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# HOMOLOGY-DEPENDENT GENE SILENCING IN PLANTS

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ABSTRACT

Homology-dependent gene silencing phenomena in plants have received considerable attention, especially when it was discovered that the presence of homologous sequences not only affected the stability of transgene expression, but that the activity of endogenous genes could be altered after insertion of homologous transgenes into the genome. Homology-mediated inactivation most likely comprises at least two different molecular mechanisms that induce gene silencing at the transcriptional or posttranscriptional level, respectively. In this review we discuss different mechanistic models for plant-specific inactivation mechanisms and their relationship with repeat-specific silencing phenomena in other species.

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## INTRODUCTION

With the rapidly increasing application of transgene technology in plants, the control of transgene expression has become an important point of concern. A common aspect of many cases of inactivation of transgenes is the presence of duplicated homologous sequences. Apparently homology serves as a signal that can trigger gene inactivation at either the transcriptional or posttranscriptional level. Homology-dependent gene silencing is the basic feature for several phenomena that apparently each have distinct regulatory mechanisms. This includes, for example, the inactivation of tandem repeats, *trans*-inactivation of allelic or ectopic copies, and the coordinated silencing of a transgene and the endogenous homologous gene. In this review we summarize the recent data on homology-dependent gene silencing, focusing on the different models for the regulation of transcriptional and posttranscriptional silencing. Most likely, transgene research has uncovered the existence of an endogenous control mechanism for multiple sequences, which does not affect transgenes exclusively. On the basis of the mechanistic models we therefore discuss the biological function of homology-scanning systems and implications for genome organization and evolution.

## HOMOLOGY-DEPENDENT SILENCING PHENOMENA

### *Inactivation of Homologous Transgenes*

The importance of homologous sequences for the induction of gene silencing was discovered when transgenic tobacco plants were retransformed with constructs that were partly homologous with the integrated transgene. In the presence of the second construct, the primary transgene became inactivated and hypermethylated within the promoter region, the site of homology be-

tween the "suppressor" and the "target" locus (76). Since this remarkable discovery, numerous cases of homology-based silencing in transgenic plants have been reported. Although single transgene copies can become inactivated (104), the integration of multiple copies enhances silencing efficiencies, particularly if repeated sequences are inserted in concatameric arrangements at one locus (5, 88), but also when homologous transgenes are located at alleles of a locus (83) or are present at unlinked sites (75). Transgene inactivation can comprise both transcriptional (83) and posttranscriptional silencing (27, 57) of marker genes.

Silencing is influenced by the length of the homology and especially by the position of the interacting sequences. Linked copies are more efficiently silenced than unlinked copies, and unlinked loci show characteristic differences in silencing capacity (132) and susceptibility to being silenced (91). The most efficient example of *trans*-inactivation is a tobacco line carrying a transgene insert with two genes driven by the 19S and the 35S promoter of CaMV, respectively. Both genes linked to the two promoters are suppressed, and this locus *trans*-inactivates newly introduced constructs that provide at least 90 bp of common homology (132).

HERITABILITY AND REVERSION OF SILENCING When interacting loci are separated in genetic crosses, reversion of the silenced state occurs slowly over several generations (91). The silenced state of concatameric transgenes, which are always inherited as a block, is preferentially transmitted to the progeny (5, 63, 88). In some transformants, silencing is progressively enhanced in subsequent generations (5, 63). Other silenced transgenes show a defined resetting phase. A silenced *rolB* transgene is reactivated and remains active in young seedlings, while silencing occurs again erratically during further development of the seedling (27).

In concatemeric transgenes, a reduction of repeats enhances the probability of reversion of the silencing event. In *Arabidopsis* transformants in which transgene copy numbers had been reduced because of intrachromosomal recombination, transgene inactivation was observed at a lower frequency than in the parental line that still contained multiple repeats (4, 5). A similar observation was made by another laboratory for an *Arabidopsis* transformant that had also lost several transgene repeats via intrachromosomal recombination. When lines were selected from the parental plant and the deletion line that had reactivated the transgene, only the deletion lines conserved the active state of the transgene during meiosis (87).

THE ROLE OF DNA-METHYLATION A correlation between gene inactivation and DNA methylation has been shown for transgenes (2, 131), transposable ele-

ments (19, 116), and some endogenous genes (123). For other genes no such correlation was observed (93). With respect to a correlation between DNA methylation and homology-based silencing, we can group the different silencing events into three classes. Silencing events that show a direct correlation between transcriptional inactivation and DNA methylation within the promoter (76, 83) or coding region (57), silencing events that are not associated with detectable changes in DNA methylation (27, 44), and silencing phenomena where hypermethylation patterns build up over successive generations (86).

An interesting aspect of DNA methylation in plants is the presence of methylated C residues outside of CG or CNG sequences, the symmetrical target sequences for maintenance methylation in plants. To date, nonsymmetrical methylation patterns, which are probably not encoded in the sequence—but more likely in the secondary structure of a sequence—have only been detected in transgenes (57, 85) and not in endogenous genes (93). It is therefore unclear whether they are specifically imposed on transgenes or genes that have been transferred into new chromosomal environments. In the latter case, they should also occur in transposable elements.

## *Paramutation*

An indication that homology-based silencing events are not specific for transgene DNA but reflect an endogenous mechanism comes from the analysis of paramutation. Paramutation was described more than 60 years ago (139) and has been examined in several species (11, 21, 48, 49). The term refers to the interaction of homologous plant alleles that leads to heritable epigenetic effects. A detailed review of paramutation, in the context of genetic imprinting, has recently been published (74). We therefore only summarize a few aspects relevant to the mechanistic models for gene silencing. A *paramutagenic* allele can cause a *paramutable* allele to undergo an epigenetic conversion to become a *paramutant* allele of lower function. The new *paramutant* state is metastable, because it can be somatically and germinally inherited in the absence of the *paramutagenic* allele, but it also reverts with different frequencies. Paramutation requires a metastable state of the *paramutable* allele, which is only amplified through its interaction with the *paramutagenic* allele. Paramutation and the frequencies of reversion are dependent on environmental and developmental factors.

