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Update on Biotechnology

How and Why Do Plants Inactivate Homologous (Trans)genes?[1]

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Gene silencing in transgenic plants has emerged in the last 5 years as a topic of intense interest for both applied and basic plant scientists. From the applied side, gene silencing has come as an unwelcome surprise. Early reviews of the prospects for plant genetic engineering did not pinpoint this as a potential obstacle. Rather, the anticipated challenge was to identify tissue- and stage-specific promoters that could be used to obtain regulated transgene expression. Yet, silencing of transgenes is turning out to be a substantial problem, as described recently in an aptly titled review, "Transgene Inactivation: Plants Fight Back!" (Finnegan and McElroy, 1994). According to this article, of 30 companies polled, nearly all reported some problems with unwanted silencing of transgenes. While companies are struggling to find ways to avoid silencing, a small cadre of basic scientists has become fascinated by the phenomenon and is analyzing a variety of silencing systems. To this latter group, the phenomenon of silencing represents more than just an unwanted response to foreign genes; rather, it has opened a door that might lead to a deeper understanding of previously unsuspected ways that plants naturally use homologous or complementary nucleic acid sequences to modify gene expression, both in the nucleus at the DNA level and in the nucleus or cytoplasm as a means to control excess production of mRNA or replication of RNA pathogens.

Initial studies of transgenic plants concentrated on those showing tissue-specific and/or high levels of expression. Plants that did not exhibit the desired expression characteristics were usually discarded. It was only a matter of time, however, until such plants would begin to be treated as objects of scientific investigation in their own right. In the late 1980s, papers started to appear that were devoted solely to describing cases of silencing that involved transgene/transgene or transgene/endogenous gene interactions. A central feature of all of these studies was that silencing was associated with multiple copies of homologous DNA sequences, which could comprise protein-coding regions, promoters, or both. Although the initial impulse was to lump these first cases together as a single phenomenon, it has since become clear that several different mechanisms are probably involved. These different mechanisms are united, however, in the sense that they all involve variations on nucleic acid interactions: DNA-DNA, RNA-RNA, and DNA-RNA. A general term that encompasses all of these phenomena is "homology-dependent gene silencing." Numerous detailed reviews of this topic are available (Jorgensen, 1992; Matzke and Matzke, 1993; Flavell, 1994; Matzke et al., 1994b; Mol et al., 1994).

MODES OF GENE SILENCING

Presently, three general modes of homology-dependent gene silencing can be defined. The first, termed *cis*-inactivation, involves the inactivation (and frequently methylation) of multiple, linked copies of transgenes, which can be arranged as inverted or direct repeats. In practice, such complex transgene inserts have usually been obtained unintentionally as a consequence of transformation techniques, particularly direct gene transfer methods.

A second type of inactivation, so-called *trans*-inactivation, can probably be viewed as an elaboration of *cis*-inactivation. A transgene insert that has become methylated and inactivated in *cis* can act as a "silencer" by somehow imposing in *trans* a similar degree of methylation and inactivation on a homologous "target" gene on a separate DNA molecule. The target gene can be at either an allelic (Meyer et al., 1993) or nonallelic (ectopic) (Vaucheret, 1993; Matzke et al., 1994a) chromosomal location relative to the silencing transgene insert. Because the silencing allele/locus independently achieves a methylated, inactivated state, *trans*-inactivation is a nonreciprocal gene interaction, i.e. a dominant silencing allele or epistatic silencing locus reduces the activity of the target allele/locus while remaining itself unchanged by the interaction.

The cases of *trans*-inactivation described so far in transgenic plants are probably most closely related to the phenomenon of paramutation, which was characterized for a handful of endogenous plant genes in the 1950s and 1960s (reviewed by Jorgensen, 1992; Matzke and Matzke, 1993). Paramutation has been defined as directed genetic change, although directed epigenetic change might be a more suitable term, since the induced alteration is often reversible.

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Abbreviations: 35Spro, 35S promoter of cauliflower mosaic virus; TEV, tobacco etch virus.

Paramutation involves an inducing ("paramutagenic") allele that weakens the activity of a sensitive ("paramutable") allele when the two are combined in a heterozygote. The weakened "paramutant" allele maintains this state for at least several generations after crossing out the paramutagenic allele, which emerges unchanged from the interaction. Paramutation transgresses Mendel's first law, which states that alleles have no residual effect on one another but segregate unchanged when passed into separate gametes.

