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Gene inactivation triggered by recognition between DNA repeats

J.-L. Rossignol and G. Faugeron

Institut de Génétique et Microbiologie, CNRS URA 1354, Bât. 400, Université Paris-Sud, F-91405 Orsay Cedex (France)

Abstract. This chapter focuses on phenomena of gene inactivation resulting from the presence of repeated gene copies within the genome of plants and fungi, and on their possible relationships to homologous DNA-DNA interactions. Emphasis is given to two related premeiotic processes: Methylation Induced Premeiotically (MIP) and Repeat-Induced Point mutation (RIP) which take place in the fungi *Ascobolus immersus* and *Neurospora crassa*, respectively. The relationships between these processes and genetic recombination are discussed.
Key words. Cytosine methylation; DNA/DNA interactions; gene silencing; point mutations; premeiotic inactivation.

Recognition in trans between complementary nucleotide sequences is used in a variety of biological processes. Whereas RNA splicing and RNA editing involve a direct recognition between complementary bases, a more complex recognition process is required by homologous recombination which involves two parental DNA duplex molecules. Hydrogen bondings between the two complementary strands within each duplex prevent direct interactions between one strand in one duplex and the complementary strand in the other duplex. Part of this issue is devoted to the study of the enzymatic machinery that leads to the formation and maturation of recombination intermediates in which the homologous DNA sequences of the two partners are put into register to give faithful recombination products (see the articles by Stasiak and Egelman, and Heyer in this issue). The cytological pairing of homologous chromosomes is treated by the contribution of Loidl.

This chapter is devoted to another group of biological processes that are triggered by the presence of DNA repeats and lead to the inactivation of genes harbored by these repeats. Homologous DNA pairing has been shown to be necessary for two, probably closely related, processes occurring in some fungi. These processes have been named 'Repeat-Induced Point mutation' (RIP; ref. 52) and 'Methylation Induced Premeiotically' (MIP; ref. 48), respectively. We will present RIP and MIP, together with the arguments showing that they depend on a preliminary DNA-DNA pairing step.

We will also briefly discuss other phenomena, in a variety of eukaryotic organisms (mainly plants and fungi), that also lead to the silencing of genes associated with the presence of DNA repeats.

Possible relationships between repeat-induced gene silencing phenomena and genetic recombination will be discussed.

I. MIP and RIP

These two related processes have been found in two different species of fungi.

RIP results in point-mutations and typically in C-methylation of duplicated sequences

RIP was discovered by Selker and colleagues[54] in a study of *Neurospora* transformants obtained via the integration of one copy of a plasmid which shared homology with the host strain for only 6kb of one uncharacterized sequence, referred to as *'flank'*. The *flank* segment was present as a tandem duplication after homologous integration of this plasmid, or as an unlinked duplication after nonhomologous integration. In the sexual progeny of such transformants, both copies of the duplicated sequences were subject to radical alterations[54], detected by gains and losses of restriction sites. Alterations of tandem duplications were observed in 100% of the progeny, whereas alterations of unlinked duplications were observed in about 50% of it. These studies and subsequent DNA sequence analyses[5] showed that these alterations corresponded to numerous C-G to T-A transitions and, typically, to methylation of cytosine residues. A detailed study of two altered duplications showed mutation of between 10% and 50% of the C-G base pairs, with a strong preference for the mutation of C belonging to CpA dinucleotides.

MIP results in C-methylation of duplicated sequences

The phenomenon which was later named MIP was first observed in *Ascobolus* by Goyon and Faugeron[14], who studied transformants harboring a 5.7 kb duplication of the *met2* gene obtained by homologous integration of a plasmid harboring the *met2*$^+$ wild-type allele in a recipient strain harboring the mutant *met2*$^-$ allele. After sexual reproduction, more than 90% of the progeny had lost the expected Met$^+$ phenotype. Genetic analysis of the Met$^-$ derivatives showed that they could revert to prototrophy after vegetative growth in selective conditions. Southern analysis confirmed that the integrated transforming DNA was still present, and indicated that the duplication had undergone C-methylation. Further study[48] of transformants having the *met2*$^+$ gene duplicated in either a tandem array, following homologous

integration, or in unlinked positions following non-homologous integration, showed that both *met2*$^+$ genes harbored by the two elements of the duplication were reversibly silenced (this was shown, without any exception, on several hundred inactivated progeny). Direct DNA sequence analysis showed unambiguously that no mutation was associated with the inactivation. This demonstrated that this process called MIP is different from RIP. Southern analysis using pairs of restriction enzymes that cut the same restriction site but which are either sensitive or insensitive to C-methylation showed that the inactivated genes displayed heavy C-methylation, not restricted to CpG or CpNpG di- and trinucleotides. As in *Neurospora*, the phenomenon occurred with a 100% efficiency with tandem duplications and, on average, with a 50% efficiency with unlinked duplications.

Thus, the gene inactivation processes occurring in *Neurospora* and *Ascobolus* show different outcomes: point mutations corresponding to C-G to T-A transitions associated with C-methylation in *Neurospora*, and only C-methylation, accompanied by stable – although reversible – silencing in *Ascobolus*. In both cases these alterations are confined, at least in general, to the duplicated segments. Both phenomena occur in the sexual cycle and both involve tandem or unlinked duplications. In both organisms, the phenomena were observed with various duplicated DNA segments including both endogenous and foreign genes (see 52, 9 for reviews).