Molecular studies in snapdragon (10, 22, 65), maize (101), and *Petunia* (83) provided insights into the mechanisms involved in paramutation. A correlation between the expression of the *paramutable* gene and its methylation state was observed for the *R* locus in maize (30) and for an *A1* maize transgene in *Petunia* (83). In contrast, no differences in cytosine methylation could be detected between *paramutagenic* and *paramutable* alleles at the *B* locus of maize, despite an extensive analysis over a distance of 12 kb (100). The latter

study does not exclude the involvement of DNA modification in paramutation, as certain types of nucleotide modifications, such as hydroxy-methylcytosine, A-methylation, or methylcytosines located in nonsymmetrical positions would have gone undetected. Nevertheless, the analysis of *B* suggests either that methylation is not the cause but a secondary effect of paramutation, or that different classes and mechanisms of paramutation exist. The latter is a possibility because *B* and *R* paramutation differ in other characteristics (100). Paramutated alleles of *B* are extremely stable, whereas *R* paramutation shows frequent reversions. Furthermore, the *B* locus contains a single allele, whereas in most *R* alleles multiple homologous genes occur at the *R* locus. A similar complexity has been found for two semidominant alleles of the *nivea* locus in *Antirrhinum majus* that show structural rearrangements such as inverted duplications or concatamerization of truncated copies of the *nivea* gene (10, 22).

### *Mutual Inactivation of Transgenes and Endogenous Genes*

The term cosuppression (59) was coined to describe the inhibition of gene expression of an endogenous gene after the introduction of a homologous transgene. This phenomenon was first described for the chalcone synthase (CHS) gene in *Petunia* (90, 129). Up to half of the transformants that contained a CHS sense copy produced white flowers or floral sectors because of the loss of CHS activity. Nuclear run-on analysis showed normal CHS transcription rates but a reduction in steady-state levels of CHS mRNA, apparently as a result of posttranscriptional effects (38, 128). Frequently, not all flowers showed the same cosuppression phenotype. Individual plants developed branches with purple, white, or sectored flowers. Among the flowers of individual branches, cosuppression patterns usually remained very similar, which suggests that cosuppression was somatically inherited and initiated during formation of the meristem of individual branches (38).

Cosuppression is not unique to CHS but appears to be a general phenomenon affecting many endogenous genes. Detection can be difficult if the inhibition of the gene does not produce a visible phenotype. Examples of genes that showed an unstable expression after the introduction of homologous sequences are dihydroflavanol reductase (129) and the homeotic *fbp2* gene in *Petunia* (3), tomato polygalacturonidase (120), phytoene synthase (40), pectinesterase (119), an *Arabidopsis* cab140 gene (13), phenylalanine ammonia-lyase (33), β-1,3-glucanase (25), chitinase (50), nitrate reductase (24), S-adenosyl-L-methionine synthetase (9), and glutamine synthetase in tobacco (G Coruzzi, unpublished data). The efficiency of cosuppression varies for individual transformants. The transfer of the same sense construct often generates cosuppressed transformants as well as transformants that overexpress the sense construct (9, 24). Individual genes show characteristic differences in their susceptibility to cosuppression. Two extreme examples are the *Petunia*

chalcone isomerase gene, for which cosuppression has not been observed thus far (28), and the nuclear-encoded cytosolic tobacco GS2 gene that is cosuppressed, to variable extents, in all transformants tested so far (G Coruzzi, unpublished data).

It is important to recognize that there are multiple steps within the expression pathway that contribute to the production and activity of a gene product. This might also explain the partly contradictory features found for individual cosuppression events. Inhibition of gene expression at the posttranscriptional level has been confirmed for many types of cosuppression (25, 38, 128), but transcriptional suppression can also be found (13). It is possible that cosuppression is mediated by different mechanisms in different species or for individual genes. Alternatively, for certain genes posttranscriptional silencing could be the primary event that induces transcriptional inactivation as a second step of a common cosuppression mechanism.

REQUIREMENT FOR TRANSCRIPTION An important aspect of cosuppression is the question as to whether the transgene and the endogenous genes need to be transcribed. Evidence for the requirement of mutual transcription comes from studies on cosuppression of polygalacturonase (PG) in tomato induced by constitutive expression of a truncated PG transcript. In ripe fruits where the endogenous gene is active, expression of both genes is reduced, and transcript levels of the constitutively expressed transgene are significantly lower in ripe fruits compared with green fruits (120). On the other hand, a promoterless CHS construct induced cosuppression effects in 15% of transgenic *Petunia* plants (128), which suggests that expression is not required for cosuppression. However, traces of CHS antisense RNA are found in these transformants (128), which allow the speculation that an endogenous promoter reads into the transgene creating an antisense-mediated inhibition.

There is some evidence that critical levels of transcription are required for efficient induction of cosuppression. For several examples, silencing was found to be enhanced or even dependent on the homozygous state of a transgene (24, 25, 50). In contrast to meiotically transmittable examples of cosuppression (60), silencing in homozygous lines was not inherited by outcross progeny now containing only one transgene. One interpretation for this effect is that the primary transgene is transcribed at a relatively high rate, and that by duplication of these rates, transcript levels in homozygous plants reach a critical threshold. Alternatively, cosuppression might be stimulated by a DNA-based interaction between the transgene alleles in homozygous lines. At least for cosuppression of the β-1,3-glucanase in tobacco, the latter assumption could be excluded. In one line, inactivation of the β-1,3-glucanase genes occurred exclusively in plants homozygous for a homologous transgene. In haploid plants of this line, suppression was observed regardless of whether the

transgene derived from homozygous or hemizygous transformants (25). This result excludes a function for allelic interactions and suggests a dose-dependent regulation for silencing that is determined by the ratio between the transgene transcripts and the copies of the endogenous genes or the entire genomes.

