytoplasm (73, 93), meiotic resetting, triggering of silencing during development, and release by viruses that counteract PTGS (C Beclin, M Fagard & H Vaucheret, unpublished data). However, run-on assays failed to detect transcription of the antisense transgene from the heavily methylated 271 locus (93). In this case, and perhaps also in promoterless sense transgenes (84, 88), silencing could result from an actual pairing of the transgene locus with the homologous endogenous genes and their subsequent modification, leading directly to the production of degradable endogenous RNA. Alternatively, aberrant sense RNA could be produced by the 271 locus, which cannot be distinguished by run-on assays from that produced by the endogenous genes.

## PTGS Mediated by Sense/Antisense Transgenes

Although the data presented in the section above point to significant differences between antisense inhibition and sense inhibition, recent models explaining PTGS predict a key role for double-stranded RNA (59, 99). These models take into account data showing that injection of double-stranded RNA in worms, flies, and trypanosomes inhibits expression of the homologous endogenous genes (45, 65, 69). In addition, intermediates of RNA degradation were identified in co-suppressed petunia plants, corresponding to a region of the RNA that could potentially form a secondary structure due to internal complementarity (59). This result led the authors to propose a catalytic model that predicts the pairing of these degradation products with endogenous RNA, followed by cleavage and self-regeneration of these small RNA molecules, which therefore increase in number at each cycle and could eventually propagate from cell to cell (59). Furthermore, small antisense RNA complementary to the targeted RNA were detected in PTG-Silenced plants (33a). However, the role of these small antisense RNA in PTGS is still not known.

In particular, whether these small RNA could propagate from a PTG-Silenced stock to a non-silenced scion through a graft-union, and whether these small RNA are still present in plants in which PTGS is released by non-homologous viruses (2, 7, 10, 44) or in PTGS-deficient *sgs* mutants (21) has not been determined.

To test the hypothesis of a role of double-stranded RNA structures, a p35S-ACC sense transgene carrying a small inverted repeat in the 5′ UTR region was introduced in tomato. Co-suppression of endogenous *ACC* genes occurred at a higher frequency in these plants than in plants carrying the regular p35S-ACC sense transgene without the inverted repeat (34). In a similar approach, sense and antisense transgenes expressing part of a viral genome that, alone, failed to trigger resistance to the corresponding RNA virus (PVY) were simultaneously expressed in tobacco (99). Although sense and antisense RNA were still detectable, plants were immune to infection by PVY. In addition, plants carrying a single copy of a p35S-PVY-aPVY transgene expressing an RNA that potentially can form a secondary structure due to the presence of homologous sequences linked together in sense and antisense orientation were also immune to infection by PVY. Similarly, a p35S-GUS-aGUS transgene silenced an endogenous p35S-GUS sense transgene more efficiently than newly introduced sense or antisense transgenes could. The authors then proposed that the production of double-stranded RNA is required to trigger PTGS, and that RdRp could be involved in such production (99).

Whether these events of co-suppression (34), *trans*-inactivation (99), or virus resistance (99) mediated by sense or antisense transgenes rely on the same mechanism as PTGS mediated by sense transgenes alone awaits the analysis of methylation, graft-transmissibility, and release by viruses that counteract PTGS mediated by sense transgenes. Nevertheless, simultaneous expression of sense p35S-GUS and antisense p35S-aGUS transgenes triggers silencing in *sgs* mutants (C Beclin & H Vaucheret, unpublished data), which suggests that at least the three steps controlled by *SGS* genes are specific to PTGS mediated by sense transgenes, and are not involved in sense- or antisense-mediated silencing.

## PTGS Mediated by DNA and RNA Viruses

As with transgenes, viruses can be either the source, the target, or both source and target of silencing. PTGS mediated by viruses can occur with DNA viruses, which replicate in the nucleus, and with RNA viruses, which replicate in the cytoplasm. These viruses can be inoculated into plants at a specific stage of their development, or can be expressed within plants throughout development by stably integrated virus-expressing transgenes.

***Viruses That Trigger Recovery*** Infection of nontransgenic *Brassica napus* plants by CaMV (a DNA pararetrovirus) leads to recovery by a PTGS-like mechanism, i.e. 19S and 35S RNA encoded by CaMV are degraded while CaMV DNA is still replicating in the nucleus. Infection of *B. napus* plants expressing a p35S-GUS transgene with a 35S terminator by CaMV leads to recovery from CaMV infection and induction of PTGS of the p35S-GUS transgene (1). CaMV is

primarily a target of the cellular silencing machinery since the 19S and 35S RNA are degraded. However, CaMV can also be considered as a source (or at least as an inducer) of PTGS for transgenes sharing homology with the virus within their transcribed regions because it activates the cellular RNA degradation machinery against them.