MIP and RIP occur premeiotically

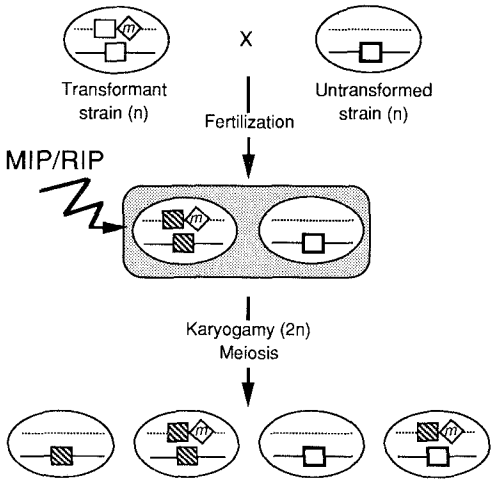
Selker et al.[54] took advantage of the observation that each RIP event led to a different restriction-enzyme cutting pattern to determine the timing of the process operating on a tandem duplication. Analysis of the four metiotic products of individual meioses indicated that the two replication products resulting from premeitoic DNA synthesis almost always display the same pattern of alteration, indicating that the mutations occur before premeiotic DNA replication. Analysis of tetrads issuing from the same perithecium showed that two tetrads could display either the same alteration pattern, showing that the event had occurred in a common ancestor, or different patterns, showing that the event occurred after fertilization, since each perithecium is issued from a single fertilization event. Thus RIP occurs between fertilization and karyogamy in the dicaryotic cells formed after fertilization. These cells, which contain both parental haploid nuclei, undergo a small number of divisions before karyogamy[8]. RIP, the acronym proposed to design this process, first meant 'Rearrangement Induced Premeiotically'[54]. The meaning of the acronym was changed to 'Repeat-Induced Point mutation' when it became clear that the 'rearrangements' of restriction sites are due to mutations[5].

In contrast to the situation in *Neurospora*, the events affecting a given duplication in *Ascobolus* are not distinguishable by restriction analysis. However, the characteristics of MIP strongly suggest that it occurs at the same time as RIP. Indeed, MIP is observed only after meiosis, and not during vegetative growth of the transformants[14]. Furthermore, MIP always involves the two copies issued from the replication of each element of the duplication, suggesting that MIP, like RIP, takes place before the premeiotic DNA synthesis. The figure indicates the four possible meiotic products obtained after MIP or RIP in the case of unlinked duplications.

MIP and RIP are triggered by duplications and always involve the two elements of the duplication

Since RIP involved sequence duplications resulting from the introduction of DNA via transformation, it was important to show that it was triggered by the duplication itself rather than by the transforming DNA. Introduction via transformation in a strain deleted for the *am Neurospora* gene of a single copy of this gene, without any bacterial sequence, nor sequence already present in the host, does not lead to RIP, whereas introduction of two or more copies of the same fragment does[55]. In conventional crosses between two strains each having a single copy inserted at unlinked chromosomal locations, the two *am* copies were not subject to RIP. In contrast, RIP occurred when strains having inherited the two unlinked *am* copies were crossed, proving that the presence of more than one copy was the triggering factor. When RIP occurred, it always simultaneously involved the two elements of the duplication.

Similar experiments were carried out in *Ascobolus*[10]. Transformants having integrated a single copy of the foreign gene *amdS* from *Aspergillus nidulans*, together with bacterial vector sequences, displayed perfect stability of transgene expression in crosses with wild type, showing that neither the foreign transgene nor bacterial sequence was responsible for MIP. No inactivation occurred either when two strains having integrated *amdS* at different locations were intercrossed. Such crosses allowed the isolation of strains harboring two unlinked *amdS* copies. When crossed with wild type these strains showed inactivation of both *amdS* copies in about half of the tetrads, and inactivation of neither in the other half. It is noteworthy that the crosses involving two parents each having one of the two copies and the crosses involving one parent with both copies and the other with none all produce after karyogamy diploid nuclei having identical genomes. Therefore, the observed differences regarding inactivation indicate that the two elements of the duplication need to be in the same haploid nucleus, both for RIP and for MIP. This constitutes a further argument that these processes are triggered before karyogamy (fig.).



Schematic representation of the sexual reproduction cycle of *Ascobolus* and *Neurospora*: timing and manifestations of MIP and RIP. Fertilization results in formation of dicaryotic cells containing each of the two parental haploid nuclei (n). These cells will undergo several mitotic divisions, followed by karyogamy. The resulting diploid nuclei (2n) will enter meiosis. The four possible meiotic products are shown. The open box indicates the wild-type allele of the gene that has been duplicated via transformation, *m* indicates the marker used to select transformants. The continuous line indicates the chromosome carrying the resident gene, the dotted line indicates the chromosome having integrated the transgenic copy. Alterations produced by MIP and RIP are indicated by hatches.