DEVELOPMENTAL AND ENVIRONMENTAL CONTROL OF COSUPPRESSION Several cases of cosuppression show developmental regulation and a dependence on environmental factors. Cosuppression of CHS genes in *Petunia* produces a variety of anthocyanin pigmentation patterns in the flower, among which highly ordered patterns can be found that are somatically heritable (90). These observations suggest a linkage between regulatory mechanisms of morphological differentiation and the induction of cosuppression (61). Frequently, silencing is triggered after a lag period, either stochastically at different stages during development (24, 50) or synchronously at a specific stage of development (25; H Vaucheret, unpublished data).

Various cases of environmental influences on silencing have been observed. Cosuppression of CHS genes in *Petunia* (129) and β-1,3-glucanase genes in tobacco (25) are stimulated by high light intensities. Silencing of chitinase genes in *Nicotiana sylvestris* (50) and nitrate reductase in tobacco (24) are dependent on germination and growth conditions.

### *Inactivation Mediated by RNA Viruses*

An unexpected link between cosuppression and transgene-mediated viral resistance was observed in transgenic plants resistant to different members of the potyvirus group (69, 89). Untranslatable constructs of the viral coat protein gene or the RNA polymerase gene of potyviruses generated a strain-specific resistance against the virus, accompanied by very low steady-state levels of the transgenic RNA. In virus-resistant lines, homologous transgenes were also *trans*-inactivated, which suggests that viral resistance is mediated by a homology-based inactivation mechanism.

## MODELS FOR HOMOLOGY-BASED SILENCING

The complexity of experimental details concerning homology-based silencing makes it difficult to allocate defined mechanistic models exclusively to certain silencing categories. We have therefore avoided linking detailed models to the description of different types of silencing presented above. In the following, we discuss several models for the molecular mechanisms involved in silencing, which are not mutually exclusive but which may apply individually or even synergistically for individual silencing types.

### *Silencing Mediated Via DNA-DNA Pairing*

The interaction of homologous DNA copies has been proposed as a mechanistic model for certain types of cosuppression (59), *trans*-inactivation (73), and paramutation (83). It was proposed (61) that silencing reflects changes in the physical state of a transgene and that mutual silencing of a transgene and an endogenous homologue are caused by regular changes in the epigenetic states of the transgene. A DNA-DNA pairing model would provide an explanation for the differences in efficiency at which silencing occurs within individual transformants, because the interaction between two homologous sequences would be determined by the probability with which the two loci associate in interphase nuclei. This probability should be higher for tandemly linked copies, compared with unlinked, ectopic copies. The tandem arrangement of transgenes may not only enhance the efficiency of DNA-DNA pairing, but the formation of stemloop structures on single strands of a region carrying inverted repeats could mediate an efficient spread of de novo methylation patterns. Foldback DNA is specifically recognized by the human methyltransferase (121).

Individual transgenes differ significantly in their capacity to *trans*-inactivate homologous copies (132), which probably reflects their potential to scan other chromosomal locations for homology. The presence of very efficient *trans*-silencers close to the telomere suggests that telomeric regions are favorable sites for the interaction with homologous sequences (72).

### *RNA-Mediated Models for Silencing*

THE ROLE OF RNA-DNA HYBRIDS The DNA pairing model suggests that epigenetic patterns, characterized by a specific state of DNA methylation or chromatin structure, are exchanged during a potential somatic hybridization (60, 75). The observation that RNA molecules can induce hypermethylation patterns within homologous DNA sequences suggested that changes in epigenetic states could also be mediated by DNA-RNA pairing. Evidence for the participation of RNA molecules in the induction of DNA methylation came from a study of tobacco transformants carrying the cDNA of potato spindle tuber viroid. Specific methylation of the viroid DNA was observed whenever viroid RNA replication had occurred (135). These data suggest that transcripts can induce methylation in the homologous DNA region, which might be especially relevant for transformants that accumulate large amounts of nuclear transcripts because of high transcription rates or imperfect RNA processing. The specific methylation of coding regions in certain posttranscriptional silencing events (57) might reflect such an RNA-mediated induction of DNA methylation.

DEGRADATION OF THRESHOLD LEVELS OF RNA The dosage-dependency of certain silencing effects (25, 27, 57) and the observation of a linkage between silencing and the onset of expression of the endogenous gene (120) suggest that silencing can be induced by the production of defined threshold levels. Such a model might especially apply for particular genes that carry target sequences for RNA degradation to control high expression levels generated by gene induction (92).

THE AUTOREGULATION MODEL Inspired by a model for cytokinin habituation (79), an autoregulation model for silencing was proposed (80). In this model, transcription of a target gene, susceptible to silencing, leads to the production of a diffusible activator that increases steady-state mRNA levels of the target gene. Expression of the target gene therefore depends on the concentration of the activator in a positive feedback loop. The model suggests that activator synthesis depends on the transcription rates of the target gene and that activator degradation is proportional to activator concentration. The activator will also stabilize mRNA levels of transgenes that are homologous to the endogenous target gene. Transcription of homologous transgenes will enhance activator concentrations. In this model, the activator therefore mediates the linked stabilization or repression of its target gene and a homologous transgene. The system is stable, when synthesis rates equal the degradation rates of the activator. Variations in transcription rates, however, will induce instabilities in the feedback control system. Development-dependent changes in transcription rates would increase the concentration of activator molecules, which would in turn enhance the activator degradation system. When transcription rates decline again, the high degradation rates would rapidly reduce the number of activator molecules, thus decreasing steady-state mRNA levels of the target gene and the homologous transgene. Steady-state mRNA levels could recover when low activator concentrations raise again. The postulation of activator molecules could explain the developmental modulation and resetting effect of certain cosuppression systems.