Infection of nontransgenic *Nicotiana clevelandii* plants by TBRV (an RNA nepovirus) also leads to recovery by a PTGS-like mechanism, i.e. TBRV RNA is degraded (77). Plants that have recovered are sensitive to infection by PVX (an unrelated RNA virus). However, they are immune to infection by a recombinant PVX virus in which TBRV sequences have been cloned. Similarly, nontransgenic *Nicotiana benthamiana* plants can recover from infection by TRV (an RNA tobravirus). Plants that have recovered from infection by a recombinant TRV-GFP virus are sensitive to infection by PVX but are immune to infection by a recombinant PVX virus in which GFP sequences have been cloned. In addition, plants that have recovered exhibit PTGS of a newly introduced 35S-GFP transgene. This indicates that viruses that induce recovery also induce PTGS against (at least partially) homologous viruses and transgenes (78).

Additional analyses are needed to determine whether PTGS mediated by viruses relies on the same mechanism as PTGS mediated by sense transgenes. Required will be analyses of transgene methylation, over-infection by viruses that counteract PTGS, introduction into *sgs* mutants, and the characterization of mutants impaired in recovery.

***Viruses That Do Not Trigger Recovery*** Infection of *N. benthamiana* by TGMV (a DNA geminivirus) is followed by high-level replication in the nucleus and accumulation of viral RNA in the cytoplasm. Infection by a recombinant TGMV virus carrying the coding sequence of the sulfur (*SU*) gene in either sense or antisense orientation leads to PTGS of the endogenous *SU* gene, i.e. the endogenous *SU* RNA is degraded (46). However, TGMV-SU RNA is not degraded, suggesting that TGMV-SU behaves only as a source of PTGS. Infection of transgenic *N. benthamiana* expressing a p35S-LUC transgene by a recombinant TGMV virus carrying the coding sequence of the *LUC* gene in either sense or antisense orientation leads to PTGS of the *LUC* transgene. In this case, both *LUC* and TGMV-LUC RNA fail to accumulate. Although viral infections are nonuniform, silencing of the *LUC* transgene seems to be complete in infected leaves, whereas silencing of the endogenous *PDS* gene is incomplete, leading to variegation. These results suggest that, in nontransgenic plants, silencing of endogenous genes requires the permanent presence of the virus. Conversely, transgenes that behave initially as targets of PTGS induced by viruses may become maintainers of PTGS through the production of a systemic silencing signal; this allows degradation of transgene and viral RNA in infected cells, and degradation of transgene RNA in noninfected cells.

Infection of *N. benthamiana* by PVX (a single-stranded RNA potexvirus) or TMV (a single-stranded RNA tobamovirus) leads to virus replication and

accumulation of viral RNA in the cytoplasm. Infection by recombinant PVX or TMV viruses carrying the coding sequence of the phytoene desaturase (*PDS*) gene in either sense or antisense orientation leads to PTGS of the endogenous *PDS* gene, i.e. the endogenous *PDS* RNA is degraded, a phenomenon called VIGS (virus-induced gene silencing) (47, 79). However, PVX-PDS RNA accumulates at a high level, suggesting that the virus is not targeted by VIGS. Infection of transgenic *N. benthamiana* expressing a p35S-GFP transgene by a recombinant PVX virus carrying the coding sequence of the *GFP* gene in either sense or antisense orientation leads to VIGS of the *GFP* transgene. In this case, both endogenous *GFP* and PVX-GFP RNA are efficiently and uniformly degraded, as in endogenous *LUC* and TGMV-LUC RNA (47). These results suggest that the continuous presence of the inducing virus is required to maintain VIGS of endogenous genes, whereas the presence of a transgene targeted by VIGS is sufficient to maintain VIGS, thus allowing the degradation of target viral RNA as well as systemic propagation of VIGS.

These results are reminiscent of data showing that RNA of endogenous genes can be degraded in nontransgenic plants grafted onto transgenic rootstocks exhibiting co-suppression of the homologous endogenous genes and sense transgenes. Here, silencing is not maintained when the source of silencing (the rootstock) is removed, which suggests that although transgenes are dispensable for the RNA degradation step of co-suppression, their presence is required to maintain silencing (72). In explanation, it was hypothesized that only some transgenes can undergo epigenetic changes that lead to re-amplification of this signal and maintenance of PTGS (72, 92), whereas endogenous genes cannot. Similarly, infection of transgenic plants by recombinant TGMV, TMV, or PVX viruses would trigger degradation of both transgene and viral RNA because transgenes would undergo epigenetic changes that allow production of the silencing signal to be maintained. Conversely, infection of nontransgenic plants by recombinant viruses would require the continuous presence of the inducing viruses to sustain silencing of endogenous genes. Therefore, the mechanism of VIGS is likely to be the same as PTGS mediated by sense transgenes, but additional molecular and genetic evidence is still required, using *sgs* mutants, for example.

