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Quelling in *Neurospora crassa*

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I. INTRODUCTION

The term homology-dependent gene silencing (HDGS) was introduced by Matzke *et al.* (1994) to include any silencing event that results from the duplication of nucleic acid sequences; however, these events can be divided into those which occur at a transcriptional level (transcriptional gene silencing or TGS) and those which occur at a posttranscriptional level (posttranscriptional gene silencing or PTGS). Quelling in the filamentous fungus *Neurospora crassa* was discovered by Romano and Macino in 1992, as a posttranscriptional mechanism of gene silencing active during vegetative growth. Quelling is distinct from the phenomenon of gene inactivation first reported by Selker *et al.* (1987), originally named rearrangement induced premeiotically (RIP), that is, active during the premeiotic phase of sexual reproduction. Both phenomena were identified as a result of transformation with transgenes which, instead of enhancing gene expression by increasing gene dosage, caused silencing of the duplicated genes. Silencing of gene expression by RIP was subsequently shown to be the result not of rearrangement, but of C-T point mutations in the duplicated homologous sequences, and was consequently renamed repeat-induced point mutation (Cambereri *et al.*, 1989), whereas quelling results in a reduction of mRNA homologous to transgenes.

Gene silencing phenomena similar to quelling have also been discovered in other organisms as a result of the introduction of transgenes or infection by viruses. The posttranscriptional silencing mechanisms which are triggered by the presence of duplicated DNA or RNA sequences, and which result in a reduction of the mRNA homologous to such sequences, have been named co-suppression and RNA interference in plants and animals, respectively.

Silencing in plants was first observed by Napoli *et al.* (1990) and van der Krol *et al.* (1990) in petunia, with genes involved in petal pigmentation. The coordinate silencing of both ectopic transgenes and endogenous homologous genes prompted the name "co-suppression." Co-suppression was demonstrated to be a general phenomenon, as it was also reported in tomato by Smith *et al.* (1990).

The more recent discovery of silencing at a posttranscriptional level in animals was made by Fire *et al.* (1998) in the nematode *Caenorhabditis elegans*. In an attempt to inactivate gene expression by injection of antisense RNA into the animal, it was found that the antisense RNA was no more effective than sense RNA in inducing gene silencing. In fact, it was discovered that small amounts of double-stranded RNA (dsRNA), present as contaminants in *in vitro* RNA preparations, had triggered the observed silencing phenomenon, which was therefore named double-stranded RNA interference (RNAi) (Fire *et al.*, 1998). RNAi was subsequently shown to be active not only in *C. elegans*, but in other animals such as *Drosophila melanogaster* (Kennerdale and Carthew, 1998; Misquitta and Paterson, 1999), *Trypanosoma brucei* (Ngo *et al.*, 1998), and *Planaria* (Sanchez *et al.*, 1999). RNAi has further been shown to be active in *Arabidopsis thaliana*

plants (Waterhouse *et al.*, 1998; Chuang and Meyerowitz, 2000) and vertebrates, namely, in mice (Wianny and Zernicka-Goetz, 2000) and in zebrafish (Li *et al.*, 2000; Wargelius *et al.*, 1999).

II. THE DISCOVERY OF QUELLING IN *Neurospora*

The first report of silencing in the vegetative phase of growth in fungi was made by Pandit and Russo (1992) in *Neurospora crassa*. They observed a loss of hygromycin resistance as a result of transformation with a plasmid carrying the bacterial hygromycin phosphotransferase (*hph*) gene fused to the promoter of the *trpC* gene of *Aspergillus nidulans*. Cytosine methylation of the transgene was correlated with silencing, suggesting possible “position effects” due to the transgene integration site; however, the fact that only stably transformed strains harboring many insertions of the *hph* gene were methylated indicated that methylation may have been due to transgenes being linked as a multicopy sequence rather than their site of insertion. Growth of *Neurospora crassa* in the presence of the cytosine analog 5-azacytidine, which prevents cytosine methylation, resulted in the reactivation of the *hph* gene, further suggesting that DNA methylation is involved in gene silencing. Moreover, gene silencing was found to be reversible, as hygromycin resistance can be recovered during vegetative growth.

While the silencing observed by Pandit and Russo involved transgenic sequences only, Romano and Macino (1992) reported silencing of both transforming DNA and homologous endogenous sequences. The new phenomenon observed in *Neurospora crassa* was called “quelling.” In the experiments carried out by Romano and Macino (1992), transformation was carried out with constructs of genes involved in the carotenoid biosynthetic pathway, as markers of silencing. Vegetative growth in *Neurospora crassa* is characterized by branching hyphae which form a mycelium that produces asexual spores called conidia. Carotenoids produced both in the mycelium and in conidia confer a bright orange color to *Neurospora*; however, mutation of any one of three genes involved in carotenogenesis determines an albino phenotype. The *albino* genes (*al-1*, *al-2*, and *al-3*) code for phytoene dehydrogenase (Schmidhauser *et al.*, 1990), phytoene synthase (Schmidhauser *et al.*, 1994), and geranylgeranyl pyrophosphate synthase (Nelson *et al.*, 1989), respectively. These genes are particularly suitable for use as markers of gene silencing, as the albino phenotype is easily visible and facilitates the identification of silenced strains, especially in the screening of large numbers of colonies. Carotenoid content can also be quantified, which is particularly useful to calculate the degree of silencing.

Silencing was observed as a result of transformation of *Neurospora crassa* with plasmids containing constructs of either the *albino-3* (*al-3*) or *albino-1* (*al-1*) gene, demonstrating it to be a general phenomenon, not restricted to a single

gene; however, both frequency and severity of silencing varied. Approximately 40% of the progeny of the *al-1* transformants had phenotypes ranging from white to dark yellow, indicating stronger or weaker suppression of gene expression, while only 0.5% of the *al-3* progeny had silenced phenotypes. This difference may be explained by the fact that *al-3* is an essential gene and therefore silenced progeny were not viable. A further demonstration that the frequency of silencing can be variable was reported by Cogoni and Macino (1997a) as a result of the transformation of *Neurospora* with a portion of the *albino-2* (*al-2*) gene. In this case the frequency of silencing was 10%, indicating that the *al-2* gene is less sensitive to silencing. Several factors may determine such variation in frequency. The first is the intrinsic characteristics of the gene determined by its nucleotide sequence. Nucleotide complementarity could lead to the formation of secondary structure in mRNA transcripts, making them more or less resistant to the action of RNAses. Second, the frequency of silencing could also be influenced by the level of gene expression. For example, one might expect that highly expressed endogenous genes could be more resistant to degradation; however, paradoxically, it seems that, at least in plants, highly expressed genes are easier targets for co-suppression, suggesting possible threshold-like effects (Elmayan and Vaucheret, 1996; Jorgensen *et al.*, 1996). Threshold effects are discussed further in Section IV.A.

Further evidence that quelling is a general mechanism, not limited to specific genes in *Neurospora*, was provided by the fact that the *qa-2* gene encoding quinic acid dehydrogenase, used as a genetic marker for transformation in the experiments carried out by Cogoni and Macino (1997a), was also silenced. This was further supported by the reports of quelling of two transcription factors in *Neurospora*, the *white-collar* genes (*wc-1* and *wc-2*) by Ballario *et al.* (1996) and Linden *et al.*, (1997), respectively, and of the *ad-9* gene involved in adenine biosynthesis by Schmidhauser (Cogoni and Macino, 1998, and references therein).

It was shown that inactivation of the *albino* genes was not due to gene disruption, as no rearrangements were found in the endogenous genes, but to a heavy reduction of the steady-state level of mRNA. It was found that over prolonged culture time, 25% of the silenced progeny tended to revert progressively to wild-type or intermediate phenotypes, demonstrating that the silencing of the *albino* genes was reversible. Reversion was unidirectional and correlated with increased levels of steady-state mRNA. Reversion also correlated with the loss of ectopic copies of the *albino* transgenes, probably due to homologous recombination during the vegetative phase (known as mitotic instability), which can result in the deletion of ectopic sequences, especially in the case of tandemly arranged transforming sequences (Selker, 1990). Thus, it seems that quelling per se is not unstable, but that the instability is a consequence of transgenes loss, suggesting that transgenes are not only required for the establishment of quelling, but are also necessary for its maintenance.

The involvement of methylation in quelling was tested. While silenced endogenous *albino* genes were never found to be methylated, it was observed that tandemly arranged transgenic loci whose appearance correlated with the occurrence of gene silencing were frequently methylated, suggesting that methylation of transgenes could somehow be required for silencing. Previous observations in which treatment with 5-azacytidine determined reversion in some silenced transformants seemed to substantiate this hypothesis (Romano and Macino, 1992); however, the subsequent observation of normal levels of quelling in *methylation-deficient* (*dim-2*) *Neurospora crassa* mutants definitively ruled out the involvement of DNA methylation in quelling (Cogoni and Macino, 1997a). The apparent incongruity between the observed reversion of some cases of gene silencing induced by 5-azacytidine and the lack of involvement of DNA methylation in quelling could be explained by the fact that another gene silencing mechanism sensitive to 5-azacytidine is active during the vegetative growth phase of *N. crassa* (C. Cogoni, unpublished results).