The third type of silencing, termed co-suppression or sense suppression, involves the coordinate silencing (and, occasionally, coordinate reactivation) of either a transgene and a homologous endogenous gene or two homologous transgene loci (Jorgensen, 1992; Mol et al., 1994; Hamilton et al., 1995). This type of interaction is reciprocal because both of the interacting genetic loci are affected in the same way by the interaction.

SILENCING CAN BE DUE TO TRANSCRIPTIONAL INACTIVATION OR POSTTRANSCRIPTIONAL PROCESSES

Although there are a number of disparate features among various silencing systems (Matzke et al., 1994b), the one with the greatest diagnostic value is whether silencing results from transcriptional inactivation or a posttranscriptional process such as RNA turnover. The most common way of differentiating between these two possibilities is to do transcription run-on experiments with isolated nuclei. If a run-on transcript is synthesized but no corresponding RNA is visible on a northern blot, then silencing is posttranscriptional. If transcriptional inactivation is involved, however, then neither run-on transcripts nor steady-state RNA on northern blots is detected.

Silencing Due to Posttranscriptional Events

It is likely that most cases of co-suppression involving a transgene/endogenous gene interaction result from a posttranscriptional process, presumably RNA turnover, as has been demonstrated directly for silencing of chalcone synthase genes in petunia (van Blokland et al., 1994). Therefore, of all the hypothetical mechanisms originally put forward to explain homology-dependent gene silencing (Jorgensen, 1992; Matzke and Matzke, 1993), a current favorite to explain at least some examples of co-suppression is the biochemical switch model suggested by Meins and Kunz (1994). According to this model, a product of gene expression, e.g. RNA, would accumulate until a sharp threshold is reached, at which point RNA degradation would be initiated. In transgenic plants, overproduction of a given RNA, resulting from transcription of an endogenous gene and a homologous transgene in addition, would thus provoke turnover of that RNA. This would account for the synthesis of run-on transcripts but the absence of the corresponding RNA in the steady-state pool, and would also explain cases in which homozygosity at the transgene locus, which presumably produces twice the amount of RNA found in the hemizygote, is required to obtain silencing (de Carvalho et al., 1992; Dehio and Schell, 1994; Meins and Kunz, 1994). The idea that high levels of transcription can be the trigger for RNA turnover via RNA-RNA interactions is discussed further below.

It is important to note that there are examples of posttranscriptional silencing that do not seem to involve excessive levels of transgene RNA (van Blokland et al., 1994). In these cases it is possible that an RNA degradation pathway can be activated by unintended antisense RNAs or defective RNAs (i.e. truncated or improperly processed) that are synthesized from complex transgene inserts.

Silencing Due to Transcriptional Inactivation

In contrast to the posttranscriptional process leading to co-suppression, other examples of gene silencing appear to be due to transcriptional inactivation. These cases include paramutation of maize *A1* alleles in transgenic petunia (Meyer et al., 1993) and at the *B* locus in maize (Patterson and Chandler, 1995). Transcriptional inactivation is also the most likely mechanism for silencing in cases of *trans*-inactivation involving unlinked transgene loci that share homology only in promoter regions (Neuhuber et al., 1994). It has been suggested for all of these systems that pairing of alleles or homologous unlinked loci is responsible for the observed silencing effects. Ways in which DNA-DNA interactions can generate inactive genetic states, through either de novo methylation or heterochromatin formation, are described further below.

Complete transcriptional inactivation is not always observed and intermediate states can be interpreted differently. For example, a 5-fold reduction in run-on transcripts was considered transcriptional inactivation in one study (Brusslan et al., 1993) but predominantly posttranscriptional silencing in another (Dehio and Schell, 1994). Because patterns of cellular mosaicism are often observed with transgene expression (Neuhuber et al., 1994), it is not always possible to assess whether reduced transcription reflects a uniform decrease in all cells or complete inactivation in some cells and normal transcriptional activity in others. Such considerations can complicate the interpretation of some run-on experiments as well as other aspects of gene silencing in plants.

