

HOMOLOGY-DEPENDENT GENE SILENCING MECHANISMS IN FUNGI

Carlo Cogoni

*Dipartimento Biotecnologie Cellulari ed Ematologia, Sezione Genetica Molecolare,
Policlinico Umberto I, Universita degli Studi di Roma La Sapienza, 00161 Roma, Italy;
e-mail: carlo@bce.med.uniroma1.it*

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■ **Abstract** Homology-dependent gene silencing (HDGS) is a ubiquitous phenomenon among fungi, plants, and animals. Gene silencing can be triggered and can affect artificially introduced nucleic acid molecules, both DNA and RNA, and/or can act on endogenous duplicated sequences. Although the various HDGS phenomena may be related each other, probably deriving from an ancestral defense mechanism, relevant differences do exist between different HDGS mechanisms. Especially in fungi, a variety of HDGS phenomena have been uncovered during the past 10 years: Gene inactivation of duplicated sequences can be achieved either through DNA-methylation and block of transcription or through sequence-specific degradation of mRNA. Moreover, duplicated sequences can also be specifically mutagenized. Studying HDGS in fungi gives us the opportunity to study such complex mechanisms in relatively simple organisms in which both genetic and biochemical approaches can be easily used.

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INTRODUCTION

During the past decade it has become evident that different organisms can react to the introduction of foreign nucleic acids by inducing gene silencing mechanisms that are based on the recognition of nucleic acid sequence homology (11, 24, 82). Gene silencing is achieved via diverse strategies: Homologous sequences can be inactivated at a transcriptional level or at a posttranscriptional level involving sequence-specific mRNA degradation. There are a growing number of indications that support the idea that various homology-dependent gene silencing phenomena correspond to host defense responses against parasitic nucleic acids such as transposons, RNA, or DNA viruses and viroids (7, 78). Moreover, HDGS can also act as a surveillance mechanism on endogenous DNA sequence duplications, having a role in genome maintenance (11, 26). Genetic and biochemical studies have suggested that the basic machinery for homology-dependent gene silencing phenomena have common features between fungi, plants, and animals (24). The filamentous fungi *Ascobolus immersus* and *Neurospora crassa*, in which it is possible to combine genetic and molecular analysis, constitute particularly suitable model organisms for the study of both transcriptional and posttranscriptional gene silencing. This review focuses on the different silencing mechanisms occurring in these two fungal systems, highlighting similarities and differences with analogous phenomena in plants and animals.

TRANSCRIPTIONAL GENE SILENCING

Repeat-Induced Point Mutations in *Neurospora crassa*

RIP, originally an acronym for rearrangement induced premeiotically (117) and subsequently renamed repeat induced premeiotically (15, 16), was discovered in the fungus *N. crassa* more than 10 years ago. It was found that duplicated sequences are mutagenized via G:C to A:T transitions. The RIP phenomenon is highly regulated, as it occurs in a specific period of the sexual cycle in the premeiotic phase (113), when the two nuclei of opposite mating types share a common cytoplasm in dikaryotic cells formed before karyogamy. RIP is able to detect any duplication with a minimal size of 400 bp (153). Interestingly, this size resembles the minimum length required for recombination both in mammals and yeast, suggesting that a recombination-like mechanism that can form a DNA-DNA pairing structure could be the substrate for RIP (106, 113). Indeed, additional evidence of the involvement of DNA-DNA interactions between homologous sequences in duplication detection by RIP comes from the observation that duplicated sequences are inactivated in a pairwise manner (114; Figure 1). When more than two homologous copies are present in a premeiotic nucleus, it was frequently found that only two copies are attacked by RIP. On the other hand, it was observed that when only two copies are present, either both or neither copy is RIPed. Moreover, linked homologous copies in a tandem array are detected by RIP with a high efficiency (nearly 100%),

whereas unlinked duplications can escape detection and inactivation (119). Altogether, these findings strongly suggest that scanning of the genome via homologous DNA-DNA interaction is a central step in the identification of duplicated DNA sequences (155).

Although the immediate consequence of gene inactivation by RIP is mutagenesis by G:C to A:T transitions, the remaining cytosines are often methylated (118). Frequently, it has been found that in a single DNA strand either Cs or Gs are mutated, but not both (153), suggesting that RIP could cause one kind of mutation only, possibly C to T transitions. This observation, together with the association of RIP with cytosine methylation, has led to the hypothesis that DNA methylation arising from DNA-DNA paired structures can cause the C to T transitions. In fact, it has been proposed that 5-methyl cytosines (5 mCs) can be deaminated, leading to conversion to Ts (46). It has been suggested that spontaneous deamination of 5 mC can be accelerated during the premeiotic phase in an environment where S-adenosylmethionine could be limiting, resulting in the accumulation of an intermediate, 5-6-dihydrocytosine, which has a higher rate of deamination than 5 mC (113). Unfortunately, it has not been possible to demonstrate the methylation of Cs in the sexual dikaryon where RIP occurs; thus, although attractive, it is still not clear whether RIP involves methylation of cytosines followed by deamination. However, it is very unlikely that the methylated cytosines associated with RIPed sequences simply derive from cytosines that somehow escaped deamination during the premeiotic phase and whose methylated state is perpetuated in the vegetative phase. This is based on the observation that RIPed *Neurospora* sequences amplified in bacteria, and therefore stripped of 5 mCs, are able to trigger de novo DNA methylation when re-introduced in *Neurospora* (84), indicating that RIPed sequences can constitute portable signals for DNA methylation (120). Although some uncertainty remains regarding the nature of the methylation signals, recent findings suggest that A:T-rich RIPed sequences and those containing high densities of TpA dinucleotides can promote de novo methylation (83).