ANTISENSE-MEDIATED RNA DEGRADATION An obvious element to account for the sequence specificity of cosuppression is the production of antisense RNA. RNA duplexes would be targets for RNAseH-like endogenous enzymes. Antisense transcripts could be generated by promoters present on the transgene DNA or by endogenous plant promoters at the 3′ end of the transgene (47). Alternatively, they could be produced by a plant RNA-dependent RNA polymerase (39, 69). If antisense RNA is only produced at particular developmental stages or if promoters located 3′ to the silenced gene are regulated by environmental stimuli, this would explain the developmental and environmental dependence of certain silencing phenomena.

The production of antisense molecules by an RNA-dependent RNA polymerase may depend on the production of specific threshold levels of sense RNA or on the accumulation of RNA intermediates during a delay in RNA transport or processing (38, 61). This hypothesis assumes that RNA-dependent RNA polymerases recognize “aberrant” transcripts, which may derive from incorrect transcription, transport, or translation of the transgene. The production of aberrant RNA may be modulated by changes in epigenetic states of a gene that influence the mode or efficiency of RNA processing (38).

Certain transgenes only cosuppress sequences that contain a homologous 3′ end, whereas genes only homologous to the 5′ region are not affected (J English, unpublished data). This observation suggests that antisense transcripts are preferentially made against the 3′ end region of the transgene. On the other hand, constructs that contained the 5′ end of one gene and a second gene at the 3′ end, efficiently silenced both endogenous genes, which argues against a general function of the 3′ end (119). Nevertheless, specific modifications at the 3′ end have been detected, such as the accumulation of processing intermediates (J Kooter, unpublished data) and incorrect splicing within the 3′ end of silenced transcripts (D Flavell and M Metzlaff, unpublished data).

## CELLULAR MECHANISMS INVOLVED IN HOMOLOGY-DEPENDENT SILENCING

Many aspects of the models listed above are still speculative, and in cases where particular molecular features, such as hypermethylation or high transcription rates, have been associated with silencing events, their general importance is still unclear. It would be premature and possibly detrimental to favor one common model for the many different silencing events, because we would narrow the scope of our investigations. From the experimental details published for various gene silencing systems we can draw two important conclusions. First, the growing number of reports on transgene silencing no longer correspond with the early assessment that we are dealing with a few rare events of minor importance. Second, gene silencing was not specifically developed for transgenes, but it reflects endogenous functions that most likely participate in the regulation of gene expression and plant development.

At present, we can formulate three major areas for future research activities: the function of chromatin in a dynamic regulatory system in plant development, the control of RNA turnover within RNA processing routes that are involved in the fine tuning of gene expression, and the importance of a homology-detection mechanism for gene expression and genome organization. We discuss the general importance of these three aspects for the regulation of gene expression in plants and other eukaryotes. We do not know whether and which of the mechanisms that have been found in other eukaryotes are also relevant

for plants. Nevertheless, the examples shown below should be helpful to define primary models for mechanisms of homology-dependent silencing in plants, and they should explain why we think that an improvement of our knowledge in the three areas mentioned above might be necessary to understand the control of gene expression in plants.

## *The Regulatory Function of Chromatin States*

It has been proposed that epigenetic patterns can be established not only by DNA methylation (54) but also by supramolecular chromatin structures (136). Our present knowledge about the formation and control of different states of chromatin in plants is still very limited. Assumptions that changes in expression patterns are based on modifications of chromatin conformation are mainly grounded in the indirect evidence that changes in DNA methylation occur and that dense methylation patterns induce the formation of highly packed chromatin (8).

The most advanced studies on the role of chromatin structure in heritable gene repression come from *Drosophila* and yeast, two species that lack C-methylation and that have proven especially suitable for chromatin studies. Their small genomes simplify the analysis of individual genomic regions and genetic analysis has identified modifiers of chromatin complexes. Yeast offers the advantages of rapid generation of mutants, easy physiological analysis, and gene replacement by homologous recombination. The polytene chromosomes in the salivary gland of *Drosophila* allow a precise localization of chromatin complexes.

POSITION-EFFECT VARIEGATION Position-effect variegation (PEV) is a partial inactivation of gene expression in *Drosophila* caused by a rearrangement that places a normally euchromatic gene near a heterochromatic region (51, 106). PEV at the *white* locus, involved in eye color, can be monitored in individual cells by the reduction of red eye pigment. Inactivation of the gene, which results from the spreading of the heterochromatic state into the euchromatic neighborhood, causes a mosaic phenotype of red and white cells that gave PEV its name (124). PEV demonstrates the differences between two kinds of chromatin: heterochromatin, which is located within the pericentric regions and which remains condensed throughout the cell cycle, and euchromatin, which is located in the chromosome arms and decondenses during interphase. Inactivation of a gene is accompanied by cytologically visible spreading of heterochromatin over 50–100 polytene bands, corresponding to hundreds of kilobases. The *Drosophila* genome consists of more than 100 loci that suppress or enhance PEV, some of which have been characterized to encode chromosomal proteins (113). A set of heterochromatin-associated genes, the products of the *Su(var)* genes, assemble cooperatively to form complexes in heterochromatin regions. These protein

complexes can continue to expand. Complex formation requires the interaction of large amounts of different gene products, and insufficiency of one of the *Su(var)* genes reduces the spreading of heterochromatin (70).

