Gene silencing and DNA methylation processes

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Epigenetic gene silencing results from the inhibition of transcription or from posttranscriptional RNA degradation. DNA methylation is one of the most central and frequently discussed elements of gene silencing in both plants and mammals. Because DNA methylation has not been detected in yeast, *Drosophila* or *Caenorhabditis elegans*, the standard genetic workhorses, plants are important models for revealing the role of DNA methylation in the epigenetic regulation of genes *in vivo*.

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Abbreviations

bp	basepair
C	cytosine
CAF1	CHROMATIN ASSEMBLY FACTOR1
CG	cytosine–guanosine
CMT	chromomethyltransferase
DDM1	*DNA DEMETHYLATION1*
DNMT1	DNA methyltransferase1
dsRNA	double-stranded RNA
GFP	green fluorescent protein
GUS	β-glucuronidase
HC-Pro	helper component proteinase
HDAC	histone deacetylase
IR	inverted repeat
MET1	METHYLTRANSFERASE1
MOF	MALES ABSENT ON THE FIRST
MOM	*Morpheus molecule*
NOSpro	nopaline synthase promoter
nptII	*neomycin phosphotransferase II*
PAI	*PHOSPHORIBOSYLANTHRANILATE ISOMERASE*
PPT	phosphinothricin
PSTVd	potato spindle tuber viroid
PTGS	posttranscriptional gene silencing
sgs1	*suppressor of silencing1*
snoRNA	small nucleolar RNA
TGS	transcriptional gene silencing

Introduction

The outcome of transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) is a reduction in the accumulation of gene transcripts. This epigenetic silencing may persist over many cell divisions or plant generations. TGS is defined as an inhibition of transcription, whereas PTGS involves the posttranscriptional degradation of RNA species but does not effect transcription rate [1]. Recent studies indicate that TGS and PTGS are mechanistically and probably functionally related because they are correlated with some of the same events, including changes in DNA methylation. TGS is associated with the hypermethylation of promoter sequences, and PTGS is associated with the hypermethylation of transcribed or coding sequences [2]. Hypermethylation can spread within promoter regions or within transcribed regions, but spreads to a lesser extent from promoter to adjacent transcribed regions and from transcribed to adjacent promoter regions. DNA–DNA interactions of multicopy sequences have been associated with methylation and gene silencing for some time. Recently, the use of constructs that produce double-stranded RNA (dsRNA) transcripts that are homologous to either promoter or coding regions has shown that RNA is an efficient trigger for methylation in association with gene silencing. Moreover, RNA-based viral and viroid systems confirm that RNA alone is sufficient to direct methylation to homologous DNA sequences.

The correlation of methylation with both TGS and PTGS suggests that it may have direct roles in establishing or maintaining silenced states. Whether methylation is a cause or an effect of epigenetic silencing, it may provide insight into common mechanisms that eukaryotes use to identify and silence target loci. The general function of DNA methylation might be conserved between plants and animals, although there are differences in the composition of DNA methylating enzymes and their target sequences between these kingdoms. Studies of epigenetic regulation in plants and mammals are yielding complementary results that reveal the evolutionary conservation of epigenetic mechanisms, including methylation [3].

In this review, we focus on selected aspects of TGS and PTGS that relate to the maintenance of gene silencing and the functions of DNA methylation. For further details on plant and mammalian DNA methylation and gene silencing more comprehensive recent reviews could be consulted [1,2,4•,5•].

TGS and DNA methylation

In mammals, methylation of cytosine (C) residues is mainly confined to symmetric cytosine–guanosine (CG) dinucleotides. Over two decades ago, this observation fostered the idea that C methylation provides a heritable epigenetic mark, which can be propagated during DNA replication by copying the existing methylation pattern into the nascent DNA strand [5•]. CG sites are also methylated in plant DNA; in addition, cytosines in the CNG context, in which N can be any nucleotide, are used as methylation substrates [4•]. As in mammals, methylation marks in both the CG and the CNG symmetric sequence contexts can easily be copied in plants. Closer inspection of methylation distribution has, however, revealed additional modifications of cytosine residues that are not linked to guanosines, which provide sequence marks for faithful reproduction of methylation patterns [6]. Thus, the simple methylation copying mechanism, which is based on

hemimethylated templates, that results from DNA replication does not always apply.