Further characterization of the quelling phenomenon exploited the characteristic coenocytic nature of *Neurospora* hyphal cells in which many nuclei share a common cytoplasm. *Neurospora* is defined as a homokaryon when genetically identical nuclei are present in the same cytoplasm, while heterokaryons harbor genetically diverse nuclei. Transformation of multinucleate *Neurospora* spheroplasts often results in the formation of heterokaryons, as not all nuclei incorporate transforming sequences. Since mutations in the *albino* genes are typically recessive, heterokaryons containing both wild-type nuclei and nuclei with mutated *albino* genes present an orange (wild-type) phenotype. In contrast, heterokaryons containing both wild-type nuclei and nuclei with silenced *albino* genes would only be albino in the event that quelling was a dominant trait. An albino phenotype was observed in forced heterokaryons between *al-1* quelled and wild-type nuclei, demonstrating that quelling is indeed a dominant trait, probably mediated by a cytoplasmatically diffusible molecule acting *in trans* (Cogoni *et al.*, 1996).

Also in plants, co-suppression was reported to diffuse from a silenced transgenic stock to a nonsilenced transgenic scion (Palaqui *et al.*, 1997; Voinnet *et al.*, 1997). The phenomenon was called "silencing acquired systemically" (SAS), due to the fact that the silencing signal was able to diffuse through plasmodesmata between cells and via the plant's vascular system to other parts of the plant (Voinnet *et al.*, 1998).

As well as being dominant, Cogoni *et al.* (1996) demonstrated that quelling in *Neurospora* is a posttranscriptional mechanism. The level of unspliced mRNA for the *al-1* gene was the same in both wild-type and silenced strains, while a dramatic reduction of spliced *al-1* mRNA was observed in quelled strains, indicating that quelling acts at a posttranscriptional level and does not influence the rate of transcription. Moreover, the fact that the nuclear levels of *al-1* mRNA were unchanged suggested that the reduction of the mRNA level was due to a

degradation process occurring in the cytoplasm. These results were in accordance with those obtained by de Carvalho *et al.* (1992, 1995) in the co-suppression of the β 1-3 glucanase gene in tobacco plants and by Smith *et al.* (1990) with the polygalacturanase gene in tomato, as in both cases an increased turnover of RNA of the duplicated genes was observed. Silencing of viral genes in plants was also observed to act at a cytoplasmic level in transgene-induced resistance to viral infections by Lindbo *et al.* (1993) and van Blokland (1994).

By transformation with various constructs Cogoni *et al.* (1996) also demonstrated that 132 bp of homology to transcribed exonic sequences was still able to trigger quelling, even though at a low frequency. Specific sequences are not required, amino-terminal and carboxy-terminal sequences are equally efficient in triggering quelling, and promoter sequences do not cause quelling. The above characteristics, together with the evidence that the signal for quelling is diffusible, suggested that it may involve an RNA molecule. Grierson *et al.* (1991) proposed that silencing could be a consequence of the unwanted production of transgenic antisense RNA (asRNA) molecules. Such asRNA could be synthesized on a transgenic DNA template integrated in the antisense direction with respect to an endogenous promoter near the integration site. Alternatively, asRNA could originate from the activation of a transgenic cryptic promoter (Baulcombe, 1996). An RNase protection assay was therefore carried out to identify anti-sense RNA in quelled *Neurospora* strains (Cogoni *et al.*, 1996); however, no asRNA could be detected. To test whether lack of detection may have been due to the highly unstable nature of the asRNA, a highly expressed anti-sense construct was made; however, transformation with this construct did not increase the frequency of quelling, indicating that in *Neurospora* silencing by asRNA does not increment an already functioning process. Instead, the unexpected finding of a chimeric sense RNA transcribed from the promoterless *al-1* transgene suggested that this molecule could have been the silencing trigger molecule. It was thought that transcription of the sense RNA may have occurred by read-through transcription from the promoter of a neighboring gene to the site of integration, or from a cryptic promoter in the plasmid, as vector sequences were identified in the 3' and 5' ends of the unexpected sense RNA. Although a sense RNA would appear to be necessary, it may not be sufficient per se to trigger quelling, as shown by the fact that high levels of a sense transcript driven by the *Aspergillus nidulans trpC* promoter were also found to be present in a nonquelled transformant, indicating that the level of transcription of the exogenous sequence is not a determining factor. It was hypothesized, therefore, that the sense RNA could possess some aberrant characteristic that confers a signal on the sense RNA to trigger the sequence of events that determines a reduction of homologous mRNA in quelled strains.

Flavell *et al.* (1994) proposed that epigenetic modifications of transgenic DNA sequences such as methylation, which can block transcription elongation, could be responsible for the transcription of a preterminated aberrant RNA. Other

aberrant characteristics could be represented by covalent modifications and/or formation of a complex with specific proteins. Alternatively, it could be the chimeric nature or abnormal structure of a sense RNA, due respectively to read-through transcription of tandem arrays of transgenes, or to hairpin structures formed by intramolecular bonding in transcripts from inverted repeats of transgenes, which confer on the RNA a quality recognized as a signal for silencing. Evidence of the silencing effect of hairpin RNAs has recently been offered by Smith *et al.* (2000) in tobacco. Almost 100% efficiency of silencing, manifested by immunity to the potato virus Y (PVY), was obtained as a result of transformation of tobacco with a construct of the viral Nia-protease (*Pro*) gene containing a single self-complementary hairpin RNA (hpRNA), while induction of silencing by normal co-suppression and antisense methods resulted in only 7% and 4% of immune transformants. Hairpin dsRNA introduced into *Trypanosoma brucei* has also been found to induce RNAi in up to 100% of transgenic progeny (Tavernarkis *et al.*, 2000).

The transcript produced from such transgenic sequences could be not only the trigger, but also the diffusible factor responsible for the dominant character of quelling; however, it has still not been demonstrated whether it is the aRNA itself, or some other RNA species produced downstream in the silencing process, that acts as the diffusible molecule.

III. THE QUEST FOR *quelling-defective (qde)* MUTANTS

A mutagenesis approach was used in the quest to isolate cellular factors involved in quelling. As in previous experiments by Romano and Macino (1992), which led to the discovery of quelling, and those of Cogoni *et al.* (1996), which proved that quelling was a posttranscriptional event probably triggered by an aberrant RNA molecule, the carotenogenic gene *al-1* was used as the exogenous transforming sequence in the experiments carried out by Cogoni *et al.* (1997a) in the search for possible *quelling-defective* mutants. It was postulated that mutations affecting a gene required for the quelling mechanism would determine the release of *al-1* silencing, resulting in the recovery of an orange wild-type phenotype. The strategy adopted was to mutagenize a stably transformed *albino* strain, i.e., one that did not present reversion, as is frequently observed in quelled strains (Romano and Macino, 1992), to exclude that the recovery of a wild-type phenotype was due to reversion.

Nineteen of 100,000 colonies were analyzed for exogenous copy number and possible rearrangements of exogenous DNA that could have accounted for the release from gene silencing. Two groups emerged: four strains had lost exogenous copies, probably the reason for the release from quelling, while the remaining 15 had neither lost copies nor had rearrangements of exogeneous DNA.

A genetic approach was used to classify the *quelling-defective* (*qde*) mutants into complementation groups. As quelling is a dominant trait, it was possible to carry out complementation tests by creating forced heterokaryons between wild-type and quelling-defective strains. Albino phenotypes were predicted in the case of complementation by the wild-type (orange) nuclei producing the *qde* gene products in heterokaryons with *quelling-defective* (orange) nuclei carrying recessive mutations. A heterokaryon between two *qde* strains (orange) was expected to show an albino phenotype if the recessive mutations were in different loci, whereas an orange phenotype would have been shown in the event the two *qde* mutants were alleles.

Three complementation groups emerged from these tests, indicating that three individual genes are involved in the quelling mechanism. The inability to restore quelling in the complementation tests between wild-type strains and members of the group that had lost transgenic copies and produced no transgenic sense RNA demonstrated that a transgenic sense RNA is essential for quelling in the heterokaryon. Confirmation that the *qde* mutants were indeed impaired in quelling was obtained by testing their ability to silence the native *al-2* gene following transformation with a transgenic copy of the *al-2* gene. None of the *qde* mutants presented additional phenotypes to quelling defectiveness, indicating that the *qde* genes are not involved in other essential biological processes.

In an attempt to identify additional loci involved in quelling that may have escaped the initial screening, more than 50 additional independent *qde* mutants were tested for complementation with strains from each of the three previously identified complementation groups; however, all were found to belong to the previously identified complementation groups (C. Cogoni, unpublished results). Due to the method of screening adopted, the 50 *qde* mutants were identified based on their ability to completely release *al-1* gene silencing (i.e., only colonies presenting a full orange color were selected). However, mutations in genes that encode other, partially or fully redundant factors for quelling which do not determine a completely reverted phenotype, together with silencing factors that also have essential biological functions, are expected to escape from this type of screening.