REPRESSION MEDIATED BY MEMBERS OF THE POLYCOMB GROUP Another example for the control of gene activity by large chromosomal complexes is the regulation of homeotic gene expression. During early embryogenesis, the maternal and segmentation gene products catalyze the assembly of inhibitory proteins of the Polycomb group (Pc-G) and activating proteins of the trithorax group (trx-G) at homeotic gene loci. Patterns of chromatin conformation, mediated by Pc-G and trx-G proteins, are mitotically transmitted and provide the basis for differential expression of homeotic genes along the anterior-posterior axis (98). Pc-G proteins can be localized at specific regions on polytene chromosomes, which implies that the complexes recognize specific regions or secondary structures. Most likely, there exists a molecular relationship between *Pc-G* genes and *Su(var)* genes, because some mutations in *Pc-G* genes affect PEV, and certain *Su(var)* genes influence *Pc-G*-mediated effects (35). Moreover, the Pc protein shares an amino-acid sequence, called the chromodomain, with the Su(var)3-9 product, the heterochromatin protein HP1 (99).

CHROMATIN-MEDIATED REPRESSION IN YEAST In yeast, at least two modes of transcriptional repression are mediated by chromatin conformation: (*a*) repression by the global regulator complex Ssn6/Tup1 (111), and (*b*) silencing of the yeast mating type loci (107) and genes at telomeres (96). The gene products of the *TUP1* gene and the *SSN6* gene are physically associated in a large protein complex, required for repression of cell type-specific genes and genes repressed by glucose or oxygen, respectively. The complex does not bind to DNA directly but is targeted to particular promoters via protein-protein interactions with specific promoter binding proteins. In this interaction, Tup1 provides the repressor activity and Ssn6 the targeting function (127). Repression by Ssn6/Tup1 is mediated by organizing repressed chromatin domains, possibly through interactions with histone H4 (111). Silencing at the yeast mating type loci, HML and HMR, and at telomeres is also regulated by the creation of a defined chromatin structure. Formation of a silencing chromatin structure is mediated by an interaction of the Sir proteins and their interaction with histones H3 and H4 (125). Targeting of the complex to specific regions is mediated by the origin-recognition complex protein (ORC), the Rap1 protein, and the Abf1 protein (111).

In summary, we can define three important aspects for chromatin-mediated gene repression: the formation of heterochromatin-related protein complexes, the targeting of these complexes to particular locations, and the role of histones as modulators for the formation of certain complexes. Not all three

aspects may be relevant for potential chromatin-mediated silencing effects in plants, but a search for modifier functions of silencing, either by mutagenesis (27) or by searching for plant proteins that share common domains with known regulators of silencing in *Drosophila* or yeast, might clarify whether chromatin-mediated repression is a universal feature in eukaryotes. Encouraging support for this assumption comes from a report about the identification of the murine *bmi-1* gene, a homologue of the *Pc-G* gene *Posterior sex combs* gene. Mice deficient for *bmi-1* show multiple posterior-directed homeotic transformations (130), caused by ectopic expression of genes of the HOX4 cluster (95).

A ROLE FOR CHROMATIN-MEDIATED REPRESSION IN HOMOLOGY-DEPENDENT SILENCING The formation of chromatin states can be relevant for two aspects of homology-dependent silencing mechanisms, the *trans*-inactivation of homologous sequences and the developmental regulation of silencing. Even a transient pairing of two homologous sequences could favor the exchange of chromatin components that have formed a repressed complex on one copy. Thus, silencing would be the result of the establishment of a repressed chromatin state in the transgene region, some part of which is transferred to other homologous transgenes or endogenes genes. Support for this assumption comes from the observations that multiple transgene copies are preferentially silenced and that transgenes can be specific targets for DNA methylation, which is associated with chromatin condensation (81). It has been proposed that DNA methylation acts as a defense mechanism against foreign DNA (7, 29). Plant genes have a relatively narrow range of AT-content and are embedded into 200-kb large chromosomal regions of a matching AT-content, termed isochores. Monocotyledonous and dicotyledonous species contain distinct isochore compositions (112). Therefore, transgenes with deviant base compositions may become specific targets for de novo methylation. A possible case of this occurs when a single copy of the GC-rich *A1*-gene from maize can become specifically methylated in transgenic *Petunia* (82), whereas its homologue, with a GC-content similar to *Petunia,* from *Gerbera* remains unmethylated (34). Because chromosomal integration sites differ somewhat in base composition, transgenes will become methylated with different efficiencies at different sites, and certain transgenes will not be inactivated at all (26). It is possible that the insertion of multiple copies will increase the probability of individual transgenes being methylated and condensed heterochromatin because the entire region is different from a typical isochore. Furthermore, multiple transgenes integrated in tandem or as inverted repeats might enhance the formation of condensed chromosomal complexes, if they provide target chromatin-associated factors, similar to repeat-induced heterochromatinization processes in *Drosophila.* Transient interactions of transgene sequences inserted at different chromosomal locations would then

induce a spread of condensed chromatin states, which explains the preferential inactivation of multiple transgene copies, even at dispersed sites.

CHROMATIN-MEDIATED REGULATION OF CHROMATIN STATES The chromatin conformation may be regularly modified during development and could also be influenced by environmental conditions. Any change could influence transcriptional activity of the gene, the efficiency of RNA transport, or the competence of the locus for somatic pairing with homologous sequences. Any of these features could explain the developmental regulation and the environmental dependence of some homology-based silencing phenomena.

It has been suggested (61) that cosuppression of CHS genes in *Petunia* reflects developmental and physiological factors that impose heritable metastable changes in the plant genome. This idea was deduced from models developed from the analysis of transposable elements in plants (12, 77). Brink proposed that the genome possesses a *paragenetic* function that is distinct from its genetic function. Based on the concept of paramutation, he defines an *orthochromatin* that harbors the DNA that is subject to mutations and a *parachromatin* that is sensitive to the cellular environment and capable of receiving, recording, and mitotically transmitting information from outside the chromosome. During development, changes in *parachromatin* would therefore condition differential activities of genetic loci in the *orthochromatin* (12). A similar concept is found in Mc Clintock's interpretation of epiallelic states of transposable elements. She observed that individual epialleles of an element showed characteristic *phases* of activity, which could be influenced by the expression of other elements. This *presetting* effect was mitotically transmitted but erased in the next generation. She proposed that individual genes are embedded or dissociated from condensed chromatin clusters in a regular manner, which regulates their differential expression during development (77). If extracellular signals induce changes in paragenetic states, this could also modify the position and association of genes within such clusters of a cell and its somatic derivatives.