In mammals, biochemical and cytological experiments have provided evidence that DNA methyltransferase1 (DNMT1) is responsible for the maintenance of methylation patterns [5•]. These experiments also showed that DNMT3a and DNMT3b are essential for changing methylation patterns by *de novo* methylation [7]. Plants have a larger variety of DNA methyltransferases at their disposal than do animals [4•]. In addition to homologues of mammalian DNMT1, DNMT3a and DNMT3b, *Arabidopsis* has a set of genes encoding a plant-specific group of methyltransferases that contain a chromodomain motif, the chromomethyltransferases (CMTs) [8]. Biochemical assays to confirm the activities of plant methyltransferase are still needed. Nevertheless, the results of genetic experiments suggest that the major *Arabidopsis* methyltransferase, METHYLTRANSFERASE1 (MET1) which is similar to DMNT1, is involved in maintaining methylation at CG sites, whereas CMT could be responsible for CNG methylation [4•,9,10]. This specificity was recently confirmed by analysis of the preferential loss of distinct methylation types in plants impaired in either MET1 or CMT activity.

Whether asymmetric methylation is significant and how its patterns are maintained remain unanswered questions. Inverted chromosomal repeats are the preferred targets for *de novo* methylation [10–12], but whether the methylation of inverted repeats (IRs) involves DNA hairpin structures or is mediated by dsRNA transcripts derived from IR structures is still under dispute (Figure 1). In either case, methylated IRs trigger the methylation of homologous sequences in ectopic positions. Remarkably, when the triggering IR is removed, the methylation of the target sequences is maintained for several generations, but at reduced levels and almost entirely in cytosines in symmetric sites [10]. These findings suggest that asymmetric methylation is inefficiently maintained. Thus, if methylation of DNA does indeed contribute to the stability and/or heritability of TGS, only modifications at CG and/or CNG are key to this process.

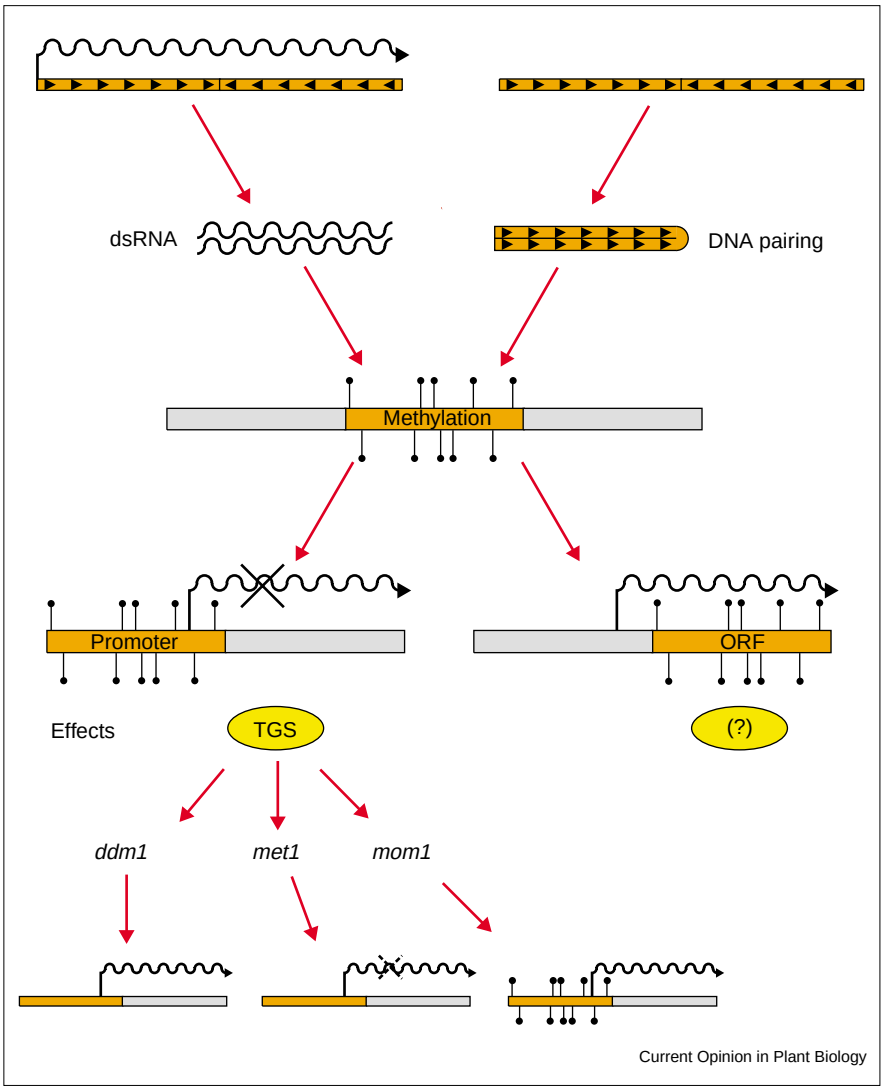
This conclusion concurs with the findings of earlier studies by Dieguez *et al.* [13] who demonstrated that methylation at CG and CNG sites is not required for the initiation of TGS, although it contributes to the maintenance of TGS [13]. A mutant form of the 35S promoter was synthesized that lacked all symmetrical methylation acceptor sites. This modified 35S promoter was fused to the *bar* gene, which encodes resistance to the herbicide phosphinothricin (PPT), and PPT-resistant transgenic lines were identified. When these transgenic lines were crossed with plants carrying the 271-silencer locus, a locus that efficiently silenced other 35S promoters [14], a significant reduction in the number of PPT resistant progeny was observed. The *bar* gene could, therefore, be silenced in the absence of symmetrical methylation acceptor sites in its promoter. TGS of *bar* in the lines carrying the 271-silencer locus was associated with the methylation of non-symmetrical sites. When silenced *bar* lines were outcrossed to remove the 271-silencer locus, progeny carrying the *bar* gene rapidly reverted to PPT resistance in the absence of the 271-silencer locus. Other studies using a wild-type 35S promoter have shown that the silenced state is maintained if the 271-silencer locus is segregated away from the transgene [15]. Therefore, initiation of TGS does not require methylation at symmetric sites but the maintenance of stable silencing may rely on it.