Mutations by RIP are generally sufficient to completely inactivate affected genes, and typically, G:C to A:T transitions result in an assortment of silent, missense, and nonsense mutations. However, DNA methylation associated with RIP-induced mutations can further contribute to the inactivation of genes attacked by RIP (126, 127), as it has been found that methylated genes resulting from RIP exhibit a strong reduction of mRNA steady-state levels (56). Normal levels of mRNA can be re-established when DNA methylation is prevented by the drug 5-azacytidine or in the presence of a mutation in the *dim-2* gene required for DNA methylation (43, 108). Interestingly, nuclear run-on assays indicate that methylation in the promoter region does not significantly affect transcription initiation. Instead, DNA methylation appears to block transcription elongation, as suggested by run-on mapping of RNA polymerase II complexes engaged on silenced genes. It would appear that DNA methylation causes the RNA polymerase to stall in the 5' portion of the genes (108). However, in *in vitro* assays, transcription elongation is not dramatically influenced by DNA methylation, indicating that methylation

per se is not able to block elongation and that it is more likely that methylation can induce changes in chromatin structure leading to the inhibition of transcription.

The finding that Trichostatin A, an inhibitor of histone deacetylase, can induce the release of blocked transcription of a gene methylated by RIP (115) strongly supports the notion that DNA methylation in *Neurospora* can act as a signal to recruit histone deacetylase, leading to chromatin changes. Thus, in fungi methyl-DNA-binding proteins, analogous to methyl CpG-binding protein 2 in animals (61), could bind methylated DNA, recruit histone deacetylase complexes, and mediate chromatin modification essential to block transcription. Surprisingly, Trichostatin A was also observed to reduce methylation levels (115), suggesting that the level of chromatin acetylation can also have an effect on methylation maintenance, resulting at least in *Neurospora*, in a self-reinforcing epigenetic state. The recent finding that the DNA methyl-transferase Dnmt1 responsible for cytosine methylation in mammals is associated with histone deacetylation activity (44) may suggest that an interdependence between methylation and deacetylation in generating stable epigenetic effects is a conserved mechanism operating across the kingdoms.

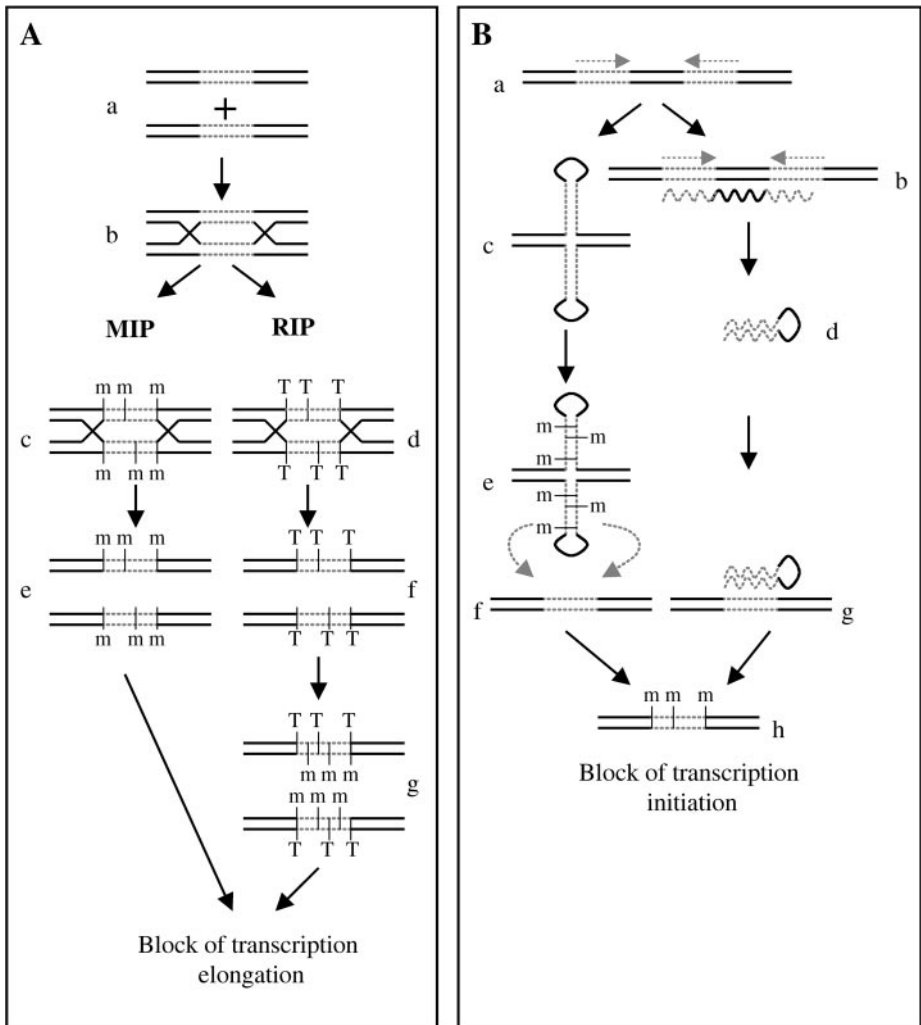
MIP: Methylation Induced Premeiotically in *Ascobolus immersus*

MIP was discovered in the fungus *A. immersus* (104) soon after the discovery of RIP in *N. crassa*. MIP and RIP seem closely related processes, at least regarding the timing and the sequence requirements for the two phenomena (107). MIP, as well as RIP, occurs specifically in the sexual phase, and a minimal size of homology of 400 bp is required (47), the same size requirement necessary for RIP. Notwithstanding these evident similarities, RIP and MIP notably differ in their consequences. Gene inactivation by RIP is irreversible because it involves local mutagenesis, whereas silencing by MIP involves only DNA methylation and is