Signals for de Novo Methylation Provide Clues for Understanding Silencing Mechanisms

The two levels at which silencing takes place, transcriptional or posttranscriptional, suggest fundamentally different mechanisms involving DNA-DNA or RNA-RNA interactions, respectively. Originally, these two mechanisms seemed to be distinct with respect to the involvement of DNA methylation, i.e. methylation was believed to be associated with transcriptional inactivation, whereas posttranscriptional processes were assumed not to involve methylation. However, this distinction is becoming blurred with mounting evidence indicating that even posttranscriptional silencing can be associated with de novo methylation of the corresponding nuclear gene, possibly via a DNA-RNA association. We will discuss silencing effects involving this third variant of nucleic acid interaction, and

its somewhat confusing implications, in the context of other signals for de novo methylation.

The trigger for de novo methylation is one of the more mysterious features of methylation in eukaryotes. Although it is generally assumed that once a sequence becomes methylated it will remain so through subsequent rounds of DNA replication via the action of maintenance methylase, the signals for the initial methylation of unmodified DNA are not completely understood. At present, there is experimental support in plants for three possibilities: a response to foreign DNA, DNA-DNA pairing, and a DNA-RNA interaction.

Response to Foreign DNA

Transgenes often become methylated in both plant and animal cells. This possibly occurs because they are recognized as "foreign" by a genomic immune function that evolved from prokaryotic restriction-modification systems (Bestor and Coxon, 1993). The best example of this in plants is a single-copy maize *A1* transgene in petunia, which became hypermethylated even though flanking plant DNA sequences remained hypomethylated (Meyer and Heidmann, 1994). In this case, methylation of the *A1* transgene could not have been due to spreading of methylation from adjacent plant DNA or repetitive elements in the transgene construct. Rather, the transgene incurred methylation because it was identified as invading DNA, perhaps because it did not have the same isochore composition (i.e. GC content) as the context plant DNA.

DNA-DNA Pairing

In principle, methylation resulting from a response to foreign DNA can affect single or multiple copies of transgenes. However, transgene loci containing repetitive elements could also become methylated and inactivated when DNA-DNA pairing serves as the methylation signal. The best evidence that pairing of homologous DNA sequences can trigger methylation de novo comes from the filamentous fungus *Ascobolus immersus*, in which duplicated sequences are readily methylated and transcriptionally inactivated, so-called "MIP" for methylation induced premeiotically (Rossignol and Faugeron, 1994). (MIP is not identical with a similar process in *Neurospora crassa* termed "RIP" for repeat-induced point mutation, which involves a high rate of C to T conversions and is thus mutagenic.) Therefore, it might be expected that repeated (trans)genes in plants would also spontaneously methylate and inactivate if they were able to pair. Support for this proposal comes from a study in *Arabidopsis*, in which an allelic series comprising different copy numbers of a transgene was generated by recombination at a single locus. Alleles containing repeats were silenced and methylated, whereas alleles lacking repeats were active and unmethylated (Assaad et al., 1993).

DNA-RNA Interaction

A DNA-RNA association can also apparently serve as a signal for de novo DNA methylation. This novel proposal came out of a recent study of viroid replication in transgenic plants engineered to contain cDNA copies of the viroid genome integrated into nuclear DNA. Only during replication of the viroid RNA genome did the nuclear cDNA copies become methylated at some sites (Wassenegger et al., 1994). These authors suggested that RNA-induced methylation of genomic DNA could provide a general means to down-regulate overexpressed nuclear genes. Although one would anticipate that the methylated viroid cDNA copies in the nucleus would also be transcriptionally inactivated, this has not yet been demonstrated for the viroid system. Contrary to this expectation, however, are two recent reports claiming that transcriptional inactivation is not necessarily an inevitable consequence of DNA methylation: posttranscriptional silencing of transgenes was correlated with increased methylation at some sites, i.e. transgenes that had become methylated continued to be transcribed (Ingelbrecht et al., 1994; Smith et al., 1994). Although these results appear at first glance to contradict the current view that DNA methylation represses transcription, it is possible that methylation had not yet achieved a density sufficient for complete inactivation of the 35S promoter (which was used in both studies). Indeed, Smith et al. (1994) proposed that the apparent RNA-induced transgene methylation that they observed represented "an attempt to reduce transgene transcription." Presumably, the transgene would eventually cease to be transcribed when a certain level of methylation was attained. Alternatively, Ingelbrecht et al. (1994) suggested that methylation did not interfere with either transcription initiation or elongation but perhaps led to the synthesis of "unproductive transcripts." In any case, RNA-mediated de novo methylation of DNA is an intriguing possibility that deserves further attention. Analyzing methylation can be tricky, however, and reports claiming a correlation (or lack of one) between methylation and silencing should be read with the following points in mind.