The importance of symmetric DNA methylation for the maintenance of TGS was further supported by studies of *Arabidopsis* mutants with reduced methylation levels. In contrast to mammals, in which loss of DNMT1 causes embryonic lethality, plants tolerate mutations or transgenic approaches that drastically alter the level and distribution of chromosomal methylation [4•,9]. Plants carrying *met1* mutations or in which *MET1* gene function has been inhibited by antisense RNA develop almost normally and set seeds. Phenotypic abnormalities in such plants accumulate only as a result of successive inbreeding [16]. Similar hypomethylation and gradual accumulation of phenotypic abnormalities are observed in *Arabidopsis* mutants of the *DNA DEMETHYLATION1* (*DDM1*) gene [17•], which encodes a plant homologue of the SWI2/SNF2 protein [18••], a component of chromatin remodeling complexes. This finding suggests that proteins involved in changes of chromatin structure are required to ensure the correct levels and allocation of methylation.

Such a role for chromatin remodeling is unexpected considering that the biochemical and cytological data derived from mammalian systems strongly suggest that DNMT1 reproduces methylation patterns instantaneously during DNA replication using hemimethylated templates. Why plants should require an additional factor(s), which probably reshapes chromatin structure, to establish methylation patterns is intriguing. Is it possible that following DNA replication chromatin first acquires (i.e. replicates) a structure that appropriately directs and correctly distributes methylation? Histone assembly and chromatin replication is accomplished with the help of the CHROMATIN ASSEMBLY FACTOR1 (CAF1) protein complex. In organisms depleted of methylation, a convincing mechanism distinguishing resident and nascent DNA strands on the basis of the presence or absence of a protein factor, the proliferating cell nuclear antigen (PCNA), has been proposed [19••]. As yet, no functional links have been established between chromatin replication by mammalian or plant CAF1 complexes, DNA methylation and gene silencing. The recently characterized *Arabidopsis* mutants in subunits of CAF1 may contribute, however, to the assignment of roles to CAF1 in the propagation of epigenetic states [20] and possibly in the maintenance of DNA methylation.

Figure 1

Selected interactions in gene silencing that involve DNA methylation. Inverted repeats (marked by an inverted set of arrows) could trigger methylation either by production of transcripts (represented by wavy lanes) that can form dsRNA or by DNA–DNA interactions. Methylation (marked by lollipops) can accumulate in the promoter or coding regions of a gene. Hypermethylation of a promoter correlates with TGS. Increased levels of methylation in a coding region (the open reading frame [ORF]) may not have immediate consequences. Maintenance of TGS requires *DDM1*, *MET1* and *MOM1* functions although mutations in each of these three genes have different effects on TGS and DNA methylation (see text for more details).



