Conservation of transgene-induced post-transcriptional gene silencing in plants and fungi

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Different types of post-transcriptional transgene-induced gene silencing have been shown to occur in Neurospora (quelling) and plants (co-suppression). Common mechanistic features include triggering by duplicated coding sequences, simultaneous silencing of the transgene and endogenous genes, reversibility of silencing, and post-transcriptional reduction of gene-specific mRNA. These shared features suggest that this type of gene silencing may have evolved from a common ancestral mechanism designed to protect genome integrity. Understanding the mechanisms involved may be particularly useful in biotechnological applications aimed at enhancing or avoiding gene silencing in transgenic plants and fungi.

Transgene-induced gene silencing was originally discovered in plants and fungi on the basis of easily scoreable colour phenotypes in these organisms. Two types of such silencing have now been uncovered in plants: one acting at the transcriptional level, when the transgenes contain sequence homologous to the promoter of the silenced gene; and the other acting at the post-transcriptional level, requiring homology in transcribed regions. The introduction of transgenes has been shown to induce two gene inactivation phenomena at different stages of the life cycle of the filamentous fungus Neurospora crassa: RIP (repeat-induced point mutation), in which the duplicated sequences are mutagenized by point mutations, occurs in the sexual phase; and quelling, consisting of a post-transcriptional inactivation of gene expression, occurs in the vegetative phase.

Post-transcriptional gene silencing in plants and quelling in Neurospora share many common features (Box 1), suggesting that they may be derived from an ancient mechanism that may also occur in animals, but has not yet been uncovered. Here we compare and contrast the present understanding of the molecular mechanisms controlling the two processes. Neurospora is a relatively simple and genetically well-characterized organism, and has provided an important tool for these studies. Dissecting the mechanism of this phenomenon has significance both at the basic research level for understanding genome maintenance and also at a biotechnological level for enabling the overexpression of foreign genes.

Transcriptional gene silencing

One of the first reported examples of gene inactivation by DNA modification in Neurospora was RIP, in which the duplicated sequences detected in the premeiotic phase are subject to point mutations. Although RIP is irreversible, all other DNA modification-based gene inactivation mechanisms (e.g., methylation) in fungi and plants are reversible. DNA methylation induced premeiotically has been shown to play a role in transcriptional gene silencing in the fungus Ascochobus immersus. The methylation of promoters has also been shown to be involved in transcriptional gene silencing in plants. Differences in DNA methylation are also associated with paramutation phenomena in transgenic petunia and with inactivation of the maize R gene family. In contrast, methylation is not required for quelling in Neurospora, and its role in post-transcriptional gene silencing in plants is equivocal (although there are several studies in which DNA methylation of the transgenes has been found to be associated with post-transcriptional gene silencing).
Common features of post-transcriptional gene silencing in plants and fungi

Reporters for studying post-transcriptional gene silencing in Neurospora

In *Neurospora crassa* the first reported observations of gene silencing induced by gene duplication were termed quelling [3]. Quelling is reversible, occurs during the vegetative phase of growth, and is a general phenomenon observed for several genes, including *al-1* and *al-3* (Ref. 1), *hph* (Ref. 16), *wc-1* (Ref. 17), *wc-2* (Ref. 18) and *ad-9* (T.J. Schmidhauser, pers. commun.). The discovery of quelling was facilitated by the easily scorable *al* ('albino') phenotype, caused by transformation of wild-type (orange) strains with an extra copy of a carotenoid transgene (*al-1, al-2* and *al-3*). This situation parallels the discovery of co-suppression in plants in which suppression of flower colour by chalcone synthase (*chs*) genes was first observed [1]. In *Neurospora*, it is possible to evaluate the level of quelling by simple visual inspection and/or quantification of carotenoid content (Fig. 1).