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Figure 1 (A) The presence of sequence duplications (a) is detected in fungi through genome-wide searches. Paired DNA hybrids (b) between homologous sequences are the substrate for specific de novo DNA methyl-transferases in *Ascobolus* during the methylation induced premeiotically process (c) or are hypermutagenized by repeat induced premeiotically in *Neurospora* via C to T transitions (d). DNA methylation is maintained even in the absence of continuous DNA-DNA pairing (e). In *Neurospora* mutagenized sequences (f) constitute substrates for methyl-transferases (g). In both *Neurospora* and *Ascobolus*, DNA methylation has been demonstrated to interfere with transcription elongation probably by inducing chromatin condensation. (B) In plants inverted repeats (a) can form cruciform DNA structures that are targets for de novo methylation (e). When a repetitive locus acquires a methylated state, it becomes a potent *trans*-silencer (e-f) able to impose the silenced state (h) on an unlinked homologous DNA sequence. Alternatively, inverted repeats could be transcribed (b), leading to the production of hairpin dsRNA molecules (d). dsRNA can interact in a sequence-specific manner (g), with DNA inducing methylation (h) and block of transcription.

always reversible (6). Methylation by MIP has been found to be coextensive with the region of homology (6), and it has been shown that MIP results in a block to transcription elongation (6). When DNA methylation is induced by duplication of the 3' portion of a gene, correspondent truncated transcripts can be detected with a size consistent with the length expected if methylation inhibited transcription elongation (6). In contrast, heavy methylation of promoter regions does not inhibit transcription initiation (37).

Thus, both RIP- and MIP-induced methylation inhibits elongation, suggesting that a general mechanism of blocking transcription elongation by DNA methylation is operating in fungi. Although the mechanism by which DNA methylation



interferes with transcription could be similar in *Neurospora* and *Ascobolus*, substantial differences do exist in both the methylation patterns and maintenance. In *Neurospora*, RIPed sequences constitute signals for de novo methylation and can be methylated even in the absence of DNA duplications. For instance, RIPed sequences are capable of inducing methylation even in nearby genes, eventually leading to their inactivation (56). In contrast, even if DNA methylation in *Ascobolus* can be maintained in the absence of duplication, DNA methylation is propagated by a maintenance mechanism different from the recurrent de novo methylation of RIPed sequences in *Neurospora* that ensures the transmission of methylation through mitotic divisions. In this regard, the methylation patterns occurring in MIP and RIP are not identical. Whereas in higher eukaryotes methylation is limited to only symmetrical CpG sites (112), in *Ascobolus* and *Neurospora* all the Cs can be methylated (48, 118, 121). However, whereas in *Neurospora* all Cs are potentially methylated equally (118), in *Ascobolus* symmetrical CpG sites are preferentially methylated, especially when the RIP targets are reduced in size (47). Methylated CpG sites appear to be essential for the maintenance of methylation in *Ascobolus* because in their absence the methylation of non-CpG sites is also lost (5). Thus, a two-step mechanism for propagation of methylation has been proposed (37): First, CpGs are methylated immediately after DNA duplication by a classic mechanism (54) employing a DNA methyl-transferase that recognizes hemimethylated DNA, and second, the presence of methylated CpG sites could trigger chromatin modifications essential for de novo methylation of non-CpG sites.

Transnuclear Transcriptional Gene Silencing in *Phytophthora infestans*

RIP and MIP phenomena both invoke a mechanism in which duplicated genes are detected and inactivated by genome scanning via DNA-DNA interactions occurring between homologous sequences (106). However, studies on an HDGS phenomenon that takes place in the diploid oomycete *P. infestans* suggest that DNA-DNA interactions may not always be required in the silencing process. In *P. infestans* gene silencing of the elicitor *infl* gene has been observed as a consequence of gene duplication by introduction of *infl* transgenes (142). Run-on nuclear assays indicated that gene silencing occurs at the transcriptional level. In contrast with RIP and MIP, gene silencing in *P. infestans* occurs during the vegetative phase and does not show the characteristic pairwise manner of gene inactivation observed in RIP and MIP, but affects all the homologous copies present. Strikingly, it was found that gene silencing acts not only on the homologous copies present in the transgenic nucleus, but also on homologous genes resident in different nuclei when these share a common cytoplasm in heterokaryosis with silenced nuclei (142). These findings strongly suggest that the initiating event of transcriptional gene silencing in *P. infestans* is radically different from the formation of a DNA-DNA hybrid proposed in RIP and MIP. The ability of gene silencing to act across nuclei implies the involvement of a cytoplasmic

diffusible molecule that can mediate gene silencing at a distance. Although there is no evidence as to the nature of this diffusible molecule, in analogy with cases of transcriptional gene silencing (TGS) described in plants (see below), it is tempting to speculate that RNA molecules, possibly double-stranded RNA produced from transgenes, could be involved in RNA-DNA interactions, which could constitute the initiating event in the establishment of the silenced state.

Similarities and Differences Between Transcriptional Gene Silencing in Plants and Fungi

In plants evidence exists that homologous genes can be inactivated at the transcriptional level either via DNA-DNA pairing (9, 130) or RNA-DNA interactions (69, 79, 80). Thus, in principle, both mechanisms of TGS shown to operate separately in different fungal species can be present simultaneously in plants. In general, transcriptionally silenced genes have been found to be characterized by hypermethylation patterns (99) and an altered chromatin structure (81, 82, 138). Whereas in fungi massive methylation of promoter regions does not appear to substantially inhibit transcription, TGS in plants is frequently correlated with methylation of promoter regions (58), indicating that methylation could block transcription initiation rather than elongation (58). Studies conducted in the plant model system *Arabidopsis thaliana* have shown that endogenous repetitive sequences such as ribosomal DNA arrays or centromere-associated regions and transgenic repeats are targets for DNA methylation and transcriptional silencing (146). Complex transgenic loci containing concatemeric arrays of several transgene copies organized in direct or inverted orientation have been frequently found to be hypermethylated (4, 75). This, in association with DNA methylation occurring in endogenous repetitive loci, may indicate that sequence repeats can be a particularly sensitive targets for methylation. Analogously with genome-wide homology search models proposed for RIP and MIP in fungi, it has been suggested (9) that a similar kind of genome search mechanism able to detect duplicated sequences could be active in plant somatic cells. In this case, tandemly arranged sequences could pair with each other with a higher probability than two unlinked identical sequences (116). Moreover, the paired structure arising from repeats, especially when in an inverted orientation, could be more stable and more easily recognized and methylated. The repeated loci not only seem to be exceptionally sensitive targets for methylation in plants, but can be particularly potent silencers of gene expression of unlinked identical genes.