It is apparent that both DDM1 and MET1 are necessary for the maintenance of TGS, although the release of TGS by interference with either of these two components differs. Demethylation caused by the depletion of MET1 generally releases TGS [21,22•], but some loci remain silent despite drastically reduced methylation levels [22•,23]. Recently, Morel *et al.* [22•] proposed the existence two modes of TGS: MET1-dependent and MET1-independent. Whether these modes relate to CG or CNG methylation, respectively, is not known. As DDM1 is required for maintenance of TGS in all of the situations examined so far, DDM1 may be epistatic to MET1 or to the methylation signal in general. This is difficult to test, however, because of the simultaneous changes in methylation and silencing caused by the *ddm1* alleles that are currently available. Only when DDM1 functions in maintenance of methylation and in maintenance of TGS are separated, can the epistatic relationship between DDM1 and MET1 be examined. Remarkably, the release of silencing by depletion of either MET1 or DDM1 interferes with the reestablishment of TGS when the depleted gene products are provided [17•], implying that the epigenetic marks directing TGS are misplaced or have vanished. Animal transient transfection experiments with methylated DNA advocate that methylation itself serves as a mark for formation of repressive chromatin [24]. Nevertheless, this function remains to be demonstrated for the methylation of chromosomal DNA.

Biochemical and cytological evidence from mammalian systems suggests that DNA methylation status is recognized by proteins that bind to the methylated DNA or by the methyltransferase itself, and translated to the corresponding chromatin structure using histone deacetylase (HDAC) complexes that influence chromatin organization [25–27]. In genetic terms, mutations in genes encoding proteins that are involved in the recognition of methylation or in HDACs should affect TGS without affecting DNA

methylation. These mutations should have similar phenotypes to those observed after DDM1 or MET1 depletion. Indeed, transgenic inhibition of *Arabidopsis HDAC1* gene expression confirms these predictions [28]. Now, it would be interesting to examine if the restoration of *HDAC1* expression rapidly reverses the acquired phenotypes or results in the formation of stable epialleles, as do MET1 and DDM1.

Methylation-independent components of TGS regulation are also being discovered. The first example of such a component was the *Morpheus molecule* (*MOM*) gene, which encodes a large nuclear protein with an interesting composition of structural domains but no general similarity to known proteins [29•]. Mutations in *MOM* release TGS without causing detectable changes in DNA methylation at transcriptionally reactivated loci. Thus, the MOM protein could be involved in transmitting the methylation signal to a chromatin structure in a manner similar to the activity of HDAC. In contrast to strains that have deficient DNA methylation or lines with HDAC inhibition, however, *mom1* plants do not acquire characteristic phenotypic abnormalities. This indicates that MOM may define a new pathway or new level of TGS regulation operating independently of methylation changes. Interestingly, MOM is not involved in the maintenance of epigenetic chromosomal marks required for the prompt establishment of TGS because the return of *MOM* by genetic crossing rapidly resets TGS. The role of DNA methylation in establishing and maintaining these marks can now be examined by combining *mom1* and *met1* mutations with transgenic loci that are resistant to the *met1* release of silencing. This combination of mutations could also be used to determine how fast reset of silencing occurs after providing MOM.

In summary, emerging genetic evidence suggests that changes in methylation might not be as tightly linked to TGS as previously anticipated. Another recent example of TGS release without effects on methylation comes from a genetic screen performed in *Chlamydomonas reinhardtii* that led to the isolation of a novel TGS component (H Cerutti, personal communication). Further mutants, their characterization and the combination of different genotypes that are impaired in TGS will be important to clarify the relationships between methylation and chromatin structure in TGS.

