Gene silencing: **Maintaining methylation patterns**

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Recent studies of an *Arabidopsis* gene family have shown that inverted repeats can be potent silencers of other identical sequences in the genome, causing them to become stably methylated at cytosine residues. From mutations affecting this process we are beginning to understand how methylation patterns are maintained.

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DNA methylation is thought to have evolved in bacteria as a defense against foreign DNA. For instance, the prokaryotic methylation–restriction systems consist of specific methylases that act at short palindromic sequences, and restriction enzymes that cleave these sequences if, and only if, they are unmethylated (as they are likely to be in the context of invading bacteriophage DNA). In eukaryotes, cytosine methylation has evolved into a mechanism that allows dividing cells to stably inherit states of gene activity. DNA methylation is involved in a myriad of epigenetic regulatory processes found in the vast majority of eukaryotes, including plants, fungi and animals. DNA methylation is absent — probably lost — in several fungal and animal lineages that include the much-used model organisms *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans*.

Plants do show DNA methylation, and the genetic manipulation of DNA methylation in the most popular model plant, *Arabidopsis thaliana*, is providing insights into how eukaryotes establish and maintain proper methylation patterns in the genome. One of the best studied phenomena involving DNA methylation, particularly in plants and fungi, is that of multiple copy gene silencing, a process by which duplicated regions of eukaryotic genomes are recognized and stably silenced. This process poses a particular problem for the biotechnological manipulation of organisms, as exogenous genes introduced into a genome, as well as the homologous endogenous genes, often become epigenetically inactivated.

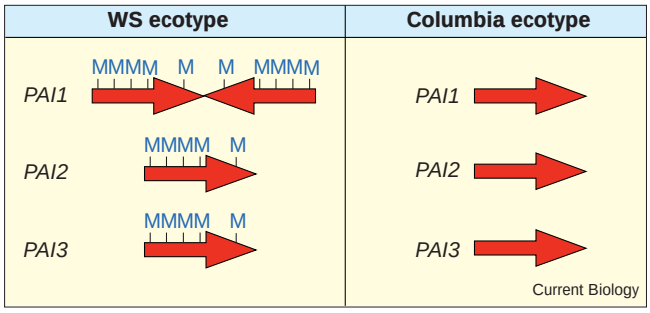
Multiple copy gene silencing also occurs within endogenous gene families. A well-studied example of this is provided by the *PHOSPHORIBOSYLANTHRANILATE ISOMERASE* (*PAI*) gene family of *Arabidopsis*. Silencing of the *PAI* genes was initially discovered during the characterization of several unstable epigenetic *pai* mutants [1]. In the genome of the wild-type ecotype WS, there are four highly homologous copies of *PAI* at three different loci (Figure 1). The *PAI1* locus consists of two complete *PAI* genes arranged as an inverted repeat, while the unlinked *PAI2* and *PAI3* loci each contain one *PAI* copy. All four of the WS *PAI* genes are normally heavily methylated.

The key observations came from analysing mutants where the inverted repeat at the *PAI1* locus had been deleted. In these mutants, the remaining, methylated *PAI2* and *PAI3* genes did not produce enough of the *PAI* gene product — an enzyme involved in tryptophan biosynthesis — so that there was a visible mutant phenotype. These *pai* mutants were found to be unstable, however, spontaneously reverting to wild-type about 3% of the time. It turned out that the revertants had lost methylation at *PAI2* and *PAI3*, elevating the levels of *PAI* gene expression to an extent sufficient for normal development. A second wild-type ecotype, Columbia, has a different *PAI* gene structure, with single *PAI* genes at all three loci, none of which are methylated (Figure 1). These observations suggested that the inverted repeat at the *PAI1* locus promotes DNA methylation within the gene family.

Several recent pieces of evidence have provided strong support for this idea. The first came when the WS and Columbia ecotypes were crossed — the results indicated that the repeated *PAI1* locus of WS can cause *de novo* methylation of the Columbia *PAI1*, *PAI2* and *PAI3* loci [2]. More direct evidence came from analysing transgenic Columbia plants containing a single ectopic copy of the *PAI1* inverted repeat — these were found to exhibit *de novo* transgene methylation, and in plants containing multiple copies of the transgene the endogenous *PAI* genes had also undergone *de novo* methylation [2]. Further circumstantial evidence came from a survey of 39 additional *Arabidopsis* ecotypes, in which six new ecotypes were found with an inverted repeat *PAI1* gene arrangement similar to that of WS. All six of these new ecotypes showed methylation within the *PAI* gene family; the remaining 33 ecotypes, however, showed a gene arrangement similar to that of Columbia, and showed no methylation of the *PAI* genes [3]. DNA methylation thus completely correlates with the presence of the inverted repeat in 41 ecotypes that have been collected from around the world.

What could be the mechanism by which the *PAI* inverted repeat induces methylation of itself and of unlinked *PAI* loci? Studies of gene silencing in eukaryotes have uncovered two broad classes of gene silencing mechanism [4]. The first is transcriptional gene inactivation, a process that is tightly correlated with methylation of the corresponding

Figure 1



The *PAI* gene families of two wild-type *Arabidopsis* ecotypes, WS and Columbia. M represents a methylated cytosine.

DNA. Transcriptional silencing is usually meiotically heritable, and is hypothesized to involve DNA–DNA pairing. The second mechanism is post-transcriptional gene inactivation, or cosuppression, which is not usually meiotically heritable and can be non-cell autonomous — that is, the silencing signals can travel systemically throughout the plant. Cosuppression is hypothesized to involve aberrant RNA structures, especially inverted-repeat-containing RNAs [5], which can autocatalytically destroy RNA products of homologous genes. There is also evidence for interaction between these two mechanisms: DNA methylation can promote post-transcriptional gene silencing, and RNA can direct methylation of homologous DNA sequences [6].