IV. ISOLATION OF THE GENES INVOLVED IN QUELLING IN *Neurospora*

The insertional mutagenesis approach was used to isolate the *qde* genes. Taking advantage of the fact that in *Neurospora crassa* plasmids are integrated at random into the genome, transformation with a plasmid was used as a simple tool to perform insertional mutagenesis. The same *al-1* stably silenced transformant strain used for UV mutagenesis by Cogoni *et al.* (1997a) to identify the *qde* genes was used as a recipient for insertional mutagenesis. Insertional mutants were isolated

by their ability to grow on media selective for transformants only and by their quelling-defective (orange) phenotype.

By complementation analysis with the three previously identified groups of *qde* strains, the mutants were assigned to each of the three complementation groups. Genomic DNA flanking the site of insertion of the transgene was used as a probe to identify cosmids containing genomic DNA capable of complementing each of the mutants. The three *qde* genes are described below in the order in which they were isolated.

A. The *qde-1* gene

Of the three classes of *quelling-defective* (*qde*) genes identified in *Neurospora crassa* (Cogoni *et al.*, 1997a), *qde-1* was the first to be isolated (Cogoni and Macino, 1999a). It encodes a protein of 1402 amino acids with significant homology to an RNA-dependent RNA polymerase (RdRP) isolated in tomato (Schiebel *et al.*, 1998). The region of homology does not involve the entire protein sequence, but is restricted to the carboxy-terminal portion, probably defining a conserved functional domain. Homology between the two proteins strongly supports the involvement of an RdRP in PTGS (Lindbo *et al.*, 1993). The importance of the cloning of the *qde-1* gene resides in the fact that this was the first experimental evidence linking a gene demonstrated to be involved in gene silencing and a biochemical function proposed to play a role in PTGS.

In vitro experiments in tomato have demonstrated that RdRP can use both single-stranded RNA and single-stranded DNA as a substrate for RNA transcription. The insensitivity of RdRP to α -amanitin and actinomycin D excluded the possibility that the enzyme was a DNA-dependent RNA polymerase. Moreover, RNA synthesis may be carried out either with or without primer extension (Schiebel *et al.*, 1993a, 1993b). The apparent versatility of this RdRP enzyme makes it a good candidate to satisfy the various roles attributed to RdRP in different PTGS models. Furthermore, it has been reported that the steady-state level of RdRP mRNA increases as a result of viroid infection in tomato (Schiebel *et al.*, 1998), and similarly in *Neurospora*, the mRNA level of *qde-1* is greater in quelled strains (Cogoni and Macino, 1999), suggesting that the surveillance system that activates PTGS in the presence of invasive nucleic acids determines a transcriptional control of RdRP required for the subsequent sequence-specific mRNA degradation (Baulcombe, 1999).

Several *qde-1* homologous genes have been identified in various species of plants and animals. Seven homologs are present in *Arabidopsis*, of which one, SDE1/SGS2, has been demonstrated to be involved in gene silencing and possibly in virus-induced PTGS (Mourrain *et al.*, 2000; Dalmay *et al.*, 2000). A *qde-1* homolog has also been identified in the yeast *Saccharomyces pombe*, indicating that a PTGS mechanism may exist in this organism. In *Caenorhabditis elegans*,

the *ego1* locus encodes a protein homologous to QDE-1 that is involved not only in RNAi (pointing out the correlation of this phenomenon with PTGS and quelling), but also in germline developmental programs (confirming studies on the *qde-2* gene and its homologs that suggest that some steps of gene silencing and developmental pathways are in common). It is interesting to point out that *ego-1* expression is limited to germinal cell lines and that *ego-1* mutants are defective in RNAi of maternal genes only, while genes expressed in the zygote remain sensitive to gene silencing (Sardon *et al.*, 2000).

The exact role of RdRP in quelling, co-suppression, and RNAi has still not been completely clarified because both the substrate and the product of RdRP enzymatic activity remain to be identified. A possible substrate for RdRP could be a transgenic RNA transcript. Recognition by the RdRP enzyme of such a transcript could be due to its aberrant characteristics. Various hypotheses have been advanced as to possible factors that may determine aberrancy. These include pretermination, covalent modifications, and read-through transcription determining a chimeric transcript and secondary structure. The “double-strandedness” of hairpin RNA could be the aberrant characteristic that signals RdRP to use such RNA as a template for polymerisation in plants (Kooter *et al.*, 1999).

The discovery by Hamilton and Baulcombe (1999) of small antisense 21–23-nt RNAs associated with co-suppression in *Arabidopsis* plants led to the hypothesis that RdRP could be involved in the generation of such molecules. This is supported by the finding that these small RNAs are depleted in an RdRP mutant in *Arabidopsis* (Dalmay *et al.*, 2000). RdRP could generate small asRNAs in two possible ways: first, small asRNA could be synthesized directly on a single-stranded aRNA template by discontinuous polymeration; second, RdRP could convert single-stranded aRNA to a large double-stranded RNA by continuous polymerization. The large dsRNA formed thereby could then be processed in small RNA molecules by a dsRNA endonuclease (see Figure 9.1). The latter hypothesis could be supported by the fact that *in vitro Drosophila* extracts, large dsRNA molecules are processed into 21–23-nt RNAs (Zamore *et al.*, 2000). Also in *Drosophila*, a temporal correlation between the appearance of small RNAs and gene silencing induced by dsRNA has been observed (Yang *et al.*, 2000). Strikingly, target mRNA has been found to be degraded by endonucleolytic cleavage at the same 21–23-nt intervals, and it has therefore been hypothesized that these small RNAs could act as “guide” molecules to mediate the degradation of homologous mRNAs (Zamore *et al.*, 2000). Moreover, the small RNA molecules have been observed to be associated with ribonuclease enzymatic activity (Hammond *et al.*, 2000).

B. The *qde-3* gene

The finding that the 1955-amino acid protein encoded by *qde-3* was homologous to a RecQ-like DNA helicase was an important discovery assigning, as in the case of

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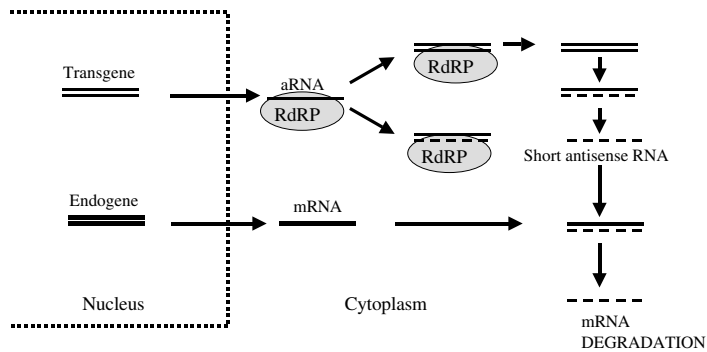


Figure 9.1. Possible mode of action of the RNA-dependent RNA polymerase encoded by the *qde-1* gene. RdRP either synthesizes a single cRNA molecule or many short cRNA molecules on an aberrant RNA template, which is subsequently cleaved into 21–23-nt dsRNA molecules (thin lines). The short RNAs mediate quelling by guiding the degradation of homologous mRNAs.

qde-1, a specific function to a factor involved in the quelling mechanism (Cogoni and Macino, 1999b). Homology from bacteria to humans demonstrates that the RecQ DNA helicase functions are highly conserved. Moreover, some organisms have several homologs: as many as five RecQs have been identified in humans, four in *C. elegans* (Kitao *et al.*, 1998, and references therein), and six RecQ proteins are expressed in *Arabidopsis* (Hartung *et al.*, 2000). All RecQ proteins are characterized by seven highly conserved DNA helicase domains, but homologs vary in size depending on the length of the N- and C-terminal nonhelicase regions. Although homology between the RecQs of different organisms is strictly confined to the helicase domains, some homology may be found in the long terminal regions, with the N-terminal region often characterized by stretches of acidic amino acids. The interaction of the nonhelicase regions with different accessory proteins may confer substrate specificity in organisms where several homologs exist.

The main role of RecQ DNA helicases is maintenance of genome stability, with activities in DNA repair, regulation of illegitimate recombination, and processing of DNA at replication forks. Defects in RecQ genes are responsible for various deleterious effects ranging from hyperrecombination to premature aging and a predisposition to cancer, characteristic of the human genetic disorders known as Werner, Bloom, and Rothmund-Thomson syndromes. The recent discovery of the *qde-3* gene, linked to posttranscriptional gene silencing, has added a new function to the possible roles of DNA helicases.