Some Pitfalls of Methylation Analyses

Although methylation is normally associated with inactive genes, expressed genes can also be methylated at some sites that might have little negative influence on gene activity (Meyer et al., 1994). Therefore, attempts should be made to identify and study cytosines that are present in essential regulatory regions. Moreover, if a critical cytosine residue is not included in a restriction enzyme site, then methylation of that cytosine will only be detectable by genomic sequencing. This method, which is the most satisfactory way to determine the methylation density throughout a given region, has also revealed in plants the frequent methylation of cytosines that are not part of a CpG or CpNpGp di- or trinucleotide (Meyer et al., 1994). The distribution of methylation in a gene can provide clues about the methylation signal. If DNA-DNA pairing or a DNA-RNA association provokes de novo methylation, then this modification should be concentrated in the paired or transcribed region, respectively. Methylation that represses initiation of transcription is often restricted to the immediate promoter region and does not extend too far

upstream or into the protein-coding sequences. Inactivation of strong promoters requires a high methylation density; weak promoters can be inactivated by sparse methylation (Bird, 1992). Any acquired methylation should be tested for reversibility following the removal of the initial stimulus for methylation. Irreversible methylation suggests a reaction to foreign DNA.

SILENCING DUE TO POSTTRANSCRIPTIONAL PROCESSES: A REFLECTION OF HOW PLANTS MIGHT DEAL WITH INFECTION BY RNA PATHOGENS?

We have mentioned RNA overproduction in two contexts. First, in the section on the involvement of posttranscriptional processes in co-suppression, we described how high rates of transcription of a transgene along with the homologous endogenous gene could produce an excessive quantity of RNA that activated a turnover pathway. Second, when discussing signals for de novo methylation, we described how abundant amounts of RNA produced by highly transcribed genes could have a feedback effect that leads, via a DNA-RNA interaction, to methylation of the corresponding gene (as discussed above, whether this eventually leads to complete transcriptional inactivation is not yet clear). In both cases, the consequence of RNA overproduction would be gene silencing, evinced by disappearance of RNA from the steady-state pool. Why do plants possess such RNA-based mechanisms for regulating gene expression? More than 90% of plant viruses have an RNA genome, as do viroids. Attempts to engineer virus resistance in plants are forging a connection between posttranscriptional silencing phenomena and cytoplasmic activities that help protect plants from viral infection. The idea emerging is that plants can somehow sense elevated levels of an RNA sequence—arising from a replicating viral genome, or a highly transcribed nuclear (trans)gene, or both acting additively—and target that RNA for destruction.

Lindbo and co-workers (1993) have proposed that resistance to TEV in transgenic tobacco plants expressing a TEV coat protein transgene is due to increased levels of TEV-specific RNA resulting from transcription of the transgene plus the replicating TEV genome in the cytoplasm. A key experiment showed that, even though the coat protein transgene was transcribed at approximately the same rate in resistant and sensitive plants, steady-state levels of transgene RNA were significantly lower in the former than in the latter. This linked the antiviral state with posttranscriptional degradation of the transgene RNA and the replicating TEV genomic RNA. Among other possibilities, it was suggested that an RNA-dependent RNA polymerase could synthesize short complementary segments of an RNA that had accumulated to intolerably high levels in the cytoplasm. Duplex RNAs formed between the short RNAs and the target would then be a substrate for double-strand RNases (Lindbo et al., 1993).

In support of this model of RNA-mediated virus resistance is another study in which plants were engineered with a cDNA encoding an untranslatable sense RNA for the coat protein of potato virus Y. Plants that transcribed the transgene at high levels accumulated only low levels of the untranslatable sense coat protein transcript and were resistant to potato virus Y, again implying the action of an RNA turnover mechanism induced by elevated coat protein RNA levels (Smith et al., 1994). The transcribed coat protein transgenes were methylated at the few sites examined. As mentioned previously, this example and other work (Ingelbrecht et al., 1994; Wassenegger et al., 1994) provides evidence for RNA-mediated de novo methylation of nuclear genes.

To What Extent Are Posttranscriptional Silencing Phenomena Dependent on Transgenes Driven by the 35S Promoter?