PTGS and RNA-directed DNA methylation

The occurrence of PTGS is tightly associated with dsRNA species and is often correlated with increased methylation levels at loci encoding PTGS-affected transcripts. Interestingly, TGS may also be associated with dsRNA. It has been possible to engineer the transgenic production of dsRNA transcripts homologous to the nopaline synthase promoter (NOSpro), which drives *neomycin phosphotransferase II* (*nptII*) expression [30•]. Transgenic production of this dsRNA induces the methylation and silencing of target NOSpro, inhibiting the transcription of *nptII* and causing plants to acquire a kanamycin-sensitive phenotype [30•]. Natural sources of dsRNA corresponding to promoter regions could come from loci that have complex arrangements of duplicated genes. For example, the endogenous TGS of the *PHOSPHORIBOSYLANTHRANILATE ISOMERASE* (*PAI*) locus of *Arabidopsis* is found in ecotypes in which *PAI* sequences are arranged as inverted repeats that are hypermethylated but selectively expressed [10,31]. Ecotypes with simple single-copy loci are hypomethylated and not silenced. Promoterless constructs of *PAI* inverted repeats used as controls suggested that transcription, and hence production of double-stranded or hairpin RNA, is not required for hypermethylation. DNA–DNA pairing *in cis* within the locus was therefore favored as the possible methylation trigger. Nevertheless, the presence of low levels of dsRNA cannot be excluded.

The cause and effect relationship between RNA and methylation can be directly established when the triggers for methylation consist of only an RNA component. RNA-directed methylation of potato spindle tuber viroid (PSTVd) transgene sequences is triggered by the infection of tobacco plants with PSTVd [32•,33••]. PSTVd is a small circular RNA molecule that does not encode a protein but is able to replicate in nuclei and to accumulate to high copy numbers in systemically infected hosts. PSTVd transgene sequences become hypermethylated at both symmetrical (i.e. CG and CNG) sites and asymmetrical (i.e. CNN) sites following infection. The heavy methylation is restricted to the PSTVd coding sequence, whereas sequences adjacent to PSTVd are methylated at a lower level. The PSTVd transgene can be pared down to 30 basepair (bp) fragments that can still function as targets for methylation [32•]. Interestingly, the minimal 30 bp target is similar in size to the 21–25-mer RNA species that are generated during PTGS [34•] and NOSpro dsRNA-mediated TGS [30•]. Plasterk and Ketting [35] have proposed that these small RNA species may guide RNAs to direct specific RNA degradation. It is possible, however, that these small RNA species could also guide DNA methylases to a homologous chromosomal sequence.

Confinement of RNA-directed methylation was also observed in replicase-mediated resistance to pea seed borne mosaic virus (PSbMV) [36]. The replicase transgene became methylated at symmetrical and asymmetrical sites in all resistant plants in which PTGS against PSbMV prevented the virus from accumulating. Subsequently, virus-induced gene silencing of a green fluorescent protein (GFP) transgene was shown to correlate with *de novo* methylation, which was restricted to a GFP-coding sequence that did not spread to adjacent promoter sequences [37•]. The hypermethylated state could, however, spread efficiently within the transcribed GFP-coding region. These findings were made using constructs that expressed either the 5′ (green fluorescent) portion of GFP or the 3′ (protein) portion. PTGS induced by either truncated form of GFP resulted in the hypermethylation of

the entire GFP coding sequence at symmetrical and asymmetrical sites. How the spread of methylation primarily becomes limited to coding regions is an interesting question to be answered in the future.

How might RNA direct the methylation of specific DNA sequences? Could it have a remote relationship to sex chromosome dosage compensation ? Dosage compensation is required to ensure the appropriate expression level for genes on the X chromosome. In *Drosophila*, dosage compensation is controlled by the dosage-compensation complex, which is specifically associated with the X chromosome. The roX1 (for RNA on the X chromosome1) and roX2 RNAs are necessary components of the *Drosophila* dosage compensation complex [38,39]. Mutations that remove both roX RNAs are lethal to male flies because the dosage compensation complex can no longer bind and superactivate the X chromosome [38]. roX RNAs bind to the chromodomain region of the MALES ABSENT ON THE FIRST (MOF) protein and facilitate the incorporation of MOF into the dosage-compensation complex through interactions with MALELESS. *MOF* encodes a histone acetyl transferase that acetylates histone H4 at lysine 16. *Drosophila* has no detectable DNA methylation, but in plants and mammals it is possible that methylation changes follow chromatin adjustments. An RNA-containing complex may achieve this step.