The demonstration of the interaction of RecQ helicases with topoisomerases and the fact that *qde-3* mutants are sensitive to type I topoisomerase inhibitors suggest a role for *qde-3* in quelling in resolving complex DNA structures between repeated transgenes to allow transcription into an aberrant RNA

molecule that triggers the sequence of events that leads to silencing of homologous mRNAs. The N-terminal region of the yeast RecQ homolog *sgs-1* has been demonstrated to interact with topoisomerase III (Gangloff *et al.*, 1994; Bennett *et al.*, 2000) to form an enzymatic complex which suppresses hyperrecombination between repeated sequences. There is also evidence in the fission yeast *Schizosaccharomyces pombe* of a similar functional interaction between the RecQ homolog *rqh-1* and a type III topoisomerase. The carboxy-terminal end of topoisomerase II, essential for faithful chromosome segregation during replication, has also been shown to interact with *sgs-1* (Watt *et al.*, 1995). Brosh *et al.* (2000) have shown a functional interaction between the single-stranded DNA-binding (SSB) protein hRPA and BLM and between topoisomerase III and BLM in humans, and likewise Shen *et al.* (1998) have demonstrated interaction and between SSBs and the WRN protein. QDE-3 is a large helicase, but lacks the N-terminal domain present in the WRN protein with 3'-5' DNA exonuclease activity, hypothesized for the removal of damaged DNA prior to repair. It cannot be excluded, however, that RecQ DNA helicases that do not have the RNaseD-like exonuclease domain in the same gene can interact *in trans* with an exonuclease. This is supported by a recent finding in *Arabidopsis* of the interaction between two of its RecQ homologs, one of which encodes an RNaseD-like exonuclease, demonstrating that the two functions of helicase and exonuclease may act *in trans*, at least in plants (Hartung *et al.*, 2000). The discovery in *C. elegans* of homology between the *mut-7* gene involved in transposon silencing and the WRN exonuclease domain encoding a RNaseD-like exonuclease (Ketting *et al.*, 1999), and in *Neurospora crassa* of homology between QDE-3 involved in quelling and a RecQ DNA helicase (Cogoni and Macino, 1999c), could be further evidence of *trans* interaction between helicase and exonuclease functions in PTGS. As shown in Figure 9.2, QDE-3, together with a topoisomerase, could therefore be important in remodeling chromatin by resolving complex DNA structures such as Holliday junctions (Holliday and Pugh, 1975) or cruciforms between inverted repeated sequences, to permit transcription. Such DNA-DNA interactions could also lead to methylation and consequently to the transcription of an aberrant RNA to trigger quelling.

C. The *qde-2* gene

While the function of *qde-1* and *qde-3* can be envisaged in a model of the action of the *Neurospora qde* genes in quelling, the collocation of *qde-2* is more difficult due to the fact that little is known regarding both the biochemistry and the function of the protein encoded by this gene. The *qde-2* locus is a member of a large novel gene family, conserved from plants to animals, involved in developmental pathways as well as in silencing processes. Sequence alignments of the *qde-2* gene family reveal a variable N-terminal portion and a conserved C-terminal region that may be involved in some specific function, probably containing a novel functional

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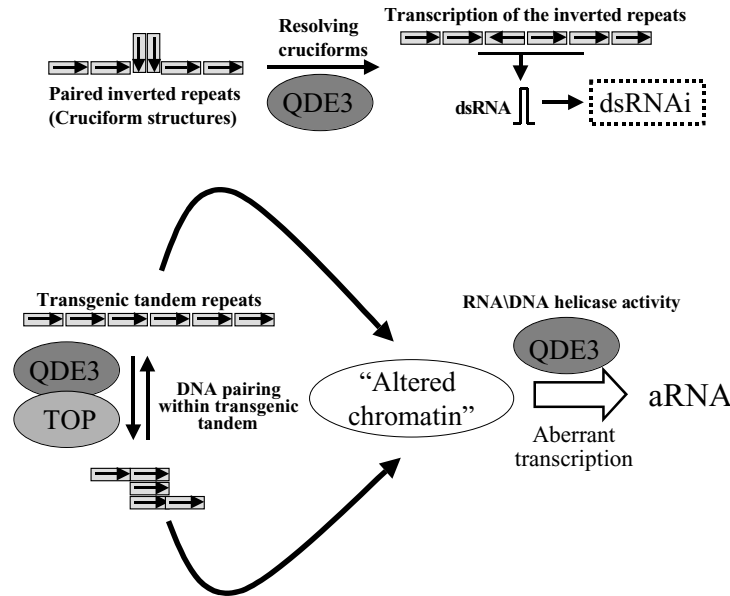


Figure 9.2. Possible mode of action of the DNA-helicase encoded by the *qde-3* gene. The DNA helicase may resolve complex DNA structures formed as a result of pairing of inverted repeated DNA sequences or transgenic tandem repeats, to enable transcription of an aberrant RNA.

domain (Catalanotto *et al.*, 2000). Interestingly, a *qde-2* homolog was identified in *S. pombe*, but not in *S. cerevisiae*. An explanation suggested by Aravind *et al.* (2000) is that functionally linked genes involved in PTGS in *S. cerevisiae* have been co-eliminated. In fact, *S. cerevisiae* lacks both QDE1 and QDE2 homologs.

In *Arabidopsis* there are seven *qde-2* homologs (Fagard *et al.*, 2000). The first and better characterized of these is AGO1 (ARGONAUTE-1), so called for the squidlike aspect of its mutants (Bohmert *et al.*, 1998). The molecular function of AGO1 is not known, but its mutant phenotype involves both leaves and flowers. It has been demonstrated that AGO-1 is involved in PTGS, as it influences the expression level of transgenes (Mourrain *et al.*, 2000). Moreover, in an AGO-1 mutant background, methylation of transgenic sequences is significantly reduced, indicating a role for AGO-1 in a control step of PTGS that involves DNA methylation (Fagard *et al.*, 2000). Another *qde-2* homolog in *Arabidopsis*, ZWILLE/PINHEAD, is required for the correct development of primary and axillary shoot apical meristems. This locus is responsible not for differentiation, but to maintain the central cells of the embryonic shoot apical meristem in an undifferentiated state (Moussian *et al.*, 1998), and is responsible for the formation of axillary meristems in postembryonic phases (Lynn *et al.*, 1999).

In *Drosophila*, the protein transcribed by the *piwi* gene seems to be a determinant in germline stem cell self-renewing division and maintenance, in both males and females, probably through a somatic signaling pathway; moreover, the presence of *piwi* mRNA in the germline is important during development in embryogenesis (Cox *et al.*, 1998). Again in flies, the STING protein controls the posttranscriptional expression level of the repetitive X-linked *Stellate* locus in the germlines of both sexes. Furthermore, this protein controls male fertility, meiotic disjunction, and meiotic drive (Schmidt *et al.*, 1998). Recently, a protein belonging to this family was cloned in rat, but with different cell-dependent localizations (either in the Golgi apparatus or in the endoplasmic reticulum). The GERp95 protein presents strong homology with AGO1, ZWILLE, and PIWI (Cikalus *et al.*, 1999).

Rabbit eIF2C (previously named Co-eIF-2A) was purified as a member of the translation machinery from a reticulocyte lysate (Chakravarty *et al.*, 1985). The eIF2C protein is implicated in the initiation of protein synthesis, stimulating the formation of a ternary complex between Met-tRNA, GTP, and the eukaryotic initiation factor eIF2 (Zou *et al.*, 1998).

Lastly, in the nematode *C. elegans* there are at least 23 genes that can be grouped in the *qde-2*-like gene family, one of which, *rde-1*, is implicated in double-stranded RNA interference. Although normally viable and fertile, *rde-1* mutants are completely defective in RNAi directed at both maternally and zygotically expressed genes (Tabara *et al.*, 1999). Interestingly, transposon mobility (Tabara *et al.*, 1999) is unaffected by loss of the RDE-1 function, suggesting that this gene (together with *rde-4*) is not required for the initiation of the silencing when the signal is an invasive DNA molecule. It can be assumed that common steps of specific mRNA degradation could be activated by two different pathways: either by an RDE-1-independent pathway triggered by invasive DNA molecules such as transposons or transgenes, or by an alternative pathway induced by dsRNA molecules, such as genomes of viruses or viral RNA intermediates or transgenic dsRNA, in which RDE-1 activity is essential. The fact that *rde-1* seems to be involved in the establishment of dsRNA-directed gene silencing was confirmed by experiments carried out by Grishok *et al.* (2000), showing that *rde-1* is essential for initiation of heritable RNAi, but is not required for the subsequent transmission of the phenomenon.