A well-characterized example of the ability of repeats to induce *trans* methylation and inactivation of identical unlinked sequences involves the PAI gene family in *A. thaliana*. The PAI genes, encoding an enzyme involved in tryptophan biosynthesis, are present in three unlinked copies in the standard *Arabidopsis* strain Columbia. However, in the Wassilewskija strain, one of the three loci is duplicated and rearranged, resulting in a nearly perfect tail-to-tail inverted repeat comprising two full-length genes (10). In the Wassilewskija strain, the duplicated PAI locus, as well as the unlinked PAI gene members, showed heavy methylation

of Cs present in both symmetric sites (such as CpG and CpNpG) and asymmetric sites. Recent findings indicate that the presence of the inverted repeated locus is required both to induce de novo methylation and to maintain a fully methylated state of the unlinked PAI genes (75). Interestingly, CpG symmetric methylation is maintained in the unlinked PAI genes even in the absence of a duplicated PAI locus, whereas methylation maintenance in nonsymmetric sites is largely dependent on the presence of the repeated PAI locus (75). This result may suggest that, as in *Ascobolus*, two different kinds of mechanisms for methylation maintenance are present: a classic mechanism employing a DNA methyl transferase that recognizes hemi-methylated CpG sites and a de novo methylation enzyme for non-CpG sites that requires DNA pairing with the duplicated PAI locus. It is not clear why repeats are so sensitive to DNA methylation or how they can transfer their epigenetic state to homologous sequences. However, both mechanisms can employ DNA-DNA interactions and can be related to homologous recombination, as demonstrated in *A. immersus*, in which during meiosis, methylation can be frequently transferred interchromosomally from a methylated to an unmethylated allele (25), involving a gene conversion-like mechanism.

Although DNA-DNA pairing seems to be implicated in several cases of gene silencing in plants, various studies have provided evidence supporting a mechanism involving RNA-DNA interactions via a signal that triggers DNA methylation and transcriptional gene silencing. The concept that RNA molecules can direct sequence-specific DNA methylation came from the observation that in tobacco plants viroids can induce methylation of homologous sequences (100, 150). Viroids are plant pathogens that consist of noncoding circular RNA folded in a rod-like secondary structure. During viroid replication, which proceeds through an RNA-RNA pathway in the nucleus, cDNA copies integrated into the plant nuclear genome became de novo methylated, indicating the existence of an RNA-directed DNA methylation mechanism (151). Following this observation, RNA-directed DNA methylation has also been observed as a consequence of infection with a cytoplasmically replicating RNA virus (60). Following virus infection, transgenes homologous to viral sequences were methylated, suggesting that viral RNAs entered into the nucleus and triggered DNA methylation. The recent finding that transcriptional gene silencing and DNA methylation can be triggered by a double-stranded RNA (dsRNA) containing promoter sequences (79) has suggested that dsRNA could be the molecule invariably required in all RNA-directed DNA methylation phenomena. In fact, not only do viroid RNAs consist of dsRNA, but the replication of both viroid and cytosolic RNA viruses also proceeds through the formation of an intermediate containing duplex RNA (149).

Transcriptional Gene Silencing: Mechanistic Considerations

To understand the mechanistic basis of homology-based transcriptional gene silencing phenomena, two general questions need to be addressed: (a) How are the duplicated sequences recognized and targeted for epigenetic changes such as

DNA methylation and/or chromatin modifications and (b) how are such epigenetic modifications propagated and how can they interfere with transcription?

Homology-based genome-wide scanning mechanisms that can detect any duplicated sequence by DNA pairing have been proposed to operate in RIP and MIP (38). Because both *Neurospora* and *Ascobolus* are haploid organisms with very few repetitive DNA sequences, any paired DNA formed in the premeiotic phase can be indicative of unwanted gene duplications. DNA methyl-transferases that specifically recognize DNA paired hybrids as a substrate could be activated only in the premeiotic phase, preventing the transmission of active repeated sequences to progeny. The only sizeable repeated sequence protected from RIP or MIP is the highly repetitive locus encoding the large ribosomal RNAs. It has been suggested that their location in the nucleolus may activate specific mechanisms that can protect these repeated sequences (114).

It is unlikely that a similar genome-wide search mechanism in which all paired DNAs are signals sufficient for DNA methylation operates in the somatic cells of diploid organisms such as plants or animals. Unlike filamentous fungi, repeated sequences are not invariably silenced in plants, but the presence of tandemly arranged complex repeated loci often appears to be required to activate gene silencing (116). These tandemly arranged repeats, especially when organized as inverted repeats, seem to be able to acquire an inactive methylated state, perhaps assuming particular conformations such as cruciform structures that could be targets for de novo methylation (Figure 1). Moreover, when a repetitive locus assumes an inactive methylated state, it becomes a potent *trans*-silencer that is able to impose the silenced state on an unlinked homologous target locus (75). It has been suggested that inverted repeats possess the special ability to pair with homologous partners and to transfer the methylated state to paired homologous sequences (116). Although inverted repeats can mediate *trans*-silencing through DNA-DNA interaction with homologous target sequences, they could also induce transcriptional gene silencing through RNA-DNA interactions (79) involving double-stranded RNAs resulting from their transcription (Figure 1). There is no evidence as yet that dsRNA molecules or any other kind of transgenic transcript could also be involved in the transnuclear gene silencing phenomenon observed in *P. infestans*.