Several pairing cycles leading to MIP or RIP may occur in dividing premeiotic cells

An *Ascobolus* strain was constructed that had three unlinked *amdS* copies. Analysis of the products of individual meioses showed that either none, or two, or all three elements of the triplication had been inactivated[10]. Inactivation of only one of the three copies was never found, consistent with the prior results with *Ascobolus* and *Neurospora* strains having just two copies of the gene assayed. Inactivation of the three copies was found as frequently as inactivation of two of them. This result suggests that inactivation occurs via a pairing process, in which one copy undergoes more than one cycle of pairing-inactivation. This implies that a sequence that has already undergone MIP can still efficiently pair with an unmodified homologous sequence and inactivate it. Indeed, in crosses involving strains carrying one (or two) methylated copies and one unmethylated copy, the unmethylated copy could interact with the methylated one and would hence become methylated[10]. This demonstrated that one methylated copy and one unmethylated copy can efficiently interact to trigger MIP, in agreement with the multiple pairing hypothesis.

The results of analyzing multiple products of RIP from a common perithecium first suggested that duplicated sequences can undergo multiple cycles of RIP[54]. This was confirmed by Fincham et al.[11] in a *Neurospora* strain harboring three unlinked copies of *am*. RIP could involve either none or two of the copies, but rarely the three of them. To explain this difference between MIP and RIP, Faugeron et al.[10] proposed that sequence divergence resulting from RIP could reduce the probability that a copy having undergone RIP will interact with a third intact copy, whereas methylation alone, due to MIP, would not decrease the pairing efficiency. This is consistent with the observation that in *Neurospora*, sequences altered by RIP show a decreasing sensitivity to RIP in subsequent generations as a function of the extent of the divergence[10]. As with MIP, RIP is apparently not sensitive to C-methylation: two copies that had suffered little mutation by RIP and showed thus only a weak divergence, but that were methylated as a consequence of RIP, were still prone to RIP. Two copies that have undergone RIP usually show less divergence between themselves than with the original pristine copy[6,15]. This agrees with the observation that duplications could sometimes continue to interact in RIP for several generations, whereas they are less prone to interact with a native copy.

Cambareri et al.[6] also showed that linked duplications accumulated more divergence than unlinked duplications. To explain why tandem copies are usually more highly mutated, they speculated that tandem copies could undergo more frequent pairing, and thus more frequent RIP. If unlinked copies, which are subject to MIP or RIP with a final frequency of about 50%, are able to undergo several pairing cycles in premeiotic cells, then, it makes sense that tandem copies which are subject to MIP or RIP with frequencies close to 100% should undergo even more frequent successive pairing cycles in the dividing premeiotic cells.

Distribution of point mutations and C-methylation along the duplications

Southern analysis of restriction digests and sequence analysis of duplicated DNA segments that had undergone RIP in *Neurospora* showed that C to T transitions were most exclusively confined to the duplicated area. However, a small number of C to T transitions were found in several instances in the unique sequence adjacent to the duplication[12]. Within the duplication, the intensity of mutation appears lighter close to its boundaries than in its middle portion. For example, in the analysis of *flank* DNA, only six changes occurred in the first 120 bp (including 60 C-G) of the duplicated segment, whereas, 83 transitions were quite regularly distributed within the more internal 443 bp (including 224 C-G) sequence, resulting in a 19% sequence divergence[5]. An analysis of a natural tandem duplication of a 800 bp segment (named the ζ–η region), the two elements of which are thought to have diverged through many cycles of RIP, also shows less frequent transitions in the regions of repeated elements the closest to the external borders of the duplication[57].

C-methylation, which is typically associated with C-G to T-A transitions, generally extends to roughly the entire length of the mutated sequence[5]. It can affect most or all cytosines in the region, but however the intensity of methylation is variable among the DNA molecules in the population, resulting in incomplete modification at most if not all sites in the overall DNA population[53]. These observations have been confirmed by direct genomic DNA sequencing (E. U. Selker, pers. commun.).

An interesting observation was that, considering an individual strand, transitions come from C to T and transitions from G to A were not randomly distributed[5]. Selker[52] reported on one case where all the 28 C to T changes occurred in the same strand. This implies that RIP operates by mutating only C to T (or only G to A).

Coextension between duplication length and C-methylation resulting from MIP was extensively documented in *Ascobolus*[3], using strains in which *met2* fragments of different sizes (1.2 to 5.7 kb) had been inserted in positions unlinked to the resident wild-type gene. The resulting duplications, which encompassed distinct parts of the *met2* gene, were all able to trigger MIP (characterized by both inactivation of the resident gene and methylation), even when the duplicated region of *met2* started 1.2 kb downstream from the start codon of the reading frame. Southern analyses showed that the extent of methylation depended on the extent of the duplication. Methylation was coextensive with the length of the duplicated fragment, although with lower intensity close to the boundaries. Direct genomic DNA sequencing of methylated segments (C. Goyon, T. Nogueira and G. Faugeron, unpubl. results) confirmed these results and also indicated that, for a given C, methylation was not maintained in 100% of the cases within the overall population of DNA molecules. Methylation at CpG was maintained best.

That MIP and RIP are coextensive with the length of the duplication means that the extent of these alterations is completely dependent on the length of homology. This constitutes a further strong argument for involvement of a direct DNA-DNA pairing step in these processes.