The large number of QDE2 homologs constitutes a family of genes that embraces functions in both PTGS and developmental pathways. QDE2 and RDE1 appear to be involved in PTGS only, while ZWILLE and PIWI control stem-cell division and maintenance, with AGO1 and STING functioning as a link between the two processes. We suggest that either common steps exist between the two processes, or the same enzymes are involved in the two different biological programs. Alternatively, a reasonable assumption could be that a posttranscriptional

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Table 9.1. Genes of the QDE2 Family and Their Involvement in PTGS and/or Development

Gene	Organism	PTGS	Development	Other function
<i>ago-1</i>	<i>Arabidopsis thaliana</i>	X	X	
<i>rde-1</i>	<i>Caenorabditis elegans</i>	X		
<i>qde-2</i>	<i>Neurospora crassa</i>	X		
<i>zll</i>	<i>Arabidopsis thaliana</i>		X	
<i>eIF2C</i>	rabbit			Translation initiation factor
<i>sting</i>	<i>Drosophila melanogaster</i>	X	X	
<i>piwi</i>	<i>Drosophila melanogaster</i>		X	
<i>Gerp95</i>	rat			

regulation mechanism acts during development to control the expression of genes involved in development. The homology of QDE2, RDE1, and AGO1 with eIF2C may lead one to believe that an association between PTGS and translational processes exists, but transgene-induced PTGS in plants (Holtorf *et al.*, 1999) and RNAi in *Drosophila* (Zamore *et al.*, 1999) are not blocked either by initiation or by elongation translation inhibitors. Due to the fact that eIF2C organizes the interaction between RNA (Met-tRNA) and protein (eIF2) molecules in a ternary complex, it would be tempting to speculate that QDE2 in *Neurospora*, RDE1 in *C. elegans*, and AGO1 in *Arabidopsis* could act as mediators in PTGS through RNA–RNA or RNA–protein interactions. A list of homologous *qde-2* genes is shown in Table 9.1.

V. A UNIFIED MODEL FOR QUELLING, CO-SUPPRESSION, AND RNA INTERFERENCE

Comparisons between co-suppression in plants and quelling in *Neurospora* have been important in the formulation of PTGS models, and although some differences do exist, a majority of common features have been revealed, supporting the notion that PTGS is indeed a conserved phenomenon. Common features include the following.

- Gene specificity is observed.
- Both endogenous and transgenes are silenced.
- Duplication of parts only of coding sequences can induce silencing.
- Homology of promoter sequences is not required.
- The introduction of transgenes per se is not sufficient to induce gene silencing.
- Tandem repeats or inverted repeats of transgenes are stronger inducers of silencing than single copies.

- The transcription of transgenes even at a low level seems to be necessary.
- Ongoing protein synthesis is not necessary.
- Varying degrees of the reduction of steady-state mRNA is a consequence of a posttranscriptional event.
- Both mechanisms are reversible in a stepwise manner.
- Both mechanisms are mediated by a *trans*-acting molecule.

See Cogoni and Macino (1997b) and Depicker and Montagu (1997) for reviews and references therein.

Many of the above features are common also to the recently discovered phenomenon of double-stranded RNA interference (RNAi). The main features of RNAi, reviewed in Tabara *et al.* (1999), are the following.

- dsRNA containing exonic sequences is necessary for RNAi.
- Small quantities of dsRNA can induce RNAi in a nonstoichiometric fashion.
- dsRNA determines a reduction of homologous endogenous mRNA.
- RNAi acts systemically.

The common features that quelling shares with co-suppression, RNAi and transposon silencing in plants and animals, makes a general model for gene silencing feasible (Cogoni and Macino, 1999c, 2000; Plasterk and Ketting, 2000; Ketting and Plasterk, 2000; Jensen *et al.*, 1999; Sijen and Kooter, 2000). The numerous genes identified in PTGS in both plants and animals, homologous to the *Neurospora crassa qde* genes (see Table 9.2), not only reinforce a unified model, but indicate that PTGS is basically similar in all organisms, probably having evolved from an ancestral gene silencing mechanism aimed at protecting the genome from invading nucleic acid molecules.

A model to explain quelling in *Neurospora crassa* is proposed in which the *qde-3* DNA helicase could be involved in the initial recognition process, maybe unwinding cruciform or other complex DNA structures which could be formed as a result of DNA–DNA pairing of inverted transgenic repeats and/or multiple copies of transgenes, to permit transcription. The aberrant sense RNA molecule transcribed from the exogenous DNA sequence, due to its inherent aberrant characteristics, could be recognised by *qde-1* RdRP and made into a double-stranded RNA molecule either by continuous polymerization or by the synthesis of many 21–23-mer asRNA molecules. These dsRNAs may then be subjected to the endonucleolytic action of a dsRNase that cleaves the dsRNA into small 21–25-nt RNA species. These small RNAs could mediate the degradation of homologous endogenous mRNAs, acting as “guide” molecules in a complex with an RNase. Furthermore, the small RNAs could act as primers on endogenous homologous mRNAs to enable RdRP to create double-stranded mRNA molecules which are subsequently processed, activating a positive feedback loop. In this way, additional

Table 9.2. Genes Involved in PTGS in Various Organisms with Putative Functions and Probable Roles

<i>Neurospora crassa</i>	<i>Caenorhabditis elegans</i>	<i>Arabidopsis thaliana</i>	<i>Drosophila melanogaster</i>	Putative function	Probable role	References
<i>qde-1</i> ⁽¹⁾	<i>ego-1</i> ⁽²⁾	<i>sgs-2/sde-1</i> ⁽³⁾		RdRP		1) Cogoni and Macino, 1999a 2) Sardon <i>et al.</i> , 2000 3) Mourrain <i>et al.</i> , 2000 Dalmay, <i>et al.</i> , 2000
<i>qde-2</i> ⁽⁴⁾	<i>rde-1</i> ⁽⁵⁾	<i>ago-1</i> ⁽⁶⁾		Unknown	Initiation of silencing activity	4) Catalanotto, <i>et al.</i> , 2000 5) Tabara <i>et al.</i> , 1999
	<i>rde-2</i> ⁽⁸⁾ <i>rde-3</i> ⁽⁹⁾ <i>rde-4</i> ⁽¹⁰⁾		<i>Dicer</i> ⁽⁷⁾	RNAse III Not cloned Not cloned Unknown	dsRNA processing Effector Initiation of silencing activity	6) Bohmert, <i>et al.</i> , 1998 7) Bernstein <i>et al.</i> , 2001 8) Tabara <i>et al.</i> , 1999 9) Tabara <i>et al.</i> , 1999 10) Tabara <i>et al.</i> , 1999
	<i>mut-2</i> ⁽¹¹⁾ <i>mut-6</i> ⁽¹²⁾ <i>mut-7</i> ⁽¹³⁾			Not cloned Not cloned RNAse D		11) Tabara <i>et al.</i> , 1999 12) Tabara <i>et al.</i> , 1999 13) Ketting <i>et al.</i> , 1999
<i>qde-3</i> ⁽¹⁴⁾		<i>sgs-1</i> ⁽¹⁵⁾ <i>sgs-3</i> ⁽¹⁶⁾		DNA helicase Unknown Unknown	Effector	14) Cogoni and Macino, 1999c 15) Elmayan <i>et al.</i> , 1998 16) Mourrain <i>et al.</i> , 2000

small RNA molecules could be produced proportionally to the abundance of the target mRNA, leading to the formation of an increased number of RNA-directed RNase complexes, thus increasing the strength of silencing.

VI. SUPPORTING EVIDENCE FOR THE PROPOSED MODEL

The proposed model is in accordance with several hypotheses that have been advanced to explain various aspects of PTGS. Both quantitative and qualitative features of the RNA trigger molecule may influence silencing, and these aspects, together with the possible roles of RNase and methylation, are discussed in the following paragraphs.

A. A threshold of mRNA

The threshold model originally proposed to explain co-suppression in plants (Jorgensen, 1991) was based on the notion that a threshold level of transcription of a transgene or virus was the eliciting factor to trigger gene silencing (Smith *et al.*, 1990). Lindbo *et al.* (1993) proposed that RNA concentrations above a threshold level induce RdRP to generate asRNAs involved in co-suppression. Several observations appear to support this model: (1) frequently, transgenes driven by strong promoters induce a higher frequency of silencing; (2) an increased silencing frequency was observed in homozygous with respect to hemizygous plants (Elmayan and Vaucheret, 1996). However, other experimental data are in contrast with these findings. Weakly or negligibly transcribed transgenes can efficiently induce PTGS (Stam *et al.*, 1998), and silencing of endogenous *chs* genes in petunia can be induced by promoter-less *chs* transgenes (van Blokland *et al.*, 1994). The above model proposed for quelling in *Neurospora* could also embrace a threshold hypothesis, i.e., only when abundant target RNA is available can a positive feedback loop be established and an efficient level of silencing be reached.

B. The possible nature of the diffusible signal for silencing

In previous models proposed by Cogoni and Macino (1997b), Stam *et al.*, (1997) and Voinnet *et al.*, (1998), the aberrant characteristics of the RNA transcript have been considered as constituting the signal to trigger the sequence of events which lead to silencing of both transgenic and homologous endogenous sequences. This would explain why not all transgenes are able to induce silencing, why only certain RNAs are the target for RdRPs, and why quelling affects only a percentage of transformants in *Neurospora*. The fact that gene silencing is diffusible from cell to cell in plants and nematodes and through the syncytium of *Neurospora* hyphae has suggested that the aberrant RNA molecule could act not only as the trigger, but also as the diffusible signal of gene silencing able to induce the cascade of

events leading to the establishment of the silenced state, even at a distance, far from the site of initiation.