The isolation of mutants defective in TGS (86) and the identification of affected genes has provided insight in the identification of the molecular components required for DNA methylation and the chromatin modifications involved in the inactivation of transcription (29). In *Ascobolus* a gene called *masc1*, essential for MIP, has recently been found (76), encoding a putative DNA methyl-transferase that contains the catalytic domain conserved in all eukaryotic methyl-transferases. However, *Masc1* also has distinctive features such as a short amino-terminal domain in contrast with the long amino-terminal domain characteristic of maintenance methyl-transferases of higher eukaryotes, suggesting that *Masc1* could be the de novo methyl-transferase responsible for MIP. It has been proposed that in vivo, during the premeiotic phase, *Masc1* recognizes paired DNA as its substrate, even though no methylation activity has been demonstrated for the *Masc1* protein

in vitro. Interestingly, homologues of *mascl* are present in *Arabidopsis*, suggesting that DNA methyl-transferases with the ability to recognize paired DNA substrates could also play a role in TGS in plants (39).

Two categories of mutants defective in TGS have been isolated in *A. thaliana*: *hog* and *ddm1* mutants in which the release of gene silencing is associated with reduced levels of DNA methylation (45, 63) and *sil* and *mom* mutants that reactivate gene expression while maintaining a normal level of cytosine methylation (1, 86, 87). The emerging picture is that DNA methylation does not directly repress transcription, but possibly works as a signal to recruit chromatin components inducing transcriptional silencing. The *mom1* gene, required for maintenance of transcriptional gene silencing of several hyper-methylated loci, was found (1) to encode a nuclear protein containing a region homologous to the SWI2/SNF2 family of proteins that are involved in chromatin remodeling (91). One attractive hypothesis is that the *mom1* gene product may act downstream from DNA methylation, mediating chromatin remodeling required for the inactivation of gene expression (1). Links between DNA methylation and chromatin modifications were also obtained in animal systems in which the methyl CpG binding protein 2 was found to recruit histone deacetylase complexes (61). The finding that the *ddm1* (*decreased DNA methylation 1*) gene encodes a protein homologous to the remodeling factor SWI2/SNF2 (59) reinforced the notion that in plants, as well as in fungi and animals, DNA methylation and chromatin remodeling can cooperate in propagating epigenetic states.

POSTTRANSCRIPTIONAL GENE SILENCING

Quelling in *Neurospora*

The fungus *N. crassa* not only has the ability to detect and inactivate duplicated sequences by RIP during the sexual cycle, but duplicated sequences resulting from transgene introduction are detected and inactivated even in the vegetative phase by a phenomenon termed quelling (105). This phenomenon was discovered about 10 years ago when wild-type (orange) strains of *Neurospora* were transformed with *albino* (*al-1*, *al-2*, and *al-3*) transgenes that are required for carotenoid biosynthesis. It was observed that transformed strains frequently showed white (*albino*) phenotypes, indicating that both endogenous *albino* genes and introduced *albino* transgenes were inactivated. Since these initial observations, quelling has been observed for several different genes, indicating that it is a general gene silencing phenomenon, not restricted to the *albino* genes (19).

Analysis of quelled *Neurospora albino-1* (*al-1*) transformants revealed drastic gene-specific reductions in levels of steady-state mRNA for the duplicated *al-1* genes. It was found that quelled strains produce the same amount of *al-1* primary transcript as the wild-type strain, indicating that quelling does not affect transcription, but acts at a posttranscriptional level inducing mRNA sequence-specific degradation (18). Moreover, the unchanged steady-state levels of precursor RNA

found in quelled strains may even suggest that quelling acts by increasing the turnover of spliced mRNA in the cytoplasm. However, other possibilities such as reduced cytoplasmatic transport and/or accelerated nuclear degradation of spliced mRNA cannot be ruled out. A notable characteristic of quelling is that it is mediated by a *trans*-acting molecule that is able to diffuse in the cytoplasm and/or into other nuclei to mediate mRNA degradation (18).

Although the nature of the diffusible silencing signal is still unknown, the finding that transcription of transgenes is somehow required for quelling has led to the hypothesis that transgenic RNA molecules could be exported into the cytoplasm, inducing sequence-specific degradation of homologous mRNA. However, the presence of transgenic transcripts is not sufficient per se to trigger quelling, suggesting that gene silencing is caused by the production of a "qualitatively different" RNA frequently referred to as aberrant RNA (19). The transgenic aberrant RNAs could be produced only in specific instances, for example as a consequence of particular arrangements or location of transgenic loci, explaining why the introduction of transgenes alone is not sufficient to induce gene silencing. The fact that the presence of transgenic tandem arrays is frequently correlated with the occurrence of quelling may indicate that tandemly arranged transgenes could be a potential source for aberrant RNAs. Complex transgenic arrays not only seem to be required to induce quelling, but their presence is a prerequisite to maintain the silenced state because the loss of transgenic copies by excision via homologous recombination results in the release of quelling (20).

By using a genetic screen based on the reactivation of a posttranscriptionally silenced *al-1* reporter gene, 15 *quelling defective* (*qde*) mutants were isolated (20). It was found that these 15 *qde* mutants belonged to 3 complementation groups: *qde-1*, *qde-2*, and *qde-3*. As the different *qde* mutants are recessive, their respective gene products act in *trans* and encode factors involved in gene silencing mechanisms. Interestingly, although all the *qde* mutants isolated are completely defective in posttranscriptional gene silencing, they do not show any additional obvious phenotypes (20), indicating that the *qde* genes are not involved in other essential biological processes.

Cosuppression in Plants

As in the case of quelling in *Neurospora*, transgene-induced posttranscriptional gene silencing in plants was discovered during attempts to over-express endogenous genes by the introduction of transcribed sense transgenes (92, 140). It was observed that transgenes could interfere with normal expression of homologous endogenous genes, resulting in the coordinate suppression (cosuppression) of both transgene and homologous endogenous gene expression. Nuclear run-on assays revealed that genes affected by cosuppression are characterized by an apparently normal transcriptional activity, whereas the mRNA steady-state levels are strongly reduced, indicating that cosuppression involves a sequence-specific RNA degradation process (30, 31, 71, 139). The observation that in several cases of gene silencing in plants the accumulation of nuclear transcripts is not affected (30)

suggests that cosuppression, as well as quelling, acts by increasing the turnover of spliced mRNA in the cytoplasm. Moreover, various studies suggest that mRNA degradation induced by cosuppression may differ from that involved in normal mRNA turnover, involving endonucleolytic cleavage followed by exonuclease digestion (55, 57, 141).