In mammals, dosage compensation depends on an RNA named X-inactive-specific transcript, which is associated with the highly methylated and inactive X chromosome [40]. A recently discovered example of plant RNAs that specifically direct the methylation of nucleic acids is the small nucleolar RNAs (snoRNAs) [41]. snoRNAs are often encoded by introns and are bound by fibrillarin proteins, which are found in many eukaryotes. The methyltransferase activity of fibrillarin proteins carries out the specific methylation of pre-ribosomal RNA (rRNA) by its methyltransferase activity. The specific site of the methylation is encoded by the stem loop structure of the snoRNA. This loop contains a short antisense region of 10 bp that is complementary to the methylated target sequence of the rRNA. On the basis of these examples, it is tempting to speculate that a protein or complex that contains, for example, dsRNA or another appropriate guide-RNA species, could carry out sequence-specific methylation of DNA directly or through an interaction with chromatin.

The roles of DNA methylation in PTGS processes and the role of RNA-mediated methylation in TGS are far from clear. It is well documented that the production of dsRNA with homology to a promoter sequence will facilitate its methylation and silencing, but is this process also a prerequisite for the natural TGS-mediated silencing of chromosomal pericentromeric repeats [21]? If it is, then mutations interfering with PTGS that could be linked to RNA-mediated methylation should also release this silencing. However, mutations that release PTGS, namely *suppressor of silencing1* (*sgs1*), *sgs2*, *sgs3* [42,43] and *argonaut1* [44], were not able to release TGS from pericentromeric targets (M Fagard, H Vaucheret, personal communication) suggesting that a PTGS-like process is not directly required for the control of this particular TGS event. The role of methylation in PTGS was addressed in a recent set of experiments using the *ddm1* and *met1* mutations [22•], which affect the maintenance of methylation and cause immediate release of TGS. When these mutations were introduced into a PTGS-silenced β-glucuronidase (GUS) line they showed sporadic reversion of PTGS, whereas parallel control lines were stably silenced. The reversion to GUS expression was not immediate and slowly occurred over multiple generations. This suggests a less direct role for methylation in the maintenance of PTGS than in the maintenance of TGS.

In addition to genetic approaches, pharmacological studies on the affect of methylation on PTGS have been conducted. 5-azacytidine failed to release the PTGS of GUS transgenes in rice cell cultures despite dramatic reductions in the methylation of the GUS transgenes [45]. In contrast, 5-azacytidine and S-9-(2,3-dihydroxypropyl) adenine caused the partial release of PTGS of *nptII* in transgenic tobacco [46]. Together, these results are similar to those of the MET1 studies in that the release of PTGS in the absence of methylation is somewhat sporadic. They further support the idea that methylation may not have a central role in PTGS.

PTGS can be uncoupled from methylation by silencing suppressors such as the helper component proteinase (HC-Pro) from potyviruses or 2b from cucumber mosaic virus. Methylation of PTGS targets is maintained in the presence of HC-Pro and 2b even though the silenced loci revert to high expression levels [37•]. The maintenance of methylation suggests that HC-Pro and 2b interfere with PTGS in a manner that is independent of the methylation status of the silenced loci. These silencing suppressors can neither revert TGS-silenced genes to genes that are expressed at high levels nor prevent the initiation of TGS [47]. Even if methylation can be triggered by similar RNA species in TGS and PTGS, the HC-Pro and 2b proteins must interfere at points after the two pathways diverge.

The question of how similar methylation in PTGS and TGS is may be resolved by the use of dsRNA promoter silencing lines such as NOSpro. The NOSpro dsRNA-silencing system has recently been shown to function in *Arabidopsis* in which forward-genetic approaches can be used to identify mutations that suppress NOSpro methylation or silencing [30•]. Mutations that inhibit dsRNA-mediated methylation of NOSpro sequences may, in turn, inhibit the methylation of coding regions destined for PTGS. These mutants would be crucial for establishing the relative importance and position of methylation in each pathway.

RNA-directed DNA methylation is an intriguing theme in gene silencing. Initially, similar types of dsRNA species may elicit the methylation of homologous DNA sequences and directly or indirectly trigger TGS or PTGS. At some point, however, the PTGS and TGS pathways must diverge to give rise to distinct mechanisms that prevent the accumulation of RNA from loci destined for silencing.

Conclusions

The identification of additional genes involved in the methylation pathway and affecting both PTGS and TGS will be necessary to link the two pathways. The growing numbers of genes with defined effects on TGS or PTGS will soon allow assay of their epistatic relationships. These experiments, with the help of biochemistry, should delineate mutual dependencies and *in vivo* hierarchies of components involved in epigenetic regulation in plants. As a consequence, the role of DNA methylation in gene silencing will become better defined.

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