However, the nature of the silencing signal is still completely obscure. It could be equally possible that RNA molecules derived from transgenic aberrant RNA could work as diffusible signals. For instance, the small double-stranded RNA species could act not only as "guide RNAs" in a protein complex capable of carrying out the degradation of homologous mRNAs in the cell in which they are generated, but could also travel from cell to cell to initiate the posttranscriptional process in neighbouring cells (Zamore *et al.*, 2000, Sharp and Zamore, 2000).

C. The role of RNase in the degradation step

Hypothetical models proposed by Bass (2000) and Yang *et al.* (2000) to explain RNAi in *Drosophila* are based on evidence found in *Drosophila* by Hammond *et al.* (2000) and Tuschl *et al.* (1999) of the presence of an RNase enzyme associated to the small guide RNAs. The models suggest that the double-stranded RNA is degraded by an RNase that remains associated with the double-stranded 25-mer RNAs that are formed as a result of degradation and which determine the specificity for the degradation of the homologous mRNAs. Zamore *et al.* (2000) have demonstrated that ATP is necessary for the various steps in RNAi. In the strand exchange model proposed by Bass (2000), a hypothetical "RNAi nuclease," with a dsRNA-binding domain, ribonuclease domains, and an RNA helicase domain, binds to the complex formed between the guideRNA and homologous mRNA, to permit strand exchange between the sense strand of the guide RNA and the mRNA and its subsequent degradation. ATP would be necessary in this process either to unwind secondary structure of the mRNA or for cleavage. These models also envisage the creation of the small dsRNAs as an amplification step; however, no involvement of an RdRP is foreseen.

The most recent evidence supporting a model envisaging a multicomponent nuclease bound to the guide RNAs as the degradation mediator of mRNA is the identification in *Drosophila* of an enzyme with both a helicase domain and RNaseIII motifs. The ATP-dependent enzyme called Dicer can bind dsRNA to create 22-mer RNAs, and it has been proposed that this could constitute the first part of a two-step process (Bernstein *et al.*, 2001). The second stage would be the base pairing of the 22-mer RNA with homologous mRNA with the RNase part of the complex responsible for cleavage of mRNAs. Interestingly, the *Dicer* gene sequence presents homology with the PAZ domain found among genes of the Piwi/Argonaute/Zwille-pinhead family of which *qde-2* and *rde-1*, both involved in gene silencing, are members (Cerutti *et al.*, 2000). It has been proposed in *Drosophila* that interaction between an Argonaute-like gene and *Dicer*, by means of their PAZ domains, could explain the formation of the RNA degradation complex (Baulcombe, 2001).

D. Maintenance of PTGS

Although the key events of posttranscriptional gene silencing in plants, animals, and fungi appear to take place in the cytoplasm, several reports suggest that nuclear events involving the homologous sequence could play an important role, especially regarding the activation and maintenance of the silenced state. Jones *et al.* (1999) have found evidence that PTGS induced by viruses is accompanied by an increased level of methylation of the nuclear homologous genes. It has been proposed that sequence-specific methylation can be induced by an RNA-directed DNA methylation (RdDM) mechanism. Indeed, clear evidence of the ability of viroid RNA to cause methylation of homologous sequences has been demonstrated in plants (Wassenegger *et al.*, 1994). Even though the role of DNA methylation associated with PTGS is still not clear, it has been proposed that interactions between the aberrant, viral or small RNAs and endogenous DNA may play a part in the maintenance of the silenced state (Wassenegger and Pélissier, 1998, 2000). In this view, methylation and/or chromatin modifications caused by an RNA-directed mechanism could be important in establishing and/or sustaining an altered epigenetic state essential for the maintenance of silencing, perhaps inducing or enhancing the production of aberrant RNA trigger molecules. Such a maintenance mechanism could be especially significant in SAS phenomena.

VII. THE SEARCH FOR ADDITIONAL PTGS COMPONENTS

Despite the fact that exhaustive screening of *Neurospora quelling-defective* mutants did not reveal any other loci involved in quelling, various experimental approaches may be used in the identification of components that participate in PTGS.

Components of gene silencing mechanisms that are active in protein complexes may be identified by two-hybrid assays, and this could be especially suitable in the case of molecules such as the *qde-2* gene product, the role of which is still unknown.

The comparative genomics approach which envisages the systematic analysis of coordinated gene loss may be used to predict new proteins implicated in silencing mechanisms. For example, homologs to all three *Neurospora qde* genes have been identified in the yeast *S. pombe*, whereas *S. cerevisiae* appears to have lost both the *qde-1* and *qde-2* homologs, retaining a single function encoded by *sgs-1*, homologous to *qde-3*. It has been hypothesized that the complete set of genes necessary for PTGS are present in *S. pombe*, but has been lost in *S. cerevisiae* (Aravind *et al.*, 2000).

Several plant viral proteins have been found to interfere with both the establishment and the maintenance of PTGS, probably interacting with specific cellular components of the silencing machinery. Thus, such viral proteins could

be used as a means to isolate new host PTGS components (Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Voinnet *et al.*, 1999). Indeed, by using such an approach a calmodulin-related gene interacting with the viral protein HC-Pro has recently been isolated (Anandalakshmi *et al.*, 2000).

VIII. CONCLUSIONS

It is now becoming clear that the PTGS phenomena identified in various organisms originate from an ancestral surveillance system aimed at maintaining genome integrity by protection from attack by invasive DNA (such as transposons and “selfish” repetitive DNA) and RNA (viruses and viroids). The notion that all PTGS phenomena have evolved from an ancestral defense mechanism is also supported by the fact that plants defective in PTGS are more susceptible to viral infections, suggesting that the same silencing mechanism operates on both transgenic and viral RNA. The extreme importance of these silencing mechanisms is shown by the fact that they are responsible not only for genomic surveillance, but also for the regulation of development and cell-differentiation, shown, for example, by the role of the *rde* genes in *C. elegans*. Moreover, an RNA silencing mechanism active in germline cells in animals and in meristems in plants would be a means of blocking transmission to progeny of viral infections.

Although the existence of homology-dependent gene silencing mechanisms has been casually discovered as a result of the experimental introduction of transgenes or dsRNA in various organisms, this discovery may also have important potential repercussions in functional genomics and in both medical and applied biotechnological fields. Despite the fact that genome sequencing projects are producing complete genomic data for several organisms, the biological function of a majority of genes remains unknown. The use of gene silencing as an instrument in reverse genetic analysis may be an effective tool in predicting gene function. For example, by using RNAi to knock out the expression of 90% of the genes located on chromosome I of *C. elegans*, it has been possible to rapidly identify the function of more than 300 individual genes, with respect to the 70 previously known functions (Fraser *et al.*, 2000). Similarly, genes involved in cell division have been identified on chromosome III of *C. elegans* by Gönczy *et al.* (2000). Recently, the regulatory interconnection between important plant functions has been identified as a result of viral-induced gene silencing in tobacco (Burton *et al.*, 2000). Medical applications of silencing phenomena can be far reaching: from gaining insight into cancer biology by the utilization of RNAi to study genes involved in cell growth and division in *Drosophila*, to the development of vectors that can resist host defense, important for somatic gene therapy (Bestor, 2000). Finally, the understanding of the fine tuning of each step of PTGS is of vital importance in agriculture to improve transformation technology. The ability to control and maybe

shut down the PTGS machinery in plants could be useful to allow more reliable expression of introduced transgenes carrying beneficial traits (Bruening, 1998).

The discovery of the genes involved in quelling in *Neurospora crassa* has been a landmark in the understanding of posttranscriptional gene silencing in this organism. The homology of these genes with those identified in other silencing phenomena, such as co-suppression and RNA interference, underlines the fact that these processes may have a common origin, and due to their importance in maintaining genome integrity have been highly conserved during evolution.