***Stably Integrated Viruses That Do Not Trigger Recovery*** Expression of a PVX-GUS recombinant virus from a stably integrated nuclear transgene, a construct referred to as an amplicon, allows 100% efficient triggering of PTGS of both PVX-GUS viruses and homologous GUS transgenes (3). Indeed, such amplicon has all the components required for efficient PTGS mediated by sense transgenes: The threshold level of transgene/viral RNA that triggers PTGS is obtained by a combination of high transcription from a p35S-driven transgene and replication of the viral RNA, whereas PTGS is maintained through transcription from a transgene, thus allowing a permanent production of the silencing signal (see above). This system therefore provides a powerful strategy for consistent silencing of endogenous genes in transgenic plants.

## Conclusions on PTGS

PTGS can be triggered by transgenes and viruses, leading to the degradation of homologous RNA encoded by endogenous genes, transgenes, and, in some cases, by the virus itself. Because some plant species can recover from infection by some viruses (caulimo-, nepo-, and tobraviruses), by a PTGS-like mechanism (1, 13, 77, 78), PTGS is likely to be primarily a defense response of the plant against viruses. Once activated against such viruses, the RNA degradation machinery of PTGS becomes naturally efficient against endogenous gene or transgene RNA if it shares homology with the targeted virus (1, 78). Other viruses for which recovery is not observed (such as gemini-, potex-, and tobamoviruses) can also trigger silencing of endogenous genes and transgenes sharing homology at the RNA level (47, 78, 79), suggesting that although recovery does not occur, these viruses activate the plant's PTGS defense machinery. Finally, viruses of two other families (poty- and cucumoviruses) can counteract PTGS of nonhomologous transgenes (2, 7, 10, 44). The fact that these viruses have developed strategies to counteract PTGS suggests that they are also targets of PTGS, a hypothesis confirmed by the observation that *sgs* mutants, which are deficient for PTGS, are hypersensitive to CMV (C Beclin & H Vaucheret, unpublished data). These results suggest that plants use PTGS as a strategy to combat viruses, and that viruses have more or less succeeded in escaping this defense: Poty- and cucumoviruses are able to knock-out PTGS; gemini-, potex-, and tobamoviruses are able to infect plants although they activate PTGS; caulimo-, nepo-, and tobraviruses are still targeted by PTGS in some species.

Why do sense transgenes trigger PTGS in the absence of viruses? Many characteristics of virus-induced PTGS (VIGS) are shared with sense transgene-mediated PTGS. Sense transgene loci that trigger PTGS likely produce an aberrant form of RNA that resembles the type of viral RNA that activates recovery of the plant from infection. This RNA is subsequently targeted for degradation (1, 13, 77, 78). The mechanistic resemblance may be related to secondary structure, cellular compartmentalization, and/or affinity for cellular components (such as RdRp), and may lead to recognition by the cellular machinery that targets this type of RNA for degradation. The characterization of the whole process of recognition and degradation will require characterization of the function of proteins encoded by genes in which mutation confers either impairment of PTGS (*SGS* genes and others to be identified) or virus resistance (to be identified).

Inhibition of gene expression by antisense RNA or simultaneous expression of sense and antisense RNA seems not to rely on exactly the same mechanism as virus- or sense transgene-induced PTGS since antisense inhibition occurs efficiently in sgs mutants (C Beclin & H Vaucheret, unpublished data). However, some steps might be common to these processes and could be revealed by identifying and characterizing mutants impaired in antisense inhibition, as well as mutants impaired in both antisense inhibition and PTGS (if any).

## GENERAL CONCLUSION: How Many Mechanisms of Gene Silencing?

As concluded in the TGS and PTGS sections, (trans)gene silencing cannot be explained by a single mechanism. Rather, multiple mechanisms involving DNA-DNA, DNA-RNA, or RNA-RNA interactions (55) may be evoked (Figure 1, see color plate). Nevertheless, there may well be common steps between these different mechanisms. Interestingly, a complex transgene locus that undergoes TGS triggers both TGS of promoter-homologous target transgenes and PTGS of coding sequence-homologous target (trans)genes (93). Similarly, a virus that undergoes RNA degradation during the PTGS-like process of recovery was shown to trigger either TGS or PTGS of homologous transgenes, depending on whether they share homology within their promoter or the coding sequence (1).