Many silencing phenomena have involved transgenes under the control of the 35Spro of cauliflower mosaic virus (Matzke et al., 1994b). What are the implications of the frequent use of this promoter? First, it is a strong promoter that could produce sufficient RNA to activate a turnover mechanism that depends on a threshold RNA concentration. Second, it is a viral promoter (although cauliflower mosaic virus is a DNA virus) and it might be subject to antiviral controls developed by plants. Third, although active in many plant organs, the 35Spro is not a constitutive promoter and its activity can follow a pattern of cellular mosaicism in leaves (Neuhuber et al., 1994). The 35Spro also comprises distinct subdomains that show cell type-specific patterns of activity in petunia petals (Benfey and Chua, 1990), where striking patterns of pigmentation have been observed in cases of chalcone synthase co-suppression (Jorgensen, 1992). It is conceivable that cases of co-suppression that seem to show developmental control actually reflect the developmental control of the 35Spro and its various subdomains, which may be represented incompletely because of deletions and/or fusions in complex transgene inserts. Although a chalcone synthase genomic clone could apparently elicit co-suppression in a few plants (Mol et al., 1994), there is still a need to test additional promoters for silencing ability.

SILENCING RESULTING FROM TRANSCRIPTIONAL INACTIVATION: A REFLECTION OF HOW REPEATED DNA SEQUENCES CAN INTERACT AND REPRESS NUCLEAR GENES?

An early hypothesis to explain silencing of homologous genes was the "ectopic pairing hypothesis," which postulated pairing of unlinked homologous DNA regions and subsequent exchange of chromatin structural components (Jorgensen, 1992). This hypothesis was derived in part from the MIP process in fungi, in which DNA-DNA pairing can lead to de novo methylation and transcriptional inactivation of duplicated genes. It also drew from the phenomenon of dominant position effect variegation in *Drosophila*, in which a rearranged, newly heterochromatinized allele can impose in *trans* the inactive heterochromatinized state on its unrearranged homolog. However, as described above, many cases of gene silencing seem to be due to posttranscriptional events. Furthermore, methylation can

apparently result from a DNA-RNA association and not just a DNA-DNA interaction. What, then, is the current status of the DNA-DNA pairing hypothesis? It is probably still viable in cases of paramutation that have been shown to result from transcriptional inactivation (Meyer et al., 1993; Patterson and Chandler, 1995). These concern allelic and not ectopic pairing, and only the case of Meyer and co-workers has been shown thus far to involve methylation. Nevertheless, direct physical contact between alleles has been suggested to account for paramutation in each respective system.

DNA-DNA pairing has also been suggested as a mechanism for two *trans*-inactivation systems comprising a silencing transgene locus and a target transgene locus that share homology only in promoter regions (Vaucheret, 1993; Matzke et al., 1994a). In the nopaline synthase promoter-based system, the extent of promoter homology was about 300 bp (Matzke and Matzke, 1993). In the 35Spro-based system, however, silencing was observed even when promoter homology comprised only 90 bp (Vaucheret, 1993). Although 90 bp is probably not enough for direct DNA-DNA pairing, it still does not eliminate the possibility of recognition of DNA sequence homology. A good distinction between direct and indirect DNA associations has been made by Rossignol and Faugeron (1994). For a direct DNA-DNA interaction depending on physical homology between repeats, homology probably must exceed a minimum length of about 300 bp. However, an indirect recognition process could affect DNA repeats that are too short for a direct DNA-DNA interaction or not perfectly homologous. For this, functional homology would be required such that DNA repeats would be recognized by the same DNA-binding proteins. This indirect process could be operating in cases of *trans*-silencing involving promoter homology: Identical promoters could be recognized by an aggregative protein, leading to clustering in the same nuclear compartment where methylation and/or chromatin changes could be imposed coordinately. Such a model might also be relevant for *trans*-silencing in a nontransgenic system in which paramutagenicity has been mapped to the 5′ flanking region of the *B′* allele in maize (Patterson and Chandler, 1995).