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References

- Anandalakshmi, R., Marathe, R., Ge, X., Herr, J. M. Jr., Mau, C., Mallory, A., Bowman, L., and Vance, V. B. (2000). A calmodulin-related protein that suppresses post-transcriptional gene silencing in plants. *Science* **290**, 142–144.
- Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H., and Vance, V. B. (1998). A viral suppressor of gene silencing in plants. *Proc. Natl. Acad. Sci. USA* **95**, 13079–13084.
- Aravind, L., Watanabe, H., Lipman, D. J., and Koonin, E. V. (2000). Lineage-specific loss and divergence of functionally linked genes in eukaryotes. *Proc. Natl. Acad. Sci. USA* **97**, 11319–11324.
- Ballario, P., Vittorioso, P., Magrelli, A., Talora, C., Cabibbo, A., and Macino, G. (1996). White collar 1, a central regulator of blue-light responses in *Neurospora crassa*. *EMBO J.* **15**, 1650–1657.
- Bass, B. L. (2000). Double-stranded RNA as a template for gene silencing. *Cell* **101**, 235–238.
- Baulcombe, D. C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. *Plant Mol. Biol.* **32**, 79–88.
- Baulcombe, D. (1999). Viruses and gene silencing in plants. *Arch. Virol. Suppl.* **15**, 189–201.
- Baulcombe, D. (2001). Dicer defence. *Nature* **409**, 295–296.
- Bennett, R. J., Noiro-Gros, M. F., and Wang, J. C. (2000). Interaction between yeast Sgs1 helicase and DNA topoisomerase III. *J. Biol. Chem.* **275**, 26898–26905.
- Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.
- Bestor, T. H. (2000). Gene silencing as a threat to the success of gene therapy. *J. Clin. Invest.* **105**, 409–411.
- Brosh, R. M., Li, J. L., Kenny, M. K., Karow, J. K., Cooper, M. P., Kureekattil, R., Hickson, I. D., and Bohr, V. A. (2000). Replication protein A physically interacts with the Bloom's syndrome protein and stimulates its helicase activity. *J. Biol. Chem.* **275**, 23500–23508.
- Bosher, J. M., and Labouesse, M. (2000). RNA interference: genetic wand and genetic watchdog. *Nature Cell Biol.* **2**, 31–36.
- Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W., and Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**, 6739–6746.
- Bruening, G. (1998). Plant gene silencing regularized. *Proc. Natl. Acad. Sci. USA.* **95**, 13349–13351.
- Bohmer, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998). AGO1 defines a novel locus of Arabidopsis controlling leaf development. *EMBO J.* **17**, 170–180.

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- Burton, R. A., Gibeaut, D. M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D. C., and Fincher, G. B. (2000). Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* **12**, 691–706.
- Cambareri, E. B., Jensen, B. C., Schabtach, E., and Selker, E. U. (1989). Repeat-induced G-C to A-T mutations in *Neurospora*. *Science* **244**, 1571–1575.
- Carrington, J. C. (2000). Moving targets. *Nature* **408**, 150–151.
- Catalanotto, C., Azzalin, G., Macino, G., and Cogoni, C. (2000). Gene silencing in worms and fungi. *Nature* **404**, 24.
- Cerutti, I., Mian, N., and Bateman, A. (2000). *TIBS* **25**, 481–482.
- Cikalus, D. E., Tahbaz, N., Hendricks, L. C., DiMattia, G. E., Hansen, D., Pilgrim, D., and Hobman, T. C. (1999). GERp95, a membrane-associated protein that belongs to a family of proteins involved in stem cell differentiation. *Mol. Biol. Cell* **10**, 3357–3372.
- Chakravarty, I., Bagchi, M. K., Roy, R., Banerjee, A. C., and Gupta, N. K. (1985). Protein synthesis in rabbit reticulocytes. *J. Biol. Chem.* **260**, 6945–6949.
- Chuang, C. F., and Meyerowitz, E. M. (2000). Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **97**, 4985–4990.
- Cogoni, C., Irelan, J. T., Schumacher, M., Schmidhauser, T., Selker, E. U., and Macino, G. (1996). Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. *EMBO J.* **15**, 3153–3163.
- Cogoni, C., and Macino, G. (1997a). Isolation of quelling-defective (*qde*) mutants impaired in post-transcriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* **94**, 10233–10238.
- Cogoni, C., and Macino, G. (1997b). Conservation of transgene-induced post-transcriptional gene silencing in plants and fungi. *Trends in Plant Sci.* **2**, 438–443.
- Cogoni, C., and Macino, G. (1998). Quelling: transgene-induced silencing in *Neurospora crassa*. In “Cellular Integration of Signalling Pathways in Plant Development” (NATO ASI Series), Vol. H 104, pp. 103–112. Springer-Verlag, Berlin, Heidelberg.
- Cogoni, C., and Macino, G. (1999a). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399**, 166–169.
- Cogoni, C., and Macino, G. (1999b). Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. *Curr. Opin. Microbiol.* **2**, 657–662.
- Cogoni, C., and Macino, G. (1999c). Post-transcriptional gene silencing in *Neurospora* by a RecQ DNA helicase. *Science* **286**, 2342–2343.
- Cogoni, C., and Macino, G. (2000). Gene silencing across kingdoms. *Curr. Opin. Genet. Devel.* **10**, 638–643.
- Cox, D. N., Chao, A., Baker, J., Chang, L., Qiao, D., and Lin, H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes & Dev.* **12**, 3715–3727.
- Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D. C. (2000). An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for post-transcriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**, 543–53.
- de Carvalho, F., Gheysen, G., Kushnir, S., Van Montagu, M., Inzè, D., and Castresana, C. (1992). Suppression of β -1,3-glucanase transgene expression in homozygous plants. *EMBO J.* **11**, 2595–2602.
- de Carvalho, F., Niebel, E., Frendo, P., Van Montagu, M., and Cornelissen, M. (1995). Post-transcriptional cosuppression of β -1, 3-glucanase genes does not affect accumulation of transgene nuclear mRNA. *Plant Cell* **7**, 347–358.
- Depicker, A., and Van Montagu, M. (1997). Post-transcriptional gene silencing in plants. *Curr. Opinion Cell. Biol.* **9**, 373–82.

- Dernburg, A. F., Zalevsky, J., Colaiácovo, P., and Villeneuve, A. M. (2000). Transgene-mediated cosuppression in the *C. elegans* germ line. *Genes & Dev.* **14**, 1578–1583.
- Domeier, M. E., Morse, D. P., Knight, S. W., Portereiko, M., Bass, B. L., and Mango, S. E. (2000). A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. *Science* **289**, 1928–1931.
- Elmayan, T., and Vaucheret, H. (1996). Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionally. *Plant J.* **9**, 787–797.
- Elmayan, T., Balzergue, S., Beon, F., Bourdon, V., Daubremet, J., Guenet, Y., Mourrain, P., Palauqui, J. C., Vernhettes, S., Vialle, T., Wostrikoff, K., and Vaucheret, H. (1998). *Arabidopsis* mutants impaired in cosuppression. *Plant Cell* **10**, 1747–1758.
- Fagard, M., Boutet, S., Morel, J. B., Bellini, C., and Vaucheret, H. (2000). AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc. Natl. Acad. Sci. USA* **97**, 11650–11654.
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.
- Flavell, R. B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl. Acad. Sci. USA* **91**, 3490–3496.
- Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325–330.
- Gangloff, S., McDonald, J. P., Bendixen, C., Aurther, L., and Rothstein, R. (1994). The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. *Mol. Cell. Biol.* **14**, 8391–8398.
- Gonczy, P., Echeverri, G., Oegema, K., Coulson, A., Jones, S. J., Copley, R. R., Duperon, J., Oegema, J., Brehm, M., Cassin, E., Hannak, E., Kirkham, M., Pichler, S., Flohrs, K., Goessen, A., Leidel, S., Alleaume, A. M., Martin, C., Ozlu, N., Bork, P., and Hyman, A. A. (2000). Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **408**, 331–336.
- Grierson, D., Fray, R. G., Hamilton, A. J., Smith, C. J. S., and Watson, C. F. (1991). Does co-suppression of sense genes in transgenic plants involve anti-sense RNA? *Trends Biotechnol.* **9**, 122–123.
- Grishok, A., Tabara, H., and Mello, C. C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494–2497.
- Hamilton, A. J., and Baulcombe, D. C. (1999). A novel species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*. **286**, 950–952.
- Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts. *Nature* **404**, 293–296.
- Hartung, F., Plchová, H., and Puchta, H. (2000). Molecular characterisation of RecQ homologues in *Arabidopsis thaliana*. *Nucleic Acids Research* **28**, 4275–4282.
- Holliday, R., and Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science* **187**, 226–232.
- Holtorf, H., Schob, H., Kunz, C., Waldvogel, R., and Meins, F. Jr. (1999). Stochastic and nonstochastic post-transcriptional silencing of chitinase and β 1,3-glucanase genes involves increased RNA turnover-possible role for ribosome-independent RNA degradation. *Plant Cell* **11**, 471–484.
- Jensen, S., Gassama, M. P., and Heidmann, T. (1999). Taming of transposable elements by homology-dependent gene silencing. *Nat. Genet.* **21**, 209–212.
- Jones, A. L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J., and Baulcombe, D. C. (1999). RNA-DNA interaction and DNA methylation in post-transcriptional gene silencing. *Plant Cell* **11**, 2291–2301.
- Jorgensen, R. (1991). How do genes interact with homologous plant genes? *Trends Biotechnol.* **9**, 266–267.