These two specific cases clearly demonstrate that both TGS and PTGS events affecting (trans)genes can be triggered as alternative (although not exclusive) responses to two important pathological conditions that plants have to face, i.e. the stable integration of additional pieces of DNA into chromosomes, and the extrachromosomal replication of a viral genome. Additional pieces of DNA can be added to chromosomes owing to the movement of transposable elements (TEs) or to the integration of (part of) the genome of pathogens like *A. tumefaciens*. These processes must be tightly regulated to avoid deleterious effects. Both TEs and T-DNA insertions can contribute to increasing the size of the genome, can deregulate the expression of neighboring endogenous genes, and could cause chromosomal rearrangements through recombination between homologous ectopic sequences. The extrachromosomal replication of a viral genome must also be regulated because viruses use the cellular machinery to their own advantage, thus limiting the availability of enzymes and subsequently of metabolites for growth.

Epigenetic silencing of plant transgenes may therefore reflect diverse cellular defense responses (56). TGS, which results from the impairment of transcription initiation by methylation and/or chromatin condensation, could derive from the mechanism by which additional pieces of DNA (TEs, T-DNA) are tamed by the genome. PTGS, which results from RNA degradation, could derive from the process of recovery by which cells eliminate undesirable pathogens (RNA viruses) or their undesirable products (RNA encoded by DNA viruses).

TGS is therefore expected to occur when transgenes insert near or within endogenous *cis*-acting silencing elements like heterochromatin, repeated and methylated elements (74, 85), or when they disorganize chromatin structure locally owing to a drastically different GC content (37, 60, 87) or the formation of secondary structures by *cis* DNA-DNA interactions between transgene repeats (4, 100). Transgenes would therefore undergo an epigenetic change (involving methylation and/or chromatin condensation) that impedes the initiation of transcription. Active transgenes could also be subjected to TGS when they are brought into the presence of promoter-homologous *trans*-acting silencing elements that may impose an

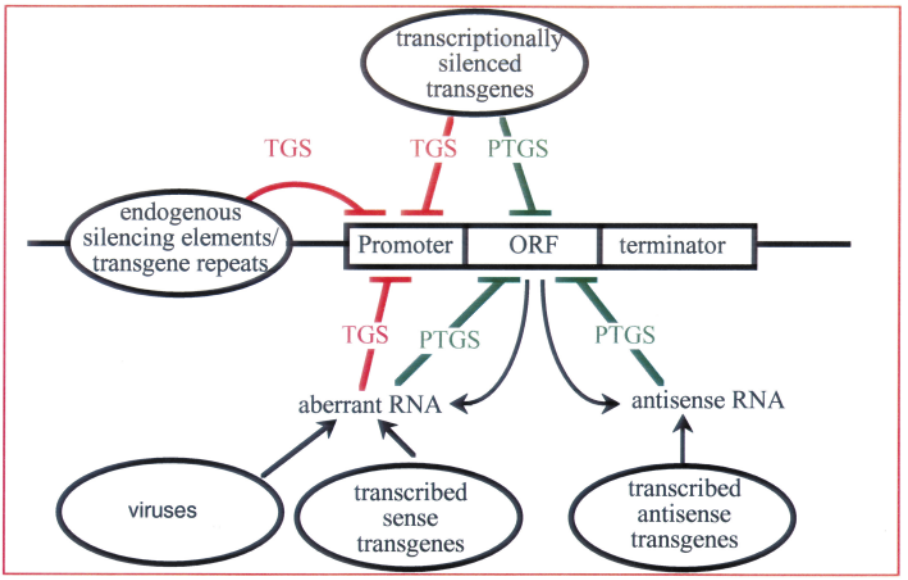


**Figure 1** Putative mechanisms of (trans)gene silencing in plants. TGS (in red) could occur in *cis* because of the presence of neighboring endogenous silencing elements or because transgene repeats can interact to create a silencing structure. TGS could also occur in *trans* when active (trans)genes are brought into the presence of homologous TG-Silenced transgenes that can transfer their epigenetic silent state through DNA-DNA interactions, or when either viruses or transcribed transgenes produce an aberrant form of RNA that impedes transcription through DNA-RNA interactions. PTGS (green) could occur in *cis* owing to the production of aberrant sense, antisense, or double-stranded forms of RNA by the transgene itself, leading to the degradation of homologous mRNA. PTGS could also occur in *trans* when the RNA encoded by active (trans)genes share homology with viruses or transgenes that themselves produce aberrant forms of RNA that activate PTGS. Alternatively, PTGS could occur in *trans* when active (trans)genes are brought into the presence of homologous TG-Silenced transgenes that can transfer their epigenetic silent state through DNA-DNA interactions, thus impairing the regular production of mRNA.