It is unknown which molecular factors regulate changes in chromatin structure, but it has been suggested that particular chromatin states are generated or conserved by changes in DNA methylation (81). Evidence that DNA methylation is involved in the determination and modulation of epigenetic states comes from the analysis of the *En/Spm* transposable element in maize (36). This autonomous element can exist in three distinct but interconvertible forms termed cryptic, programmable, and active. These forms can be distinguished by the methylation levels of GC-rich sequences in the downstream control region (DCR), near the promotor. Cryptic elements are almost stably inactive and exhibit somatic reversion frequencies to active states of $10^{-5}$: They are highly methylated. Active elements are unmethylated. Programmable elements

that revert more frequently than cryptic elements and that can be *trans*-activated by an active element have an intermediate methylation state. The modulation of epigenetic states is mediated by TpnA, an autoregulatory protein encoded by the element. TpnA has three distinct functions. It is required for transposition of the element (41), it activates methylated promoters of programmable elements, and it represses the unmethylated promoter of active elements (115).

Besides the determination of their phases of activity, transposable elements are regulated by a developmental control mechanism that determines the heritability of the phases and the activity of the elements during development. Therefore, changes in the epigenetic state influence the activity of the element in the next generation (37). A related observation has been made for the activity of an *A1* transgene in *Petunia* that was also correlated with changes in DNA methylation. In an F1 progeny from one transgenic line homozygous for *A1,* plants derived from pollination of the first flowers showed a significantly more stable expression of the marker gene, while in progeny from pollination of older flowers the transgene became inactivated and methylated at high frequencies (84). These data also suggest a developmental regulation of epigenetic patterns that can be transferred to the next generation.

## *Posttranscriptional Control of Gene Expression*

Posttranscriptional regulatory mechanisms have an important function in the control of gene expression (43, 52). The efficiency at which a gene will be expressed depends on mRNA processing, transcript stability, nucleocytoplasmic transport, translation efficiency, and protein modification and half life. We do not discuss these items in detail but concentrate on a few examples that indicate how changes in RNA stability might be involved in gene silencing.

RNA TRANSPORT In recent years several partly contradictory models have emerged about the transport of RNA from the site of transcription to the cytoplasm. In analogy to the established model of an organized movement of newly synthesized polypeptides through the cytoplasmic secretion machinery (105), it was proposed that transcripts move to the cytoplasm in an ordered fashion, passing through localized spots that harbor individual steps of the processing machinery (16, 139). This model is supported by reports of the localization of splicing components in subnuclear domains, called speckles and foci, and by observations that intron-containing RNAs are targeted to speckles upon microinjection into mammalian nuclei (122).

In contrast with these reports, other studies argue against a compartmentalization of RNA processing, because splicing occurred at the sites of transcription. These sites were not coincident with intranuclear speckles that harbor components of the splicing machinery (142). Assuming that the position of

certain genes within the interphase nucleus determines the entrance of the transcript into defined processing routes, transcripts of multiple transgenes localized at different positions may enter common or separate processing routes. Local concentrations of homologous transcripts would be enhanced significantly, if transcripts of the transgene and the homologous endogenous gene pass the same processing track. We therefore not only have to consider the general quantities of steady-state RNA levels within the nucleus but also have to account for the local concentrations of homologous RNA molecules within the processing track.

RNA STABILITY RNA stability is influenced by a number of factors. The 5′ cap structure and the 3′ poly(A) tail stabilize mRNA against degradation. The poly(A) tail also regulates the efficiency of translation, but only when the transcript is capped (42). Other posttranscriptional modifications have been proposed to serve as signals for degradation. Adenine residues can be methylated or converted into inosines that might serve as a tag for RNA degradation (64). Destabilizing and stabilizing sequence elements have been identified in specific mRNAs. These elements either provide target sequences for RNA degradation (92) or binding sites for stabilizing factors (17).

RNA stability is also influenced by the efficiency of translation. An interesting linkage between translation efficiency and mRNA metabolism has been detected for the human β-39 mRNA, a mutation of the β-globin gene that carries a stop codon at position 39 (6). The nontranslatability of the β-39 mRNA induced a significant reduction in mRNA accumulation, although transcription, splicing, and polyadenylation of the β-39 mRNA are not altered. This observation prompted the ideas that either a nuclear mechanism exists that is capable of sensing nonsense mutations or that there is a feedback communication from the cytoplasm to the nucleus. It has been suggested that this feedback interaction occurs at the nuclear membrane at points of contact with the rough endoplasmatic reticulum, where translation can occur (6).

PRODUCTION OF ANTISENSE RNA As mentioned earlier, it has been proposed that antisense transcripts are involved in posttranscriptional silencing. Antisense transcripts could be produced by promoters located in the 3′ region of a gene or by an RNA-dependent RNA polymerase. Several examples exist for the use of endogenous antisense transcripts for transcriptional (94) or posttranscriptional (53, 55) control of expression. In barley, a lack of alpha-amylase expression coincides with the appearance of a transcript complementary to the alpha-amylase mRNA (108). RNA-dependent RNA polymerases are widely distributed among plants, although there has been a dispute about the possible contamination of the material by viral RNA polymerases (39). These enzymes are usually present in low amounts, which can be significantly increased upon viral infec-

tion. The enzymes in different plants are clearly distinctive in size and template specificity. The host-specificity is conserved after induction of higher enzyme levels by infections with the same virus, and this supports the hypothesis that the enzymes are not derived from the virus but are encoded by the host plant. The biological role of RNA-dependent RNA polymerases has not been fully elucidated, but it is obvious that they can create antisense molecules against existing cellular transcripts.