Silencing of the transgene and the endogenous gene in quelling and co-suppression

In *Neurospora*, functional *al-1* transgenes cause an *al* phenotype, indicating that both the transgene and endogenous gene are silenced. This situation parallels co-suppression in plants, where functional flower colour genes suppressed colour in petunia flowers [2-3]. After these initial observations, cases of co-suppression have also been shown to occur in a variety of plant species and for several genes, including *β-1,3-glucanase* [3], *β-glucuronidase* [4], neomycin phosphotransferase [5], *chitinase* [6], *rol* [7] and nitrite reductase [8], as well as for viral transgenes [9,10]. Thus both quelling and co-suppression are general phenomena.

Reduced numbers of mRNA transcripts through post-transcriptional mechanisms

Analysis of quelled *Neurospora* *al* transformants revealed drastic gene-specific reductions in amounts of steady-state mRNA for duplicated albino genes. This effect is post-transcriptional, with quelled strains producing the same amount of *al-1* primary transcript as the wild-type strain [11]. Possible mechanisms for post-transcriptional reduction of mRNA include:

- A splicing defect, resulting in degradation of partially spliced intermediates and small amounts of correctly matured mRNA.
- Reduced cytoplasmic transport and/or accelerated nuclear degradation of mature mRNA.
- Cytoplasmic instability of mature mRNA.

In view of the apparently unchanged steady-state levels of precursor RNA in quelled and nonquelled strains, cytoplasmic instability of mature mRNA appears to be the most likely explanation.

In plants, a specific post-transcriptional decrease of amounts of steady-state mRNA of silenced genes has also been observed in cases of gene suppression. This conclusion is based on run-on transcription assays and analysis of nuclear RNA levels [2,26,27]. Moreover the post-transcriptional nature of mRNA reduction in co-suppressed strains is supported by evidence that transgenic plants containing viral transgenes have reduced levels of viral RNA during subsequent viral infection. As the viral cycle for replication and transcription is cytoplasmic, this also indicates a post-transcriptional phenomenon.

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**Fig. 2.** Schematic representation of the *al-1* ('albino') locus. Below the complete locus are several deletion constructs containing different portions of the *al-1* gene used in transformation experiments. The ability to induce gene silencing is indicated on the right. Homology in the promoter region is insufficient to induce gene silencing (construct 2). However, the duplication of both the 3' and 5' ends (constructs 3 and 4) or the duplication of 132 bp of the coding region (construct 5) can induce quelling.

**DNA sequence requirements for silencing**

In *Neurospora*, deletion studies have shown that small portions of *al* transgene coding sequences can cause quelling, indicating that neither the promoter regions nor the transgene protein product are required. DNA sequence homologies in coding regions are important, because any portion of the coding region can function in quelling and

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**Fig. 3.** Schematic representation of heterokaryotic hyphae. Nuclei in which *al-1* ('albino') is mutated or silenced are represented as open ovals. Shaded ovals represent nuclei containing a wild-type *al-1* allele. The *al-1* mutations are recessive, and hence the heterokaryon containing *al-1* and wild-type nuclei shows an orange phenotype represented as a shaded region (a). The presence of a quelled nucleus in the heterokaryon also triggers the silencing of *al-1* genes resident in wild-type nuclei, producing a heterokaryon with an *al* phenotype represented as an open region (b). The arrows indicate that the quelling acts in trans between nuclei.
there is a minimal size (132 bp) requirement for this homology\(^1\) (Fig. 2). In plants, coding regions or portions thereof can also cause co-suppression\(^{26}\), indicating that, like quelling, neither promoters nor functional gene products are required for co-suppression.

**Copy number and stability of gene silencing**

Quelling is unstable as silenced *Neurospora* transformants revert progressively to wild-type or intermediate phenotypes over a prolonged culturing time. The reversion of quelling for the al-1 gene appears to be unidirectional. Revertant isolates show a strong reduction in copy number of exogenous sequences. Mitotic instability of ectopic sequences is a naturally occurring phenomenon in *Neurospora* and is independent of quelling\(^{25,29}\). Thus, the instability of quelling may be explained by tandem repeats, whose presence was found to correlate with quelling, being more prone to excision.