epigenetic change and impedes the initiation of transcription. These *trans*-acting elements could be allelic or ectopic (trans)genes already subjected to TGS if their epigenetic silent state is transferred through direct or protein-mediated DNA-DNA interactions (52, 54, 60, 86). They could also be viral RNA (1) or (aberrant) RNA produced by transcribed transgenes that resemble viral RNA (58), and that are able to impose a transcriptionally repressive state on the homologous promoter sequences through DNA-RNA interactions.

On the other hand, PTGS is expected to occur when transgenes produce an aberrant form of RNA that mimics either viral RNA or viral RNA degradation products after infection. Transgene RNA would therefore be targeted for degradation, as is RNA from viruses inducing recovery (1, 13, 77, 78). Endogenous genes, transgenes, or viruses that are not themselves able to activate PTGS or recovery could also be subjected to PTGS when their RNA shares homology with the targeted RNA sequences of transgenes that induce PTGS (25, 34, 68, 99), or of viruses that induce a PTGS-like response (1, 77, 78). Surprisingly, endogenous genes can also be subjected to PTGS when brought into the presence of TG-Silenced transgenes that could transfer their epigenetic silent state through DNA-DNA interactions. The newly imposed epigenetic state would fail to inhibit transcription initiation because of the absence of homology within the promoter region, but would impair the regular transcription of mRNA and thus lead to degradation (84, 93).

Since PTGS is a mechanism leading to the degradation of viral RNA, it is not expected to involve any step at the DNA level (78). However, the fact that not all transgenes induce PTGS probably means that not all produce aberrant RNA, or at least not in sufficient quantities. PTGS mediated by sense transgenes most likely involves an additional step or steps at the DNA level compared to PTGS mediated by viruses. The production of the aberrant form of RNA could depend on the ability of a transgene locus to undergo readthrough transcription, transcription from a cryptic promoter, premature termination, and/or unintended production of antisense RNA. Alone, or in combination with regular mRNA, these types of molecules could therefore activate PTGS, as does viral RNA. In some cases, the plant RdRp enzyme (11) could be required to amplify these molecules in order to reach a threshold level of aberrant molecules capable of activating PTGS.

Whether the production of aberrant RNA relies only on the primary structure of DNA, i.e. the arrangement of transgene copies within the genome, or also depends on epigenetic changes is unclear. Changes in the methylation state of PTG-Silenced transgenes have been observed (21, 25, 38, 41, 84), but whether as a cause or a consequence of PTGS is not known. Introgression of PTG-Silenced transgenes into the Arabidopsis *ddm1* and *ddm2* mutants (or in plants expressing antisense *MET1* RNA will be critical in determining whether methylation (28) and/or chromatin remodeling (39, 40) play a role in PTGS. If an effect of *ddm1* and/or *ddm2* on PTGS were found, the hypothesis that epigenetic changes affecting transgenes play an active role in the triggering and/or the maintenance of PTGS would be

confirmed. The role of such changes was already suggested by the requirement for the presence of a transgene to maintain grafting-induced PTGS in plants (72) and to degrade viral RNA in VIG-Silenced plants (46, 79). Only epigenetic changes (such as methylation) occurring through interactions between aberrant/viral RNA and the corresponding transgene DNA (92, 97, 98) could explain the maintenance of RNA degradation after the initial source of silencing (virus or PTG-Silenced rootstock) has been eliminated (72, 79). Similarly, only DNA-DNA interactions allowing transmission of an epigenetic silent state from TG-Silenced transgenes to homologous endogenous genes could explain the impairment of regular transcription and the subsequent degradation of endogenous RNA (93).

As mentioned throughout this review, we do not yet have enough information to understand the mechanisms of gene silencing in plants. The identification of viruses that are targets or sources of TGS and/or PTGS, and of Arabidopsis mutants impaired in TGS and/or PTGS will help to classify silencing events on a genetic basis, and determine how many mechanisms exist and the steps common to the different silencing pathways.

## ACKNOWLEDGMENTS

We thank Judith Bender, Ian Furner, Rich Jorgensen, Jan Kooter, Peter Meyer and Eric Richards for communicating unpublished results. We also thank our colleagues from the lab and colleagues of the European Network on Gene Silencing for fruitful discussion.



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