Minimum duplication size triggering MIP and RIP

What is the minimal size necessary to trigger MIP and RIP? Using smaller and smaller *met2* fragments disposed in tandem with regard to the resident *met2* gene, it was shown in *Ascobolus* that a 450 bp long segment still efficiently triggers methylation of the corresponding segment in the resident gene, whereas no MIP could be detected after duplication of a 320 bp segment (C. Barry, G. Faugeron and J.-L. Rossignol, unpubl. results). A 600 bp unlinked duplication still triggers MIP, but in a low fraction of premeiotic cells. In *Neurospora*, unlinked 1 kb duplications are sufficient to trigger RIP[52]. Spontaneous mutations in the *mtr* gene of *Neurospora*, resulting from tandem duplications of 400–1000 bp, reverted to mtr^+ with a high frequency but became fully stable as soon as they went through a sexual cross, suggesting that they were prone to RIP[62]. DNA sequencing of these mutants indicated that the shortest which showed RIP had a duplication of 446 bp, while the longest duplication which did not show RIP was 380 bp long (D. Stadler, pers. commun.). Altogether, the observations indicate that MIP and RIP may be triggered by small duplications and that the minimal required size is less than 450 bp, which is close to the minimal size of homology required for homologous recombination in mammals[31] and for ectopic recombination in yeast[22].

MIP, RIP and natural repeats

Natural DNA repeats probably constitute the actual target of MIP and RIP. To this regard, the finding in *Neurospora* of the $\zeta-\eta$ tandem duplication[57] is particularly instructive. Each element of this 0.8 kb tandem duplication contains a 5S rDNA gene which has diverged by C-G to T-A transitions from an ancestral 5S rDNA gene. Some *Neurospora* strains do not contain the duplication and have at the same position a 5S rDNA gene, θ, embedded in unique sequences. All but one of the 267 mutations in $\zeta-\eta$ relative to θ are transition mutations polarized such that C-G base pairs in the θ region are T-A base pairs in the $\zeta-\eta$ region. This strongly suggests that this 5S rDNA gene was involved in a tandem duplication and subsequent mutagenesis by RIP. Selker[52] also suggests that RIP could be responsible for the single-copy status of *Neurospora* genes that are represented as small families of identical or closely related genes in other organisms. The few repeated sequences that have been identified in *Neurospora* show evidence of RIP (E. U. Selker, pers. commun.). In *Ascobolus*, three natural repeats were isolated on the basis of the strong hybridization signal they gave when probed with total DNA (C. Goyon, G. Faugeron and J.-L. Rossignol, unpubl. results). Two of them, which are several kb long, correspond to repeated sequences present in more than ten copies in the *Ascobolus* genome and show homology with retroposon and transposon genes. They are strongly methylated as expected if they were subject to MIP. The third one corresponds to 5S rDNA and is not methylated, as in *Neurospora*[58]: this was expected, since the size of this element (about 120 bp) is much smaller than the minimal size required to trigger MIP or RIP.

28S–18S rDNA genes are arranged in tandem array in both *Ascobolus* and *Neurospora*. They are obviously expressed, and thus presumably immune to inactivation by MIP and RIP. Both *Ascobolus* (C. Goyon, G. Faugeron and J.-L. Rossignol, unpubl. results) and *Neurospora*[45] rDNA genes show methylation. The in-

tensity of methylation in the *Ascobolus* rDNA is lower than expected if it were subject to MIP. The basis for the immunity of rDNAs to MIP and RIP is not known, but preliminary experiments in *Neurospora* suggest that the immunity is due to the particular position of the rDNA rather than its nucleotide sequence[52].

MIP and RIP in other fungi

MIP and RIP are not the rule in fungi. They were not found in *Sordaria macrospora*[29] nor *Podospora anserina*[7], two species that are closely related to *Neurospora*, nor in *A. nidulans* (C. Scazzocchio, pers. commun.). The observation of premeiotic de novo C-methylation in haploid nuclei of *Coprinus cinereus* containing either a triplication, a tandem duplication or an ectopic duplication led Freedman and Pukkila[13] to name this phenomenon MIP. In contrast to MIP in *Ascobolus*, methylation in *Coprinus* does not necessarily result in gene inactivation, does not extend on the whole length of the repeat, is weaker and preferentially involves CpGs[13].

MIP and RIP: a unified view

All the evidence accumulated in experiments using *Ascobolus* and *Neurospora* strongly suggest that MIP and RIP constitute closely related processes. Indeed,

- both occur premeiotically. RIP (and probably also MIP) takes place in dicaryotic ascogenous tissues;
- both involve homologous DNA segments larger than a few hundred base pairs;
- both lead to the simultaneous alteration of the two elements of the duplication, and extend almost exactly to its length, suggesting that they involve direct DNA-DNA interaction; and
- both occur with very similar frequencies, and may involve multiple pairing cycles, which might be the rule when the two elements are in tandem array.

The only significant difference between MIP and RIP is the nature of the alteration. To understand the reason for this difference, it is important to know what is the causal relationship between point-mutation and methylation observed in RIP. If MIP and RIP are closely related, it would appear simpler to assume that C-methylation takes place first, and that – in *Neurospora* only – there is a second deamination step transforming the methylated cytosines into thymines; this step should be enzymatically driven to account for the apparent speed of the process. However, no example of the postulated DNA-cytosine deaminase has been described so far. Selker and coworkers showed that the change in sequence resulting from point mutations by RIP itself serves as a signal for de novo methylation in vegetative cells. Sequences altered by RIP and then stripped of their methylation by propagation in *Escherichia coli*, become specifically and reproducibly remethylated in vegetative cells when reintroduced into *Neurospora*, even when the sequence was engineered to be unique in the genome[6,56]. This means that products of RIP bear a portable signal for methylation. Therefore the methylation observed in vegetative cells could be a secondary consequence of RIP. If this explains RIP-associated methylation, it does not account for the origin of RIP-induced point mutations nor for methylation provoked by MIP (since it is not associated with any mutation). However, Selker (pers. commun.) notices that methylation might not only be the consequence, but also the cause of the point-mutation.