In plants, reversion of co-suppression is also associated with a reduction in transgene copy number resulting from intrachromosomal recombination during mitosis or meiosis\(^{20,33}\). Thus, in quelling and in co-suppression, persistence of transgene copies seems to be required for the maintenance of a silenced state. However, the maintenance and inheritance of silencing in plants is more complex and cannot always be predicted on the basis of the presence of transgenes. Environmental conditions and developmental state both seem to play a role in establishing and maintaining silencing in plants\(^{21,22}\).

**Dominance of silencing and action through the cytoplasm**

In *Neurospora*, heterokaryon analysis has shown that quelling involves a cytoplasmic signal. Heterokaryotic strains containing both al-1-silenced and nonsilenced nuclei exhibit an al (quelled) phenotype. This indicates that quelling is dominant and acts in *trans* to silence genes in both transformed and untransformed nuclei (Fig. 3). This finding has two important implications. First, the presence of the transgene and endogenous gene in the same nucleus is not a prerequisite for silencing. This strongly argues against models in which ectopic pairing and DNA–DNA interactions are required for gene silencing. Second, as quelling is not restricted to the nucleus, this indicates the involvement of a diffusible trans-acting molecule. This molecule must be able to diffuse into nuclei and/or to act in the cytoplasm to mediate mRNA degradation.

In plants, there are also indications that post-transcriptional gene silencing acts in the cytoplasm. Support for this notion derives from experiments in which co-suppressed plants are resistant to subsequent viral infection\(^{24,25}\). Transgenic tobacco plants containing either full length or truncated tobacco etch virus (TEV) coat protein are resistant to infection with TEV and contain low levels of transgenic coat protein mRNA and viral RNA. As TEV replicates exclusively in the cytoplasm, this indicates that gene silencing is a cytoplasmic event in which specific RNA sequences (TEV and coat protein mRNA) are degraded. Also consistent with a cytoplasmic site of action is the observation that co-suppression of the β-1,3-glucanase genes in tobacco does not affect accumulation of transgene nuclear mRNA (Ref. 27). However, the accumulation of polyadenylated endogenous *chs* mRNA fragments deriving from endonucleolytic degradation in silenced petunia plants\(^{28}\) also suggests that nuclear events of post-transcriptional gene silencing can occur.

**Sequence duplication and the induction of gene silencing**

The introduction of al-1 transgenes in *Neurospora* is necessary, but not sufficient, to trigger gene silencing; only a portion of transformants (typically 30%) containing duplicated sequences show silencing. Both high copy number and a tandem sequence arrangement are important for triggering quelling. In plants, there is also evidence that copy number and a tandem arrangement of transgenes are required to trigger co-suppression\(^{24,27,28}\). In *Neurospora*, the level of transgene expression seems to be irrelevant to the establishment of quelling, as demonstrated by the effectiveness of promoterless constructs\(^{11}\). In plants too, silencing of the *chs* gene was shown to be independent of the level of transgene expression. In this case, a promoterless construct was able to induce gene silencing just as effectively as a construct carrying strong promoters\(^{36}\). However, even if high levels of transgene expression are not important for inducing quelling in *Neurospora*, the expression of the transgene is still required. A correlation between the production of an unexpected transgenic sense transcript from a promoterless construct and the occurrence of silencing in *Neurospora* has been described\(^{11}\). This unexpected transgenic sense RNA may be aberrant in some feature and able to induce gene silencing. In the case of silencing of the *chs* gene in petunia, it has been suggested that the promoterless transgene may interact by DNA–DNA pairing with the endogenous gene inducing the production of aberrant RNAs (Ref. 35).

**Possible mechanisms of post-transcriptional gene silencing**

The evidence presented reveals that *Neurospora* and plants share several features in common concerning post-transcriptional transgene-induced gene silencing. Three possible models for the mechanisms involved in the silencing are:

- Involvement of an unexpected production of an antisense RNA (model 1).
- The 'threshold' hypothesis (model 2).
- The production of aberrant RNA induced by the presence of transgenes (model 3).