Not all transgenes induce cosuppression because in a typical transformation a variable number of transgenic plants show silenced phenotypes. Thus, transgenic loci able to trigger cosuppression must have some special characteristic (32, 42). Whereas in *Neurospora* high levels of transgene transcription are not required because promoter-less transgenic constructs are efficient silencing inducers (18), in plants the frequency and the extent of cosuppression is, at least in some cases, dependent on the level of transgenic transcripts. Several reports indicate that transgenes transcribed from strong promoters and highly transcribed transgenes or those producing more stable RNA can increase the efficiency of silencing (35, 102). However, the finding that even in plants cosuppression can be induced by transgenes without promoters indicates that high levels of transgenic transcripts are not a general requirement for the activation of gene silencing. Similar to observations in *Neurospora*, cosuppression is often associated with repetitive transgenic loci either organized as direct or inverted repeats (52, 70, 131, 146). A role of repetitive transgenic loci as potent inducers of cosuppression is also consistent with the observation that transgenic repeats constructed *in vitro* induce a higher frequency of silenced transformants (52). It has been proposed that in plants both highly transcribed transgenes and repetitive transgenic loci could activate RNA degradation through the production of aberrant RNA transcripts (131).

Interestingly, it was found that posttranscriptional gene silencing (PTGS) of endogenous genes or transgenes can also be triggered by infections with recombinant RNA viruses containing homologous sequences (3, 71, 109), suggesting that viral RNA also possess some aberrant characteristic recognized by the cosuppression machinery. Although aberrant RNA molecules are frequently evoked to explain either quelling or cosuppression, their characteristics still remain elusive, as is the nature of the silencing signals that mediate gene silencing at a distance (152). As already described in fungi, the existence of such signal molecules has been spectacularly demonstrated in plants. By using grafting experiments, Vaucheret's group demonstrated that silencing can be systemically transmitted in plants (systemic acquired silencing) from a silenced stock to unsilenced scions (95–97) and that the silencing signal can travel over a long distance through the plant's vascular system (62, 143, 145).

RNA Interference

It was recently observed that double-stranded RNA (dsRNA) molecules, when injected into *Caenorhabditis elegans*, specifically interfere with the expression of homologous resident genes (41). Similar examples of dsRNA interference (RNAi) were subsequently documented in a number of invertebrate species, including planaria (110), trypanosomes (93, 123), insects (65, 66, 85), hydra (74), and more

recently vertebrates such as zebrafish (72, 94, 148) and mammals (133, 154). As with transgene-induced PTGS in plants and fungi, RNAi also appears to act post-transcriptionally to reduce the accumulation of processed mRNA (88, 89). The fact that in order to induce gene silencing, the dsRNA introduced into an organism must contain exonic sequences indicates a postsplicing target for RNAi and that RNA degradation occurs mainly in the cytoplasm (40, 122).

An interesting characteristic of RNAi is that the injection into the worm of only a few molecules of dsRNA per cell is sufficient to completely silence expression even of an abundantly expressed gene (41), suggesting that dsRNA molecules do not act stoichiometrically to silence gene expression. Furthermore, the effect appears to be non-cell autonomous because the injecting dsRNA into worm intestine (41) or feeding worms with bacteria expressing dsRNA (136) is as efficient as direct injection into the germ line. This finding, together with evidence that injection of dsRNA in *C. elegans* hermaphrodites generates RNAi that can be stably inherited into the F2 generation (41), has led to the hypothesis that dsRNA might be transported by an active mechanism across tissues and cellular boundaries to act catalytically and/or to be replicated by cellular proteins (12).

Mechanistic Basis of PTGS

Although quelling, cosuppression, and RNAi show consistent differences because they are activated, respectively, by transgenes, transgenes or viruses, and double-stranded RNA, these phenomena also present clear similarities. In all cases introduction of nucleic acid, either RNA or DNA, induces sequence-specific degradation of homologous mRNA, and in most cases gene silencing is non-cell autonomous, suggesting the existence of a specific silencing signal able to mediate gene silencing and the presence of mechanisms that can amplify those signals. These considerations have led to the idea that the various PTGS phenomena indeed reflect the existence of a single gene silencing mechanism conserved among different organisms or, at least, that PTGS phenomena are strictly related and share a common mechanistic basis. In the past year this theory has been dramatically confirmed by genetic and biochemical approaches that have greatly increased our comprehension of PTGS mechanisms.

The development of in vitro systems (53, 137) to study RNAi in *Drosophila* has provided important insight into the biochemistry of RNAi. It was found that addition of dsRNA to cell-free extracts from *Drosophila* embryos reproduces the RNA interference phenomenon in vitro, inducing sequence-specific degradation of a cognate mRNA.

The most exciting observation was that large dsRNA molecules are promptly processed into small 21–23-nucleotide-long RNA molecules when added to the extracts (157). The processing is independent of the presence of homologous target RNA, and both strands of dsRNA are processed, implicating a dsRNA nuclease in the process. Even more dazzling was the finding that the cleavage sites of the target mRNA also occur every 21–23 nucleotides and span almost exactly the region of homology with the dsRNA (157). These results clearly indicate that the

small RNA molecules guide mRNA destruction. In an independent study (53), a nuclease called RISC (RNA-induced silencing complex) was purified from RNAi-competent extracts obtained from a *Drosophila* embryonic cell line called S2. It was found that the RISC nuclease has integral nucleic acid components constituted of 21–23 nucleotide RNAs that are required for its activity, strongly supporting the notion that the small RNAs work as a guide to direct nuclease complexes to the target RNA. The involvement of small RNA molecules in PTGS has previously been demonstrated by Hamilton & Baulcombe. In their fundamental work (51), a small RNA species of about 25 nucleotides identified in plants correlated with the occurrence of PTGS induced either by transgenes or viral infections. Consequently, the presence of small RNA appears to be a general feature of PTGS in different systems, independent of the triggering events: transgenes, virus, or dsRNA.