FUTURE PROSPECTS; AVOIDING SILENCING

We have discussed three possible gene-silencing mechanisms that are based on different nucleic acid interactions: DNA-DNA, DNA-RNA, and RNA-RNA (Fig. 1). Current research concerning silencing is leading in several directions: (a) DNA-DNA pairing. Does this occur between homologous sequences at allelic and/or ectopic locations in somatic plant cells? How frequently does pairing serve as a signal for de novo methylation and/or heterochromatin formation in plants? Can pairing be developmentally regulated? (b) DNA-RNA associations. Does this link overproduction of RNA with de novo methylation and transcriptional inactivation of nuclear genes? Alternatively, are "unproductive" RNAs transcribed from methylated genes? (c) RNA metabolism. How is a threshold RNA concentration sensed in the cytoplasm and/or nucleus? Do RNA-RNA interactions and/or defective RNAs trigger RNA turnover? What enzymes are required (double-strand RNases, RNA-dependent RNA polymerases, etc.)?

In addition to these questions that arise from mechanistic differences, there is a common interest in isolating genes whose products modify the degree or timing of all types of silencing (Dehio and Schell, 1994) and in determining T-DNA modifications and/or configurations that are associated with silencing loci (A. Matzke et al., 1994; van Blokland et al., 1994). Finally, there is a pressing need to clarify the involvement of methylation in silencing by increased use of genomic sequencing and to study alternative forms of stable gene repression, such as chromatin condensation.

Strategies to minimize silencing could be tailored to different silencing mechanisms. Eliminating repeated elements from transgene constructs should alleviate problems with DNA-DNA pairing and de novo methylation. To avoid RNA turnover induced by excess RNA production, moderate transcription rates might be preferable to extremely high ones. Because the 35Spro seems to be associated frequently with silencing effects, it might be wise to avoid using it altogether. Targeting transgenes into compatible isochores and/or including flanking scaffold attachment regions in constructs might dampen the "foreign DNA response."

GENERAL SIGNIFICANCE

Gene silencing that results from interactions in the nucleus between homologous (trans)genes may reflect a natural tendency for repeated DNA sequences to interact in eukaryotic genomes. If so, then silencing that results from DNA-DNA pairing should occur in organisms other than plants. This has been demonstrated not only for some filamentous fungi, as described above, but also more recently for *Drosophila* (Dorer and Henikoff, 1994). In both cases, repeated sequences provide an excellent substrate for de novo methylation or heterochromatin formation, respectively. Therefore, pairing of repeats may provide a universal means to inactivate large portions of genomes and/or form heterochromatic regions. This mechanism might also help limit the expression and spread of transposable elements (Flavell, 1994). It is important to note that multigene families can escape this type of silencing by either increasing the degree of sequence divergence or decreasing the length of sequence homology by dividing coding sequences into exons that are too short to pair efficiently (Kricker et al., 1992). A factor contributing to inbreeding depression may in fact be the pairing of identical alleles, which could lead to methylation and silencing.

RNA turnover triggered by RNA overproduction could have originally evolved in plants as a defense against RNA viruses and viroids and then subsequently been put to use as a means to posttranscriptionally silence developmentally regulated and inducible plant genes. This mechanism may be particularly well developed in higher plants or even unique to the plant kingdom, as suggested by the fact that co-suppression resulting from RNA turnover has not