All three models are based on the notion that the transgene directly or indirectly produces RNA molecules able to trigger and/or mediate the degradation of target homologous mRNAs.

The models attempt to incorporate the common features of post-transcriptional gene silencing in *Neurospora* and plants, which include:

- Generality: The expression of many different genes was found to be suppressed in transgenic plants and fungi transformed with homologous transgenes. This feature invokes a mechanism involving the detection of DNA or RNA sequence homology.
- Mediation in *trans* in a post-transcriptional process. The data suggest the involvement of a diffusible molecule that mediates the degradation of target mRNA in a sequence-specific way.
- Occurrence in only a portion of transgenic *Neurospora* or plant lines, and somatic instability.

**Model 1**

Unintended antisense RNA could be produced by read-through transcription from a promoter adjacent to the site of chromosomal integration of the transgene. The observation that gene silencing occurs at less than 100% efficiency
The silencing of duplicated gene sequences is a widely occurring phenomenon in plants and fungi, and is potentially derived from a common ancient mechanism. Two alternative views can account for the existence gene silencing: it may be an 'accident' resulting from the perturbation of complex regulatory mechanisms of RNA metabolism; or it may be a response of the cell to invasive DNA.

The introduced transgene could possibly mimic invading DNA such as transposable elements, viruses and other repeated elements that can accumulate in the genome. Multiple mechanisms could have evolved to protect genomes from invasive DNA, such as DNA modification (transcriptional gene silencing) or post-transcriptional gene silencing. According to this interpretation, the two types of mechanisms for gene inactivation in *Neurospora* may have evolved to suit the different vegetative and sexual phases. Quelling, the post-transcriptional mechanism, acts to silence transposons and virus genes, inhibiting their expansion and replication in the vegetative phase. By contrast, RIP (repeat-induced point mutation) acts in the sexual phase through irreversible mutagenesis of duplicated sequences to prevent their transmission to progeny. These two mechanisms are therefore not redundant, but can be thought of as synergistic to protect the individual and its progeny.

could reflect the frequency with which transforming DNA integrates near an active antisense-oriented promoter. The possibility that antisense RNA may promote mRNA degradation is also consistent with a post-transcriptional process. Antisense transgene transcripts have been detected in *chsc*-co-suppressed petunia lines. However, these are not clearly correlated with gene silencing and a direct role in gene suppression remains to be investigated. It is unlikely that an unexpected antisense RNA plays a role in quelling in *Neurospora*. This is firstly because antisense RNA products are not detected in quelled *al* transformants. Secondly, transgenic constructs that produce antisense RNA gave the same quelling efficiency as promoterless constructs.

**Model 2**

The threshold hypothesis is based on the notion that transgenic organisms are able to detect abnormally high amounts of an mRNA species, and if these exceed a determined threshold level, gene-specific mRNA degradation mechanisms are induced. This model implies the existence of a regulatory circuit that is extremely sensitive to the abundance of individual mRNA species. The hypothesis is consistent with studies on transgenic plants in which co-suppression is only observed in tissues in which the transgene mRNA is expressed at high levels. In accordance with the model are numerous examples where co-suppression is associated with high copy numbers of transgenes. The threshold hypothesis is also compatible with post-transcriptional gene silencing of the highly transcribed *uidA* transgene in tobacco and single copies of *chs* transgenes in petunia. In contrast, in *Neurospora*, quelling is not associated with high expression levels of the transgenes. Promoterless transgenes were found to induce gene silencing as well as a transgene under the control of a strong promoter. In plants, there are also examples of promoterless transgenes causing co-suppression. Even if the absolute level of normal transgenic mRNA is unimportant, the expression of the transgene was found to be necessary for gene silencing in *Neurospora*, as indicated by the correlation between the accumulation of unexpected transgenic sense RNA from promoterless constructs and the occurrence of quelling. This unexpected sense RNA may be qualitatively distinct from the normal mRNA and so be able to trigger gene silencing.