The isolation of mutants defective in PTGS (20, 34) and the subsequent cloning of genes coding for cellular components of the PTGS machinery in different systems such as *N. crassa*, *C. elegans*, and *A. thaliana* has definitely demonstrated the existence of a common genetic base for these phenomena. The first gene involved in PTGS to be identified was *qde-1*, which is essential for quelling in *Neurospora* (21). The *qde-1* gene is highly homologous with a tomato gene encoding a protein exhibiting RNA-dependent RNA polymerase (RdRP) activity (111), providing the first experimental evidence for previous models in which RdRPs were proposed to play a key role in PTGS (73). More recently, with the cloning of the *SDE1/SGS2* gene (28, 90) required for PTGS in *Arabidopsis* and showing homology with *qde-1*, RdRP was also demonstrated to be involved in gene silencing in plants. Although the RNA polymerase activity of both the QDE-1 and SDE1/SGS2 proteins remains to be established and the nature of the RNA templates used by these proteins still awaits clarification, two models have been envisioned concerning the role of RdRPs in gene silencing (24). Because PTGS can be initiated by a single-stranded transgenic RNA both in plants and fungi, it has been proposed that RdRP can convert abnormal highly expressed transgenic RNAs or aberrant transgenic RNA into double-stranded RNA. The dsRNA molecules thus produced could induce molecular events both in plants and fungi similar to those occurring in other organisms such as *C. elegans*, in which dsRNA initiates gene silencing directly. For instance, the large dsRNA formed could be processed into small RNAs that could direct sequence-specific RNA degradation.

The finding that the introduction of transgenes able to express dsRNA as a hairpin structure induces gene silencing in plants with very high frequency (129) may confirm the above model. In this case it is of course expected that the direct expression of dsRNA can bypass the requirements of a functional RdRP. In other words RNAi should be effective even in a *SGS2/SDE1* mutant background. However, the fact that the *ego-1* gene necessary for RNAi in *C. elegans* (128) also shows homology with RdRPs suggests that the function of RdRPs is not merely the production of an initial dsRNA from a single-stranded RNA template. Owing to the extreme effectiveness of both transgene-induced PTGS and RNAi, RdRP could also be involved in amplification of the silencing effect, for example by

copying dsRNAs, increasing their concentration, or converting part of the target mRNA into dsRNA (24). Alternatively, small antisense RNA molecules in plants and fungi could be the direct products of RdRP using a single-stranded sense RNA as a template (Figure 2).

The finding that *qde-2*, essential for quelling in *Neurospora* (17), is homologous to both *rde-1*, which is necessary for RNAi in *C. elegans* (134), and *AGO-1*, which is involved in PTGS in *Arabidopsis* (36), further supports a common genetic mechanism for PTGS phenomena. The homology of these genes with the rabbit translation initiation factor eIFC2 (158) suggests a potential connection between PTGS and translation. However, inhibitors of translation initiation and elongation are not effective in blocking gene silencing either in transgene-induced PTGS (55) in plants or RNAi in *Drosophila* (157), arguing that ongoing translation is not essential for PTGS.

Although the presence of homologous nuclear genes has been shown to be unnecessary for the inheritance of RNAi in *C. elegans* (50), homologous transgenes are required not only for PTGS initiation in plants and fungi but also for the maintenance of gene silencing (20, 131). For instance, in systemic acquired silencing, in which silencing is transmitted by grafting from silenced stock to unsilenced scions, it has been found that only scions containing transgenic loci are competent to maintain silencing when removed from silencing stock (97). This observation suggests that unlike RNAi, a nuclear step is necessary in transgene-induced PTGS. For example, as described for TGS phenomena, RNA molecules could interact with transgenes, inducing DNA and/or chromatin modifications necessary for gene silencing maintenance. The finding that a RecQ DNA helicase, QDE3, is necessary for quelling in *Neurospora* (23) can be of help in understanding the epigenetic effects on transgenic loci that could be required both for initiation and maintenance of transgene-induced PTGS phenomena.

Further insight into sequence-specific mRNA degradation mechanisms comes from data indicating that mutations in the *smg* genes, required for nonsense-mediated mRNA decay, affect the persistence of RNAi in *C. elegans* (33), suggesting that RNAi and nonsense-mediated decay pathways may share some common components. Another potential candidate as a constituent of the mRNA degradation machinery is the product of the gene *mut-7*. Although its function has not been well defined, the homology between MUT-7 and 3'-5'-exonucleases such as bacterial RnaseD may suggest a role of MUT-7 as a nuclease in the degradation step of RNAi (67).

HOMOLOGY-DEPENDENT GENE SILENCING AS A GENOME PROTECTION MECHANISM

It is evident that homology-dependent gene silencing (HDGS) mechanisms have not evolved to respond to transgenes or artificially introduced dsRNA molecules, but presumably reflect natural processes aimed at host genome defense. In the