To evaluate the importance of posttranscriptional control mechanisms for silencing we still need to answer several key questions. We do not know whether transcription, translation, or polysome-association is a prerequisite for silencing. We need to define what determines the "aberrant" state of RNA and whether this induces RNA degradation or the production of antisense transcripts. We also need to understand how transcripts are transported into the cytoplasm and whether and which factors exist that induce feedback responses at the DNA level when RNA processing or translation is disturbed.

## *Repeat-Specific Control Mechanisms*

The participation of repeated sequences in gene inactivation phenomena is not limited to plants but can be found in several other eukaryotes. A comparison of homology-dependent silencing in plants with other eukaryotic silencing systems illuminates some interesting similarities that suggest common biological functions.

THE RIP- AND MIP-MECHANISMS OF FILAMENTOUS FUNGI In the filamentous fungi *Neurospora crassa* and *Ascobulus immersus,* the presence of DNA repeats triggers methylation and inactivation of the repeated regions. In *Neurospora* transformants that contain linked or unlinked duplicated sequences, a mechanism named Repeat Induced Point Mutation (RIP) induces methylation of C-residues followed by mutation of C to T, preferentially at CA dinucleotides (14, 117). In *Ascobolus,* gene duplication leads to de novo methylation and premeiotic inactivation because of a mechanism termed Methylation Induced Premeiotically (MIP) (45). The efficiency of MIP depends on a gene's location and on the length of homologous repeats. Clustered repeats of a critical length always become methylated, whereas efficiencies for ectopic homologues vary considerably (110). Methylation not only encompasses C residues within CpG dinucleotides, it also extends to C residues located in nonsymmetrical sequences, which implies a novel type of methyltransferase activity (46). Similar nonsymmetrical 5mC patterns have been observed for sequences that had undergone RIP in *Neurospora* (118) and for plant transgenes that had become transcriptionally (85) or posttranscriptionally silenced (57), respectively.

An inverse correlation between copy number and the expression of transgenes was also shown for the asexual cycle of *Neurospora* (97). After transfor-

mation with a resistance marker, vegetative and reversible inactivation of the marker occurred in multicopy transformants. Inactivation was accompanied by hypermethylation of marker genes. Treatment with 5-azacytidine induced a stable reactivation of the marker in some, but not all, transformants, which suggests a functional impact of DNA methylation on gene expression. Methylation might also be involved in a unidirectional silencing event in *Neurospora,* termed quelling (109). The expression of endogenous genes was impaired when several homologous copies were integrated at an ectopic site. Reversion of the quelling effect was correlated with a reduction in the number of ectopically integrated gene fragments. These data demonstrate that *Neurospora* and *Ascobolus* contain homology-searching mechanisms responsible for the specific methylation of repeated sequences. These show some similarities to silencing phenomena in plants. Because filamentous fungi are excellent subjects for mutation analysis, it is very likely that endogenous genes involved in the regulation of silencing will be first identified in *Neurospora* or *Ascobolus* before they are found in plants.

HOMOLOGY-MEDIATED REPRESSION IN *DROSOPHILA* In *Drosophila,* repeated sequences are involved in the initiation of position-effect variegation, in *trans*-effects of PEV, and in transvection. PEV-like effects were induced when tandemly linked copies of a P-transposon carrying a *white* transgene were integrated into the *Drosophila* genome (31), which suggests that pairing of repeats contributes to heterochromatin formation. Homologous chromosomes of nondividing nuclei are physically paired in somatic cells of *Drosophila,* providing the basis for the transmission of heterochromatin states to homologous alleles. One example for pairing-dependent *trans*-inactivation is dominant PEV at the *brown* locus. Heterochromatin that was imposed on a rearranged *brown* allele was transmitted to the unrearranged homologous copy, which also became inactivated (32).

Other pairing-dependent modulations of gene expression have been described under the term transvection (68). Like paramutation, transvection depends on the interaction of susceptible alleles, but the effect is not preserved after segregation of the interacting alleles during meiosis. A well-characterized example for allelic interaction is the regulation of the *white* gene by the product of the *zeste* gene (103). The *white* gene carries a set of *zeste* binding sites in its promotor, in the eye enhancer region. A lack of *zeste* function leads to a moderate decrease in *white* activity. The $z^1$ mutation of *zeste* is responsible for the *zeste* product forming hyperaggregates (20), and this causes severe repression in lines that contain two paired copies of *white.* Repression is not observed for single *white* copies, but if a homologous *white* gene, together with the *zeste* binding sites, is inserted at ectopic positions, about one third of these lines show repression in a $z^1$ background. This observation is interpreted as indicating that specific loci can interact with the *white* locus to form $z^1$-spe-

cific aggregates. *Zeste-white* interactions are further modified by several genetic loci, most of which are members of the *polycomb group.*

REPEAT-INDUCED DNA METHYLATION IN MAMMALS In contrast to the large number of repeat-induced inactivation events found in transgenic plants, it has been unclear whether a similar mechanism exists in mammals. Sense inactivation of an endogenous gene (15) and an inverse correlation between the number of transgene copies and their methylation state have been reported (78). Because such events are rare, it was doubtful whether these events reflected the presence of a homology-search mechanism as has been postulated for plants and fungi.