**Model 3**

Post-transcriptional gene silencing has been proposed to be caused by the production of 'qualitatively different' sense RNA, which is also termed aberrant RNA. This hypothesis predicts that an aberrant RNA produced directly from the transgene or induced by the transgene can trigger gene-specific mRNA degradation. What might be the nature of such aberrant RNA? An aberrant RNA could be aberrant either in *cis* characteristics (e.g. base modifications or absence of a standard 5'-cap or polyadenylated tail) and/or in *trans* characteristics (e.g. forming aberrant ribonucleoprotein complexes as consequence of an abnormal location of the transgenes in the nucleus). Chromosomal location or tandem repeats could each lead to the production of aberrant RNAs. It has been proposed that tandem repeats, especially inverted repeats, could by DNA–DNA interactions lead to
the formation of cruciform structures that act as a template for aberrant RNA transcription. DNA–DNA pairing between the transgene and endogenous genes may also explain cases in which nontranscribed transgenes are able to induce post-transcriptional gene silencing. The instability of the DNA–DNA pairing could also account for the erratic nature of post-transcriptional gene silencing often observed in plants. Finally, it was proposed that methylation of transgene DNA often found in association with post-transcriptional gene silencing in plants may play a role in the production of aberrant RNA (Ref. 15).

The second question regarding the aberrant RNA model concerns how an aberrant RNA could induce the degradation of the endogenous mRNA counterpart. In Lindbo’s model, transgenic RNA is proposed to be recognized by RNA-dependent RNA polymerase, leading to the production of complementary RNA, the formation of double-stranded RNA and eventually to mRNA degradation. This model explains why endogenous and transgenic RNA are simultaneously degraded in a sequence-specific fashion, and is also consistent with the presence of an RNA-dependent RNA polymerase activity in plants. The fact that no complementary RNAs have been detected in plants or Neurospora might be explained by their instability or their small size. Other mechanisms by which aberrant RNAs could induce the degradation of endogenous mRNA can be envisioned. For example, the aberrant RNA could be recognized by a protein complex defining a footprint used as a template to detect and/or degrade RNAs with a similar structure. Alternatively, it has been suggested that the aberrant RNA could interfere directly with the metabolism of the target RNA (Ref. 33). There is evidence for some kind of aberrant RNA in Neurospora. An unexpected sense RNA produced from the transgenes in quelled strains is correlated with the occurrence of silencing (Fig. 4). This aberrant RNA could be produced as a consequence of tandem repeat organization of a transgene, or from a cryptic promoter in the plasmid, or in the sequences flanking the transgene integration site. As transgenic RNA contains vector sequences at both ends, this may constitute an aberrant signal in the cell, triggering responses leading to gene silencing. The aberrant RNA model fits all the other characteristics of gene silencing in Neurospora, including it being a cytoplasmic factor acting in trans and in a dominant fashion.

**Future prospects**

The fact that quelling in Neurospora and co-suppression in plants share so many common features suggests that the mechanisms for post-transcriptional gene silencing in plants and fungi may have evolved from a common ancestral mechanism (Box 2). However, the precise mechanisms underlying this silencing are still largely unknown. As molecular and biochemical approaches have failed to uncover the exact post-transcriptional mechanism involved, a genetic approach to identify components of this machinery is attractive. In plants, Arabidopsis mutants have been isolated in which the timing of rolB transgene silencing is altered. The sophisticated molecular and genetic tools available in Neurospora make it an important model for dissecting the gene silencing mechanism, and mutants defective in quelling have recently been isolated. Already, gene silencing is helping to disclose some features of gene regulation that might otherwise remain unknown, and to clarify aspects of genome organization in plants and fungi.

Elucidation of the mechanisms of gene silencing will also be of great help in transformation technology, and will provide the capability both to avoid and to enhance gene silencing in transgenic fungi and plants.

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