Selker proposed another hypothesis, which appears the most attractive[52]. He pointed out that the enzymatic mechanism of C-methylation is thought to go through an intermediate (5,6-dihydrocytosine) that is at least 10^4-fold more prone to spontaneous deamination than cytidine[59]. He suggested that a low level of S-adenosyl methionine (SAM), necessary to complete the reaction from dihydrocytidine to 5-methylcytosine, might prolong the life time of the intermediate and thus result in deamination. If so, a difference in the chemical environment in ascogenous tissues (i.e., a higher concentration of SAM in *Ascobolus*) could be sufficient to account for the different outcomes of MIP and RIP, as was suggested[48]. The finding that HpaII methyltransferase induces a high frequency of C to U transitions (leading to C to T transition mutations after reintroduction in *E. coli*), when DNA is incubated in presence of a low concentration of SAM[61], is consistent with this type of interpretation.

That MIP and RIP strictly operate during a precise step of the respective life cycles of *Ascobolus* and *Neurospora* means that they should reflect tightly regulated developmental processes. Induction of these processes may be related to the recognition between the nuclei of each mating-type, which can proceed only in ascogenous tissues, since both in *Ascobolus* and *Neurospora*, fusion of hyphæ of opposite mating types leads to death during vegetative growth (ref. 44; D. Zickler and H. Jupin, pers. commun.). The two processes must imply a mechanism of search for homology that involves any sequence, or most of the sequences in the genome. They are likely to go through some type of pairing structure which is recognized by a methylation machinery leading directly to C-methylation in *Ascobolus*, and leading to both C to T transitions (possibly through prior C to U deamination) and C-methylation in *Neurospora*.

II. Other examples of Repeat-Induced Gene Silencing in plants and fungi

Many situations have been described in plants which lead to the silencing of transgenes. Although they reflect a large variety of situations, they all result from the introduction of one or several transgenes. For this reason, it was proposed to name them by the common

acronym: RIGS, for 'Repeat-Induced Gene Silencing'[2]. These phenomena have been extensively reviewed[24,25,28,34,35,36] and we therefore give a limited number of examples here.

Multiple integrations and transgene silencing

An inverse correlation between the copy number and the expression of transgenes was described in tobacco[20], in petunia[30] and in *Arabidopsis*[2,40]. Mittelsten Scheid et al.[40] observed that 50% of the homozygous plants transgenic for a *hpt* gene confering hygromycn resistance failed to transmit the resistance phenotype to the progeny. This inactivation was reversible and happened only with plants that contained multicopy (five to ten) integrations. A somewhat similar observation was made in *Neurospora*[43], after transformation with a plasmid harboring a *hph* gene (homologous to *hpt*). In those transformants harboring a single copy of the transgene, the resistance phenotype remained unaltered, whereas several multicopy transformants displayed vegetative and reversible inactivation of resistance (note that this is different from the premeiotic RIP-associated inactivation).

A correlation between the number of integrated copies, the inactivation status and C-methylation was observed in most[2,20,30,43] but not all[40] of these cases. When the relative positions of the multiple integrations were known[20,40], they could be either in clustered or at unlinked sites. In several instances, a decrease in the number of copies was accompanied by transgene reactivation.

Unidirectional trans-silencing

Another type of RIGS is the silencing in trans of one locus triggered by the presence of another unlinked locus. We will call it unidirectional trans-silencing. Matzke and colleagues[32,37,38] transferred two unlinked partially homologous transgene complexes into tobacco, using either two sequential transformation steps, or sexual crossing to combine the interacting transgenes. They found that one transgenic locus (the silenced locus) could be inactivated and methylated by the presence of the other (the silencing locus), which continued to be expressed. The inactivation of the first transgene was dependent on the presence of the second. Indeed, the observed inactivation and methylation of the first transgene was reversed when the two transgenic loci were segregated in the progeny. However, this reversion was gradual, occurring over several generations[33]. In this example, the two interacting transgene loci comprised different genes. If one excludes the homology corresponding to the common T-DNA vector, the stretches of homology corresponded to 300 bp of the nopaline synthase promoter which was present upstream from the two genes constituting the silenced locus and upstream from one of the two genes in the silencing locus.