More compelling evidence that repeat-induced inactivation is also present in mammals came from the analysis of human genetic diseases associated with the amplification of triplet repeats (67). Most of these genes, which encode transcription factors, carry multimers of CAG- or CCG-triplets that are responsible for poly-glutamine or poly-proline stretches within the coding sequence. The triplet repeats are variable in length and can expand or contract in somatic cells or when passed to the next generation. As the repeat expansion is not uniform, individuals carry a mosaic pattern; individual cells harbor various repeat lengths in the affected gene (102). Excessive repeat amplification causes disease. The onset of disease is developmentally regulated; for most diseases there seems to be an inverse relation between the number of triplet repeats and the age at which the first symptoms of the disease become manifest (56, 126). Because the probability and the length of repeat extension significantly increase in cells that show deficiencies in DNA repair (1), it is unclear whether diseases are caused by a defective mismatch repair or whether the repeat elements are causally involved. For triplets located within protein-coding regions, repeat amplification results in a significant increase in the length of the single amino acid stretches. It has been shown that long poly-glutamine stretches reduce the activity of transcription factors (18), and the disease phenotype could reflect a lack of function of the protein. Amplified triplets are not restricted to protein-coding regions, however, but are found in the 5′ noncoding region (141) or in 3′ untranslated DNA of some genes (138). For these diseases, the repeat-amplification does not affect protein function but transcription. The expansion of triplet repeats in the *FMR1* gene (140) actually leads to a reduction of gene expression. The increase in CGG repeats makes the CpG island-type promoter of the FMR1 gene, which is normally unmethylated, susceptible to methylation. The level of methylation is not uniform. Individuals have different levels and the mental retardation phenotype correlates with the extent of methylation (71). In analogy to repeat-induced heterochromatinization in *Drosophila* (31), it is tempting to speculate that amplification of triplet repeats in humans induces heterochromatin formation, which is induced or followed by cytosine methylation, converting the gene into condensed and transcriptionally impaired chromatin.

Indirect evidence for the existence of a mechanism for homology-based DNA methylation came from a study of the distribution of CpG dinucleotides in the mammalian genome (66). Based on the criterion that CpG depletion and TpG overrepresentation for a particular genomic region indicates a prior history of high methylation, repetitive sequences were identified as preferred targets for methylation. These data suggest that sequence repeats are specifically recognized to become methylated. This would not only affect highly repetitive DNA but also homologous members of gene families and pseudogenes. It was proposed (66) that the parental sequences are protected from the methylation mechanism, because the insertion of introns into their sequence masks the sequence homology with their pseudogenes.

A ROLE FOR REPEAT-INDUCED METHYLATION IN GENOME EVOLUTION If we consider that many plant genomes carry a large proportion of duplicated loci (137), homology-dependent silencing mechanisms should influence the expression of endogenous duplicated genes. It is conceivable that duplicated sequences escape silencing if they are embedded in noninteracting chromosomal environments or if they contain a significant degree of sequence divergence. In this respect, the presence and efficiency of repeat-dependent silencing mechanisms should influence the potential of the plant genome to develop new allelic variations during evolution. In this context, repeat-induced methylation counterbalances DNA amplification processes generating heterogeneous epigenetic patterns in repeated sequences that can be further modulated by environmental stress. In mammalian tissue cultures, certain resistance genes can be amplified under selection pressure (114). In plants, the most dramatic examples for DNA amplification are the environmentally induced morphological changes in flax. Flax plants treated with high levels of fertilizers amplified specific genomic subsets (23). It has been proposed (58) that changes in heterochromatin content and DNA methylation are also associated with this phenomenon. Epigenetic states are also responsive to changing environmental conditions as demonstrated by the activation of transposable elements (133, 134) or the change of DNA methylation patterns in tissue culture (62) or in field-grown plants (84). A combination of environmentally regulated mechanisms of gene amplification and epimutation would provide the cell with an efficient system to adapt to changing external conditions. Because of the stochastic character of amplification and epigenetic modification mechanisms, the plant would be a chimera of distinctive somatic sectors subject to selection. Novel phenotypes of the genome could be manifested but also corrected in subsequent generations. In mammals the separation of germline and somatic cells, as well as the high degree of complexity of differentiated cells, puts severe constraints on the efficiency of such a mechanism. For plants cells, however, totipolence and the production of germ cells late in somatic development, a balanced activity of amplification and epimutation should have a considerable evolutionary advantage.

## CONCLUSIONS AND OUTLOOK

The term homology-dependent silencing refers to inactivation events at the transcriptional or posttranscriptional level that differ in efficiency, resetting, and heritability. Besides the requirement for homology, a common feature of all homology-dependent inactivation events is the disturbance of the sequence context because of DNA rearrangements. This applies for the inactivation of transgenes that integrate randomly into the genome via illegitimate recombination and for nontransgenic silencing events such as transposon inactivation and paramutation. On the other hand, not every illegitimately recombined transgene is subject to silencing, which suggests that the chromosomal location plays an important role. The efficient silencing activity of certain loci may result from a particular sequence context, a specific DNA rearrangement, or a secondary structure. Alternatively, the position of a locus within the nucleus may determine the efficiency of homology pairing or RNA processing.

To clarify the underlying mechanisms of gene silencing and to understand their importance in the regulation of plant development, we must expand our very limited knowledge about the location of genes within the nucleus, the factors involved in chromatin conformation, and the regulation of transcript transport and processing. Finally, we have to understand how developmental programs and environmental conditions influence the formation of epigenetic patterns at the DNA level and whether there are feedback control signals in the RNA processing pathway. Most certainly, we can expect more than one mechanism of gene silencing to exist, and it is unlikely that the silencing phenomena reported so far already represent a complete selection. For the scientific community, it will be rewarding to note and pursue silencing events with greater emphasis than in previous years, when silenced genes were out of the scope of many scientists. Understanding the molecular basis of gene silencing will not only improve the control of the application of transgene technology, it will most likely unveil new endogenous control mechanisms involved in the regulation of plant development and genome evolution.

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