- Jorgensen, R. A., Cluster, P. D., English, J., Que, Q., and Napoli, C. A. (1996). Chalcone synthase co-suppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single copy vs. complex T-DNA sequences. *Plant Mol. Biol.* **31**, 957–973.
- Kasschau, K. D., and Carrington, J. C. (1998). A counter defensive strategy of plant viruses: suppression of post-transcriptional silencing. *Cell* **95**, 461–470.
- Kennerdell, J. R., and Carthew, R. W. (2000). Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat. Biotechnol.* **18**, 896–898.
- Ketting, R. F., Haverkamp, T. H. A., van Luenen, H. G. A. M., and Plasterk, R. H. A. (1999). *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**, 133–141.
- Ketting, R. F., and Plasterk, R. H. (2000). A genetic link between co-suppression and RNA interference in *C. elegans*. *Nature* **404**, 296–298.
- Kitao, S., Ohsugi, I., Ichikawa, K., Goto, M., Furuichi, Y., and Shimamoto, A. (1998). Cloning of the new human helicase genes of the RecQ family: Biological significance of multiple species in higher eukaryotes. *Genomics* **54**, 443–452.
- Kooter, J. M., Matzke, M. A., and Meyer, P. (1999). Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.* **4**, 340–347.
- Li, Y. X., Farrell, M. J., Liu, R., Mohanty, N., and Kirby, M. L. (2000). Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev. Biol.* **217**, 394–405.
- Lindbo, J. L., Silva-Rosales, L., Proebsting, W. M., and Dougherty, W. G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus expression. *Plant Cell* **5**, 1749–1759.
- Linden, H., and Macino, G. (1997). White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. *EMBO J.* **16**, 98–109.
- Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P., and Barton, K. (1999). The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. *Development* **126**, 469–481.
- Matzke, M. A., Matzke, A. J. M., and Mittelsten Scheid, O. (1994). Inactivation of repeated genes – DNA-DNA interaction? In “Homologous Recombination and Gene Silencing in Plants” (J. Paszkowski, Ed.), pp. 271–307. Kluwer, Dordrecht.
- Misquitta, L., and Paterson, B. M. (1999). Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. *Proc. Natl. Acad. Sci. USA* **96**, 1451–1456.
- Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikić, S., Picault, N., Remoue, K., Sanial, M., Vo, T. A., and Vaucheret, H. (2000). *Arabidopsis* SGS2 and SGS3 genes are required for post-transcriptional gene silencing and natural virus resistance. *Cell* **101**, 533–542.
- Moussian, B., Schoof, H., Haecker, A., Jürgens, G., and Laux, T. (1998). Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. *EMBO J.* **17**, 1799–1809.
- Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**, 279–289.
- Nelson, M. A., Morelli, G., Carratoli, A., Romano, N., and Macino, G. (1989). Molecular cloning of a *Neurospora crassa* carotenoid biosynthetic gene (*albino-3*) regulated by blue light and the products of white collar genes. *Mol. Cell Biol.* **9**, 1271–1276.
- Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* **95**, 14687–14692.
- Pandit, N. N., and Russo, V. E. (1992). Reversible inactivation of a foreign gene, *hph*, during the asexual cycle in *Neurospora crassa* transformants. *Mol. Gen. Genet.* **234**, 412–422.

- Palauqui, J. C., Elmayan, T., Pollien, J. M., and Vaucheret, H. (1997). Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* **16**, 4738–4745.
- Plasterk, R. H. A., and Ketting, R. F. (2000). The silence of the genes. *Curr. Opin. Gen. Dev.* **10**, 562–567.
- Romano, N., and Macino, G. (1992). Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol. Microbiol.* **6**, 3343–3353.
- Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **6**, 937–946.
- Sanchez Alvarado, A., and Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* **96**, 5049–5054.
- Schmidhauser, T., Lauter, F. R., Russo, V. E. A., and Yanofsky, C. (1990). Cloning, sequence and photoregulation of *al-1*, a carotenoid biosynthetic gene of *Neurospora crassa*. *Mol. Cell Biol.* **10**, 5064–5070.
- Schmidhauser, T. J., Lauter, F., Schumacher, M., Zhou, W., Russo, V. E. A., and Yanofsky, C. (1994). Characterisation of *al-2*, the Polytoene synthase Gene of *Neurospora crassa*. *J. Biol. Chem.* **269**, 12060–12066.
- Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sanger, H. L. (1993a). RNA-directed RNA polymerase from tomato leaves. I: Purification and Physical Properties. *J. Biol. Chem.* **268**, 11851–11857.
- Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sanger, H. L. (1993b). RNA-directed RNA polymerase from tomato leaves. II. Catalytic *in vitro* Properties. *J. Biol. Chem.* **268**, 11858–11867.
- Schiebel, W., Pelissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sanger, H. L., and Wassenegger, M. (1998). Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell* **10**, 1–16.
- Schmidt, A., Palumbo, G., Bozzetti, M. P., Tritto, P., Pimpinelli, S., and Schafer, U. (1998). Genetic and molecular characterization of *sting*, a gene involved in crystal formation and meiotic drive in the male germ line of *Drosophila melanogaster*. *Genetics* **151**, 749–760.
- Shen, J. C., Gray, M. D., Oshima, J., and Loeb, L. A. (1998). Characterization of Werner syndrome protein DNA helicase activity: directionality, substrate dependence and stimulation by replication protein A. *Nucleic Acids Research* **26**, 2879–2885.
- Selker, E. U., Cambareri, E. B., Jensen, B. C., and Haack, K. R. (1987). Rearrangement of duplicated DNA in specialized cells of *Neurospora*. *Cell*. **51**, 741–752.
- Selker, E. U. (1990). Premeiotic instability of repeated sequences in *Neurospora crassa*. *Annu. Rev. Genet.* **24**, 579–613.
- Sharp, P. A., and Zamore, P. D. (2000). Molecular biology. RNA interference. *Science* **287**, 2431–2433.
- Sijen, T., and Kooter, J. M. (2000). Post-transcriptional gene-silencing: RNAs on the attack or on the defense? *Bioessays* **22**, 520–531.
- Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N., and Maine, E. M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* **10**, 169–178.
- Smith, C. J. S., Watson, C. F., Bird, C. R., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Mol. Gen. Genet.* **224**, 477–481.
- Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G., and Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* **407**, 319–320.
- Stam, M., Mol, J. N. M., and Kooter, J. M. (1997). The silence of genes in transgenic plants. *Annu. Botany* **79**, 3–12.
- Stam, M., de Bruin, R., van Blokland, R., van der Hoorn, R. A., Mol, J. N., and Kooter, J. M. (2000). Distinct features of post-transcriptional gene silencing by antisense transgenes in single copy and inverted T-DNA repeat *loci*. *Plant J.* **21**, 27–42.

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- Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C. C. (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132.
- Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A., and Driscoll, M. (2000). Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genetics* **24**, 180–183.
- Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev.* **13**, 3191–3197.
- van Blokland, R., Van der Geest, N., Mol, J. N. M., and Koote, J. M. (1994). Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J.* **6**, 861–877.
- van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N., and Stuitje, A. R. (1990). Flavonoid genes in *Petunia*: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2**, 291–299.
- Vaucheret, H., Beclin, C., Elmayer, T., Feurbach, F., Godon, C., Morel, J.-B., Mourrain, P., Palaqui, J.-C., and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. *Plant J.* **16**, 651–659.
- Voinnet, O., and Baulcombe, D. C. (1997). Systemic signaling in gene silencing. *Nature* **389**, 553.
- Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**, 177–187.
- Voinnet, O., Lederer, C., and Baulcombe, D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**, 157–167.
- Voinnet, O., Pinto, Y. M., and Baulcombe, D. C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA* **96**, 14147–14152.
- Wargelius, A., Ellingsen, S., and Fjose, A. (1999). Double-stranded RNA induces specific developmental defects in zebrafish embryos. *Biochem. Biophys. Res. Commun.* **263**, 156–161.
- Wassenegger, M., Heimes, S., Riedel, L., and Ranger, H. L. (1994). RNA-directed *de novo* methylation of genomic sequences in plants. *Cell* **76**, 567–576.
- Wassenegger, M., and Pelissier, T. (1998). A model for RNA-mediated gene silencing in higher plants. *Plant Mol. Biol.* **37**, 349–362.
- Wassenegger, M. (2000). RNA-directed DNA methylation. *Plant. Mol. Biol.* **43**, 203–220.
- Waterhouse, P. M., Graham, M. W., and Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci. USA* **95**, 177–187.
- Watt, P. M., Louis, E. J., Borts, R. H., and Hickson, D. (1995). Sgs1: a eukaryotic homolog of *E. coli* RecQ that interacts with topoisomerase II *in vivo* and is required for faithful chromosome segregation. *Cell* **81**, 253–260.
- Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nature Cell Biol.* **2**, 70–75.
- Yang, D., Lu, H., and Erickson, J. W. (2000). Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in drosophila embryos. *Curr Biol.* **10**, 1191–1200.
- Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33.
- Zou, C., Zhang, Z., Wu, S., and Osterman, J. C. (1998). Molecular cloning and characterization of rabbit eIF2C protein. *Gene* **211**, 187–194.