Another case of trans-silencing was observed in tobacco with a transgenic locus (the silencing locus) carrying two genes, one under the 19S promoter of the cauliflower mosaïc virus (CaMV), and the other under the 35S promoter of the same virus. The genes in the silencing locus were not expressed and they were highly methylated. The expression of a series of genes all different from those harbored by the silencing locus and integrated at different loci was inhibited when they were introduced (either by transformation or by sexual cross) in the strain containing the silencing locus (cf. ref. 69). The silencing effect was observed everytime the genes that would undergo silencing were expressed from the same 19S or 35S CaMV promoter as in the silencing locus, and whatever their location in the genome. Silencing was not observed when the genes were expressed from a promoter different from the CaMV promoters present in the silencing locus. Interestingly, silencing was observed for a transgene under a deleted 35S CaMV promoter, sharing only 90 bp homology with the 35S promoter in the silencing locus. The whole observation suggests that the silencing locus acts as a general silencer for 19S and 35S promoters and imprints a silent state to the transgenes expressed from these promoters. Segregation of the silencing and the silenced loci (through sexual cross) led to a progressive re-expression of silenced genes. This ectopic unidirectional effect is reminiscent of silencing processes which do not typically correspond to RIGS, and where the two interacting copies are allelic: the presence of one inactivated allele (silencing allele) triggers the silencing of the other formerly active allele (silenced allele). This type of process was termed 'paramutation' to describe interactions between two resident allelic genes[4]. More recently, paramutation was observed for a pair of transgenic alleles inserted at homologous positions[39].

Another situation that might be related to unidirectional trans-silencing was described in *Neurospora*[50]. A large proportion of transformants showing an albino phenotype were recovered following transformation of a wild-type strain by fragments of an albino gene. This inactivation of the resident albino gene was associated with the integration at an ectopic site of several copies of the gene fragment. Inactivation was reversible. Reversion was stable (no inactivation occurred in revertants) and was associated with a reduction in the number of integrated gene fragments.

Co-suppression

Interlocus effects are not limited to transgene interactions. There are many examples of interactions between a transgene and homologous resident genes in plants (see 24, 36 for reviews). An intriguing feature of these interactions is that commonly the expression of both the transgene and the resident genes is suppressed, even though the genes are driven by unrelated promoters.

Thus, in contrast with previous trans-silencing phenomena characterized by a unidirectional effect, the transgene and the homologous resident genes mutually interact to silence each other in a coordinate manner. This phenomenon is termed 'co-suppression'. A striking example involves the loss of flower colour in petunia. This was obtained in one instance[41] by introducing a chalcone synthase gene by transformation: in some transformants, both the transgene and the two homologous resident copies were silenced, leading to white flowers. In another case[68] the same phenotype was obtained as the result of the introduction of a transgenic dihydroflavonol reductase gene, leading to the silencing of both the transgenic and the resident copies. Interestingly, revertant sectors were found on some flowers in which both transgenic and resident genes were re-expressed. Furthermore, re-expression was found as soon as the transgene was segregated via sexual cross. Co-suppression has also been found in tobacco[17] and in tomato[63].

Does Repeat-Induced Gene Silencing in plants result from DNA-DNA interactions?

The grouping of RIGS phenomena in categories could be somewhat artificial, because present information is still incomplete. Especially, studies made on different systems do not share the same types of observations, preventing relevant comparisons. Extensive comparative analyses on a limited number of systems is needed to allow unquestionable classification. However, although data are still incomplete, we do not think it is likely that all RIGS phenomena are related by a mechanistic relationship in such a way that the situations described might reflect a continuum of phenotypes. Rather, the emerging picture suggests that RIGS phenomena reflect distinct groups of effects caused by distinct mechanisms. For example, several features distinguish unidirectional trans-silencing and co-suppression. Unidirectional trans-silencing is an asymmetrical process, associated with methylation, where the silenced locus will not recover expression at once after the silencing locus has been segregated away. In contrast, co-suppression is a symmetrical process where the endogenous and transgenic copies are coordinately silenced and reexpressed[23], and which does not seem to be associated with methylation of the silenced copies. Furthermore, some observations suggest that gene expression might be necessary for co-suppression[63], and that inactivation associated with co-suppression could result from a post-transcriptional process (see ref. 24 for review).

Several types of hypotheses (reviewed and discussed in ref. 24) have been proposed to account for these silencing effects. We will discuss here only the hypothesis based on a homology sensing mechanism resulting in recognition between the repeats, and triggering inactivation. Such a hypothesis, inspired by RIP and MIP, was proposed and discussed[24,34,36]. Before discussing this hypothesis, it is important to stress that recognition between DNA repeats may be mediated by two types of interaction: direct DNA-DNA interaction depending on physical homology between repeats (i.e., identical base sequences), or an indirect recognition process that could require functional homology (i.e., two repeats could either not share identical nucleotide sequence, or have too short a length of homology to allow direct DNA-DNA interaction, but may be recognized by the same DNA binding proteins). For example, genes sharing the same promoter could be recognized by an aggregative protein binding to the promoter, leading to their clustering in the same nuclear compartment. Such clustering, in turn, could promote chromatin changes associated with gene inactivation, or gene activation, depending on the location in the particular nuclear compartment. Such a scenario, which may account for some of the unidirectional trans-silencing effects, could be related to the transvection and dominant position effect variegation phenomena described in *Drosophila*[47,19]. That homologous DNA segments could be clustered in the interphasic nucleus is suggested by the observation in mammals that two distinct repeated chromosomal segments which alternate along the chromosome as a consequence of DNA amplification, can be clustered in two distinct sets[67].