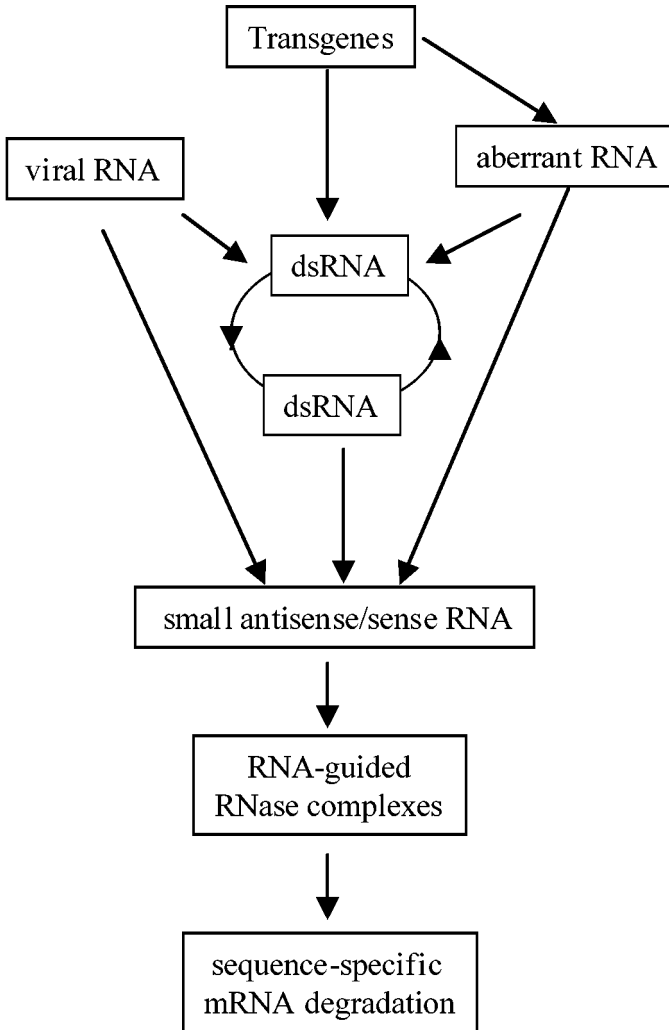


Figure 2 Aberrant single-stranded RNA transcribed from transgenes or viral RNA can be converted to double-stranded RNA (dsRNA) by the action of RNA-dependent RNA polymerases (RdRPs). dsRNA could also be a substrate of RdRP, leading to an amplification cycle. Small 20–25 nt sense and antisense RNAs result from the processing of large dsRNA or, alternatively, are produced by RdRP directly on aberrant or viral RNAs. These small RNAs can work as a guide to direct nuclease complexes to target mRNA.

past few years convincing evidence has accumulated that transposable elements and viruses represent targets for both transcriptional and posttranscriptional gene silencing mechanisms. Fungi appear particularly well equipped to fight against parasitic sequences (22). For instance, in *Neurospora* at least two gene silencing mechanisms exist, quelling and RIP, which act during the vegetative and reproductive cycles, respectively. Transposable elements appear to be particularly rare in *Neurospora* because most of the natural *Neurospora* strains isolated are devoid of active transposons. Only one natural strain out of a hundred analyzed has been found to possess an active retroelement, called Tad.

Interestingly, relics of Tad retrotransposons were found in several *Neurospora* strains, but these relics represent Tad elements showing sequence degeneration with G:C to A:T transitions characteristic of the outcome of the RIP process (14, 68). Moreover, in *Ascobolus* the small fraction of repetitive DNA is composed of full-length retroelements or relics of retroelements that are all methylated, indicating that in this case they are also the natural targets of MIP. Although DNA methylation by MIP or hyper-mutation by RIP seem to be principally directed to the inactivation of gene expression of potential dangerous invasive elements, these DNA modifications could also have another important function for genome stability and protection. DNA methylation was found to suppress homologous meiotic recombination in *Ascobolus* (77), and nucleotide divergence resulting from RIP is predicted to prevent or reduce recombination between repeats. Thus, both RIP and MIP could protect the genome against deleterious rearrangements produced as a result of events of homologous recombination between dispersed DNA repeats (26). Duplications of single endogenous genes (49) and even large duplications (101) produced as a consequence of chromosome rearrangements are also sensitive to RIP, indicating that RIP is not only proficient to combat "selfish DNA," but can also have a central role in genome stability (26).

Although transcriptional gene silencing can act directly on DNA sequences leading to gene inactivation and/or the blocking of recombination, posttranscriptional gene silencing mechanisms act on RNA molecules, inducing their degradation. At least in plants, viral RNA molecules appear to be one of the elective targets for PTGS (144). Indeed, numerous observations support the hypothesis that PTGS in plants is related to a virus resistance mechanism (103). Viruses have been shown to be both initiators and targets of PTGS. Plants transformed with a transgene homologous to the genome of a given virus were resistant to this virus, and the resistance was associated with PTGS of the transgene (135). Gene silencing of a nuclear plant gene can also be activated by infection with a virus carrying a sequence homologous to this plant gene. Subsequently, it was shown that in nontransgenic plants resistance to different viruses occurs via an RNA degradation process that resembles PTGS, suggesting that viruses can also trigger PTGS in the absence of homologous nuclear sequences. The finding that several plant viruses encoding PTGS suppressors (2, 8, 13) adopt a counter-defensive strategy (64) further suggests that PTGS is a defense mechanism (27). This hypothesis was

definitively proved by the recent evidence that plant mutants defective in PTGS also show hyper-susceptibility to virus infections (90).

There is a growing amount of data indicating that viruses may not represent the only target for PTGS. The finding that mutants defective in PTGS both in *C. elegans* and in the alga *Chlamydomonas reinhardtii* show an increased level of transposition (67, 156) indicates that transposable elements can also be subjected to posttranscriptional control. PTGS could therefore represent a mechanism operating in a synergetic fashion or in addition to TGS on repetitive DNA sequences. Transposons could be detected and inactivated at the DNA level for their repetitive nature, but their transcripts could also be recognized as aberrant and consequently degraded.

It has been proposed that dsRNA molecules may be generated either as a consequence of viral replication (151) or from the transcription of tandemly arranged multicopy transposons (67). Thus, cells may recognize dsRNA as a universal signal indicative of a threat associated with the presence of transcriptional active transposons and/or viral infections. The fact that dsRNA molecules are well-known elicitors of interferon-inducible antiviral responses in mammals (132) could further indicate a general role of dsRNA in defense mechanisms conserved throughout evolution.

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