The weight of evidence suggests that *PAI* gene silencing acts through a transcriptional inactivation process. *PAI* silencing is associated with dense methylation at both the symmetric — CG and CNG — and asymmetric cytosines, and this methylation is meiotically stable. Furthermore, recent findings indicate that DNA–DNA pairing itself may be the trigger for methylation. DNA methylation is coextensive with the repeated segments of DNA. The transfer of the methylation from the inverted repeat to homologous loci shows chromosome-position effects, with the linked *PAI1* locus becoming methylated faster than the unlinked *PAI2* locus. And a *PAI1* inverted repeat transgene can trigger methylation even when its promoters have been deleted, so that no *PAI1* RNA is produced [2]. It thus seems likely that DNA–DNA pairing of the inverted repeat, with itself and/or with other related sequences, may be sufficient to cause *de novo* methylation and gene silencing. At this point, however, the alternative hypothesis — that undetectable amounts of RNAs are made from the promoterless inverted repeat constructs, and that these RNAs trigger '*trans*' silencing of the endogenous *PAI* loci — cannot be completely ruled out.

What proteins are responsible for DNA methylation and silencing at the *PAI* loci? One approach to answering this question is to study the effect of *Arabidopsis* methylation mutations on *PAI* gene activity. This has recently been done with a mutation at the *DDM1* locus. The recessive *ddm1-2* allele, which causes overall hypomethylation of the genome [7], abolishes both methylation and gene silencing at *PAI2* [8]. The *DDM1* gene was recently cloned [9] and found to encode a putative SWI2/SNF2 class chromatin remodeling protein. This makes an interesting connection between DNA methylation and chromatin structure. *PAI* gene methylation and silencing was also found to require the activity of the *Arabidopsis* DNA methyltransferase METI — a homolog of the mammalian maintenance methyltransferase Dnmt1 — as an antisense-*METI* transgene was found to cause hypomethylation and reactivation of *PAI2* (J. Bender personal communication). Both *ddm1* [7] and antisense-*METI* [10,11] lesions cause a loss of DNA methylation in repetitive elements of the genome — such as the rDNA and centromeric DNA repeats — and of repetitive transgenes [12], further establishing the similarity between *PAI* gene silencing and other multicopy gene silencing phenomena.

Another example of a meiotically stable gene silencing event in *Arabidopsis* is provided by the hypermethylated *superman* alleles [13]. These alleles, which are also heavily methylated at both symmetric and non-symmetric cytosines, have very different — and in some ways opposite — qualities to the hypermethylated *PAI* alleles. Whereas the *PAI* genes are methylated in wild-type plants and become demethylated in *ddm1* or antisense-*METI* plants, the *SUPERMAN* locus is initially unmethylated in wild-type plants, but becomes densely methylated in *ddm1* or antisense-*METI* mutants ([13] and my unpublished observations). After outcrossing, these hypermethylated *superman* alleles are stable in wild-type backgrounds for several generations but, like *pai* mutants, revert to wild type about 3% of the time.

How can *ddm1* and *METI* mutants cause hypomethylation of some genes and hypermethylation of others? One hypothesis is that there are two classes of DNA methyltransferases in plants: the METI-type enzymes that are responsible for the maintenance of duplicate gene silencing, as seen at the *PAI* loci; and a second class of enzymes that become hyperactivated in *ddm1* and *antisense-MET1* mutants, causing stable methylation of genes such as *SUPERMAN*. Candidates for this second class are the chromomethylases, plant DNA methyltransferases containing a so-called chromodomain [14]. At least two genes for chromomethylases exist in the *Arabidopsis* genome: the first, *CMT1*, is a pseudogene in many common laboratory strains [14], but the second, *CMT2*, appears from its sequence to be functional (accession number AL021711 [15]).

Recent evidence suggests that a key difference between the METI-type and chromomethylase-type enzymes is

their methylation site specificity, with METI-type enzymes acting primarily at CG sites and the chromomethylase-type enzymes acting at CNG sites. Several lines of evidence indicate that METI acts preferentially at CG sites. Firstly, in antisense-*METI* plants methylation is reduced preferentially at CG sites in repetitive DNAs [10]. Secondly, although the hypermethylated *superman* alleles are stable in an antisense-*METI* background, most CG methylation, but not CNG methylation, is lost [13]. And lastly, in the absence of the *PAI1* inverted repeat, methylation at the *PAI2* locus occurs primarily at CG sites [2,8], and this methylation is abolished in antisense-*METI* plants. The chromomethylases seem to be CNG-specific enzymes, as a *cmt* loss-of-function mutation in maize was found to cause a decrease in CNG methylation, but not in CG methylation, at repetitive loci (C. Papa, N. Springer and S. Kaeppler, personal communication). Because the most heavily methylated positions at the *SUPERMAN* locus are CNG sites [13], it follows that the chromomethylases could be responsible for maintenance of this methylation.

The METI-type and chromomethylase-type enzymes may thus have complementary roles in gene silencing and methylation, with both types acting at some genes simultaneously, such as at the very densely methylated *PAI1* and *SUPERMAN* loci. METI might be the primary determinant of stable *PAI* gene methylation, acting mainly on CG sites, while the chromomethylases might be more important in silencing genes such as *SUPERMAN*, acting mainly on CNG sites. The *PAI* and *SUPERMAN* genes also have a significant fraction of asymmetrically methylated sites as well, but it is not clear which methyltransferase(s) are responsible for this. What is clear is that *Arabidopsis* is the ideal model organism for testing these ideas, given the known stable epimutations such as *pai* and *superman*, a growing number of methylation mutants and the relative ease with which genetic screens can be performed.

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