We will comment on the possibility often proposed[24,34,36] that some RIGS processes acting in plants are related to MIP/RIP in that they involve a direct DNA-DNA interaction. Two basic observations led to the concept that MIP/RIP really depends on direct homologous DNA-DNA interactions: inactivation always occurs by pairs and there is co-extension between the length of homology and the length of the sequences affected by methylation and point-mutations. Until now, to our knowledge, there is no situation in plants where co-extension could be shown between the length of the repeat and that of an induced DNA-modification accompanying the inactivation. Nor is there a clear example in plants of pairwise inactivation of repeated sequences. For example, in co-suppression, where a direct DNA-DNA interaction is often invoked, no situation has been documented where inactivation would involve the transgene and either one or the other copy of the resident gene. Inactivation in co-suppression actually involves all the homologous copies, which are at least three copies (the two allelic resident copies and the transgenic copy) and possibly more (in polyploid organisms like tobacco). When three copies are present in *Ascobolus* or *Neurospora*, MIP/RIP frequently involves two of them only. If however, co-suppression actually does depend on pairing, this pairing must be permanently maintained, whereas in MIP only transient DNA-DNA pairing is required. Indeed, maintaining inactivation resulting from co-suppression needs the

continuous presence of the transgene, whereas in *Ascobolus*, each copy stays inactivated after the other copy has segregated away. In co-suppression, permanent association via direct DNA-DNA interaction is hardly conceivable if all three copies (the transgene and the two resident copies) must be involved simultaneously.

It is striking that homologies as small as 90 bp (cf. ref. 69) still allow completely efficient trans-silencing. If a direct DNA-DNA pairing process is involved in this case, it should be extraordinarily efficient, as compared to the situation in fungi where no MIP/RIP is detected when the size of homology falls below 400 bp. It would also mean that the minimal efficient processing segment (MEPS) for pairing in plants would be much smaller than that defined in mammals for homologous recombination (around 300 bp; ref. 31).

In conclusion, RIGS phenomena in plants are likely to reflect several families of biological processes. For none of these processes is there an indication at the present time that they could involve direct DNA-DNA pairing as inferred for MIP and RIP. More investigations focused on a limited and well chosen number of model systems will be required to get a final answer to that question.

III. Premeiotic inactivation processes and genetic recombination

Since the only processes in which good evidence for direct DNA-DNA pairing has been documented are MIP and RIP, we will focus this discussion on the possible relationship between these premeiotic inactivation processes and homologous recombination.

Search for homology in meiotic recombination and in MIP/RIP

The high efficiency of MIP/RIP on repeated sequences in ectopic positions means that premeiotic *Ascobolus* and *Neurospora* cells have the capacity to search the entire genome for homologous partners. This capacity may be compared to that of *Saccharomyces cerevisiae* meiotic cells which display similar frequencies of gene conversion between homologous sequences whatever the allelic or ectopic relative positions of these sequences[16], showing that the predominent way in which homologous sequences interact during recombination in yeast is largely independent of chromosomal position. This observation led Haber and coworkers[16] to suggest that an analogous 'homology sensing machinery' would act in MIP/RIP and in yeast, except that the yeast machinery would be associated with a recombination enzyme complex and the MIP and RIP machineries would be associated with a methylase, ensuring that the paired homologous DNA segments will be processed differently to give either recombinant products or products of MIP/RIP.

During meiosis, double-strand breaks (DSBs), which appear early in prophase[64], are good candidates to be primary events in recombination, that could lead to single-stranded substrates[65] allowing the formation of joint molecules further processed in recombination products[66]. Could DSBs also trigger search for homology in MIP/RIP? Meiotic DSBs in yeast occur in specific locations corresponding to intergenic regions[71]. The observation that all the DNA sequences tested (even those corresponding to gene fragments, or those as small as a few hundred bp) are able to undergo MIP/RIP when they are duplicated implies that they can all initiate a homology sensing process. Thus, if DSBs were involved in this initiation, they should affect a much larger number of sites in MIP/RIP than in meiotic recombination. In addition, for a given sequence, MIP/RIP acting on ectopic repeats is much more frequent than allelic, as well as ectopic meiotic recombination: MIP/RIP involves about half of the cells, whereas meiotic recombination usually involves a few percent of them. The high frequency of MIP/RIP would lead to a systematic and extensive fragmentation of the genome. Since most of the genome in *Neurospora* is constituted by sequences which are not duplicated[52], almost all these lesions should be repaired without a homologous partner. In conclusion, if DSBs are indeed the primary events triggering the search for homology in meiotic recombination, it seems unlikely that MIP/RIP and meiotic recombination are initiated by a common search for homology mechanism.

The homology sensing machinery common to meiotic recombination and MIP/RIP, suggested by Haber and coworkers[16], could operate prior to DSBs (that would then constitute the specific branching toward recombination). Indeed, presynaptic chromosome alignment during early meiosis occurs before the appearance of DSBs[18,42,50]. Kleckner and coworkers suggested that homologous DNA segments may undergo a genome wide DNA/DNA homology search, and that such a search should involve unstable, paranemic interactions between intact duplexes that would specify presynaptic chromosome alignment in early meiosis[26]. The observation by B. de Massy, F. Baudat and A. Nicolas (pers. commun.) that DSBs normally take place in haploid meiosis indicates that these putative paranemic unstable interactions between homologous segments on homologous chromosomes are not required for DSBs, and thus, probably, do not take part in initiating meiotic recombination. The observation that allelic and ectopic copies equally interact in meiotic recombination[16] also argues against a role of presynaptic chromosome alignment in initiating meiotic recombination events. In contrast, similar paranemic DNA-DNA interactions could constitute the homology sensing mechanism playing in MIP/RIP. They could be associated with MIP/RIP by activating the specific enzymes responsible for

methylation and point mutation which are present in premeiotic cells.