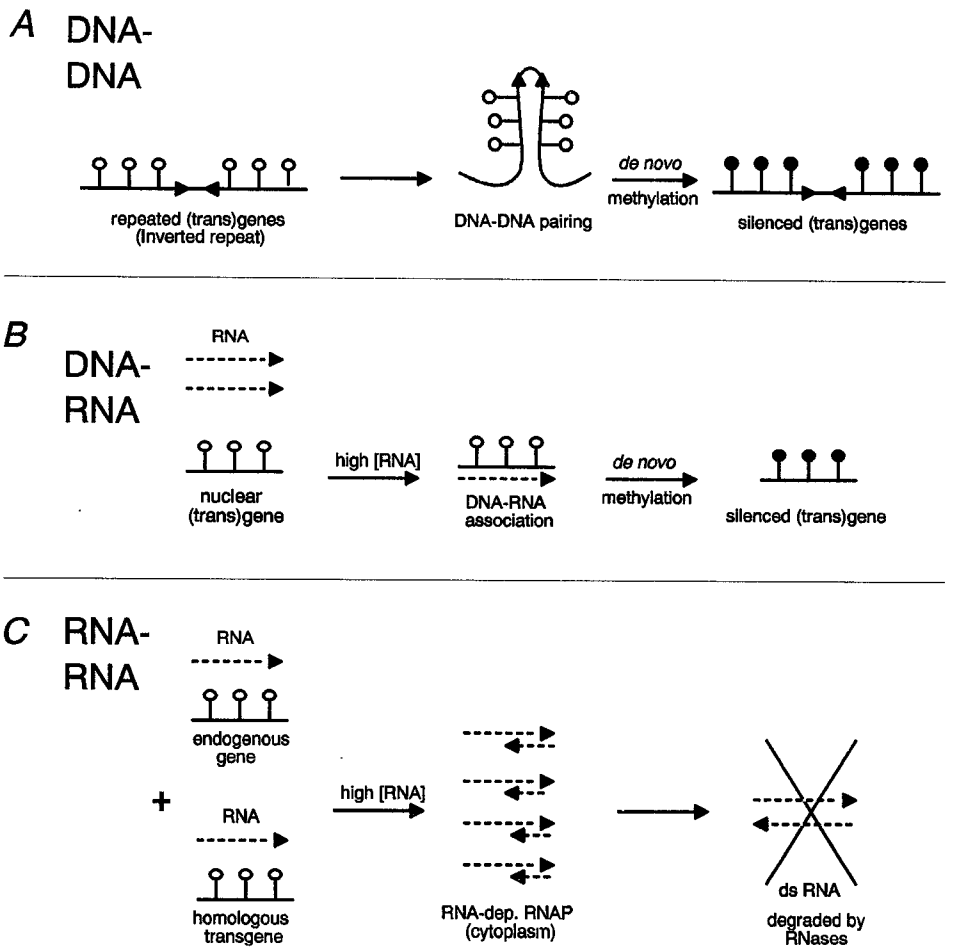

Figure 1. Hypothetical mechanisms of gene silencing based on different nucleic acid interactions in transgenic plants. A and B are conceived as nuclear processes that lead to de novo methylation of DNA and transcriptional inactivation. The posttranscriptional RNA turnover mechanism in C takes place in the cytoplasm and would not necessarily result in DNA modifications (Meins and Kunz, 1994). However, B and C might be connected if elevated RNA levels lead to both RNA turnover in the cytoplasm (C) and de novo methylation of nuclear genes (B). Whether such methylation eventually leads to complete transcriptional inactivation is still not known; a modest degree of apparent RNA-induced methylation does not necessarily result in complete transcriptional inactivation (Ingelbrecht et al., 1994; Smith et al., 1994). Additional ideas about silencing mechanisms and different points at which it can occur in the pathway from DNA to protein have been discussed by Flavell (1994). Lindbo et al. (1993) and Smith et al. (1994) have elaborated on the connection between RNA virus resistance and silencing of nuclear transgenes. An inverted repeat is shown in A, but direct repeats or more than two copies can also pair (Dorer and Henikoff, 1994). Open and closed circles designate unmethylated and methylated cytosines, respectively. A locus that has become silenced in *cis* (A) can potentially *trans*-inactivate homologous, unlinked loci in a manner that might rely on DNA-DNA pairing (Matzke et al., 1994a). ds RNA, Double-stranded RNA; RNA-dep. RNAP, RNA-dependent RNA polymerase.

yet been reported for transgenic animals. This could change, however, as the plant phenomena become better known and animal transgeneticists begin to examine their systems more carefully for silencing. A defense strategy against RNA pathogens, which replicate in the cytoplasm, might also have been co-opted as a way to repress transcription of nuclear genes via methylation, because an RNA-DNA association may be similar enough to DNA-DNA pairing, which is a known signal for de novo DNA methylation in the fungus *Ascobolus*.

A final point to emphasize is that, regardless of the mechanism(s) of gene silencing, it can generate heritable epigenetic variants (Jorgensen, 1993). "Epigenetic inheritance" can be broadly defined to include mechanisms based on either chromatin marking, steady-state systems, or structural inheritance (Jablonka et al., 1992). The first is exemplified by DNA methylation, which is not always erased completely during plant sexual reproduction (Matzke and Matzke, 1993; Vaucheret, 1994). Perhaps not so well known is that the heritability of steady-state systems based on self-regulatory feedback loops can also be quite high (Jablonka et al., 1992). This might have relevance for silencing phenomena involving RNA turnover. Because the immediate and lingering effects of

homology-dependent gene interactions can potentially create substantial epigenetic variability, both in plant populations and in cell populations in meristems of individual plants, such interactions are likely to have important implications for plant evolution and adaptation, respectively.

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