Hence, the premeiotic homology sensing mechanism required for MIP/RIP might be completely different from that required for initiating meiotic recombination.

Premeiotic recombination and MIP/RIP

Genetic recombination may happen through different pathways: even if MIP/RIP does not share a common step with meiotic recombination, this does not mean that it cannot be mechanistically related to other recombination processes, especially premeiotic recombination. Indeed, we know that dicaryotic premeiotic cells in fungi may be prone to frequent events leading to the excision by homologous recombination of one or several copies of direct tandem repeats. In *Neurospora*, excision of one copy of a tandem duplication involving 6 kb of homology was observed in about 2/3 of the progeny; a consistent portion of these excisions were shown to involve intrachromatid events and occur after fertilization and prior to meiosis[54]. In a fraction of the excised progeny (4 out of 13), the remaining copy was mutated by RIP, indicating that excision followed (or was cotemporal with) the RIP event. In *Podospora*, a more than 20 kb long tandem duplication was shown by genetic analysis to excise premeiotically with an extremely high frequency[46]. Interestingly, the frequency of premeiotic excision increased from 39% to 60% with the age of the fruiting body. The simplest way to account for this observation would be to assume that multiple cycles of excisions occur in the ascogenous tissue, leading to an increasing likelihood of excision in the dicaryotic cells which have undergone several divisions before meiosis. The possibility that multiple cycles of excision of tandem duplications might occur in premeiotic dicaryotic cells is to be connected to the finding that multiple cycles of pairing are involved in MIP/RIP. Thus, there may be a relationship between premeiotic excision of tandem duplications and MIP/RIP. Premeiotic excision may be a homologous recombination process basically distinct from meiotic recombination not only because it occurs at a different time in the sexual cycle, but also because it could be driven by mechanic and enzymatic processes completely distinct from those occurring in meiotic recombination. This opens the possibility that identical processes operate in premeiotic cells for recognition of homologous sequences leading to excision and to MIP/RIP, whereas an entirely different process of sequence recognition operates in meiotic recombination. The premeiotic search for homology process may lead to different outcomes, depending on the available enzymatic machinery: this would be consistent with the existence of only excision in *Podospora*, MIP in *Ascobolus*, RIP and excision in *Neurospora*.

Little is known about a possible relationship between MIP/RIP and premeiotic recombination when the duplication is constituted by two ectopic copies. Three situations may characterize such a relationship. 1) MIP/RIP may be associated with ectopic recombination; 2) the two events may occur independently; 3) the two events may be mutually exclusive. No recombination was reported to be associated with MIP/RIP between dispersed repeats. However, since the frequency of ectopic recombination is not known in *Ascobolus* and *Neurospora*, no conclusion can be drawn.

Consequences of modifications resulting from MIP and RIP on genetic recombination

RIP-induced point mutations are likely to impair genetic recombination. In eukaryotes, the amplification of DNA sequences creates identical repeats in ectopic positions that threaten the integrity of the genomes by generating chromosomal scrambling via ectopic recombination. Genome stability may be reached by accumulating sequence divergence between the repeats. Sequence divergence drastically reduces the frequency of homologous recombination in bacteria[60] and mammalian cells[70]. Kricker et al.[27] argued that a RIP-like process could be efficient in eukaryotic germ lines, allowing rapid divergence of DNA repeats, hence preventing chromosome rearrangements by ectopic recombination. They tested a model based on methylation and subsequent mutation of CpG dinucleotides specifically affecting duplicated DNA segments. They showed that the frequency of CpGs was much smaller in repeated DNA segments than in unique genes. Their results supported the conclusion that mammalian genomes possess a general mechanism for speeding the divergence of repetitive sequences by predominent transitions from CpG to TpG (or CpA) when the size of the repeat is longer than 0.3 kb. This value is close to the estimated minimum efficient size for MIP and RIP, which would range between 0.3 and 0.4 kb. Kricker et al.[27] suggest that RIP-like processes may act in many different organisms including mammalian cells. Segments larger than 0.3 kb (such as distinct copies of the same pseudogene) able to recombine would be subjected to a RIP-like process preventing recombination. The fragmentation of the coding sequences into exons (the size of which is usually smaller than 0.3 kb) would protect active genes from both recombination (the 0.3 kb size is also the minimum efficient processing segment (MEPS) for recombination) and from RIP-like interactions with processed pseudogenes.

MIP may indirectly lead to a slow accumulation of point mutations (mimicking RIP) via deamination of methylated C. Indeed, the CpG deficiencies characteristic of mammalian genomes are thought to reflect such a process[49]. Methylation by itself could also prevent homologous recombination. Site-specific recombination leading to immunoglobin gene maturation is blocked by C-methylation[21]. High frequencies of chromosome rear-

rangements are observed in hypomethylated mammalian cells, with exchange points located in the heterochromatic segments close to the centromeres[1]. C-methylation resulting from MIP may be sufficient to prevent recombination even in the absence of point mutations introducing sequence divergence. Premeiotic methylation of new repeats would then allow an immediate protection against meiotic ectopic recombination, comparable to the effect of the sequence divergence by RIP.

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