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Epigenetic inheritance in *Arabidopsis*: selective silence

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Eukaryotic organisms have the remarkable ability to inherit states of gene activity without altering the underlying DNA sequence. This epigenetic inheritance can persist over thousands of years, providing an alternative to genetic mutations as a substrate for natural selection. Epigenetic inheritance might be propagated by differences in DNA methylation, post-translational histone modifications, and deposition of histone variants. Mounting evidence also indicates that small interfering RNA (siRNA)-mediated mechanisms play central roles in setting up and maintaining states of gene activity. Much of the epigenetic machinery of many organisms, including *Arabidopsis*, appears to be directed at silencing viruses and transposable elements, with epigenetic regulation of endogenous genes being mostly derived from such processes.

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Introduction

When Conrad Waddington developed the idea of epigenetics in the early 1940s, he was attempting to create a new conceptual framework for the study of development [1]. Epigenetics is a derivative of epigenesis, a theory stating that animal development entails a gradual increase in complexity. Epigenesis stood in opposition to preformation, which argued that all the complexity of a fully developed organism was present in the zygote, or even in the gametes. In epigenetics, Waddington aimed to synthesize preformation and epigenesis into a single theory by combining genetics — the study of the hereditary material (pre-existing complexity) found in the zygote — and developmental biology — the study of changes undergone by the zygote (epigenesis) [1]. The concept of epigenetics has undergone a great deal of change in the last sixty years. In 1994, for example, Robin Holliday offered the following definition: ‘[epigenetics is]

the study of the changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression’ [2]. Ten years later, epigenetics is most commonly described as ‘the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence’ [3].

The major reason for the changing view of epigenetics is that the original concept was meant to be a broad theoretical framework to guide studies in an area in which not only were the mechanisms not understood but the nature of the processes was unknown. In this spirit, Timothy Bestor offered the following light-hearted description at the 1995 Gordon Research Conference on Epigenetics: ‘known gene, known product: molecular biology; known gene, unknown product: genetics; unknown gene, known product: biochemistry; unknown gene, unknown product: epigenetics’ (T Bestor, unpublished). In recent years, both genetic and biochemical studies have greatly advanced our knowledge of two epigenetic processes: siRNA-mediated gene silencing; and chromatin-based inheritance of gene activity states. What has begun to emerge from under the almost mystical epigenetic umbrella is a picture of an ancient system of cellular and genomic immunity predating the divergence of plants, ciliates, animals and fungi. Here, we focus on recent work with the model plant *Arabidopsis thaliana* that has significantly contributed to our understanding of this system and how it has been co-opted to influence development.

The epigenetic inheritance of *FWA*

FWA was first identified fifteen years ago as a dominant mutation that caused a delay in flowering time [4]. In every genetic test, *FWA* behaved as a typical Mendelian trait: the phenotype was stable over many generations and segregated with the expected 3:1 ratio. The mutation was mapped to a single gene encoding a putative homeodomain transcription factor [5]. However, no DNA sequence changes could be found in the mutant allele. Additionally, introducing a wild type transgenic copy of the gene would occasionally recreate the mutant phenotype. Clearly, there was something odd about *FWA*.

An unusual feature of the *FWA* gene is that its promoter and transcription start site are encompassed within two pairs of almost perfect direct repeats [5]. Repeated sequences were known to be associated with DNA methylation and gene silencing, suggesting a possible epigenetic mechanism to explain the behavior of *FWA*. Additionally, plants that have lost most of their DNA

methylation as a result of the *ddm1* mutation often exhibit a dominant late-flowering phenotype, which consistently mapped to the vicinity of *FWA* [6]. Indeed, the *FWA* repeats were found to be methylated in wild type plants, keeping the gene off in all tissues except the endosperm — an extra-embryonic tissue akin to the placenta that nourishes the developing embryo [5]. In the endosperm, the maternal *FWA* allele is selectively de-methylated through the action of the DNA glycosylase *DEMETETER* [7^{*}]. The *FWA* mutation is caused by loss of DNA methylation in all tissues, resulting in inappropriate expression throughout the plant and the late-flowering phenotype [5].

The methylated *FWA* epiallele is present in numerous locally adopted populations (ecotypes) of *Arabidopsis*, suggesting that it has been stably inherited for thousands of years. The unmethylated form of *FWA* appears to be equally stable [5,8,9^{*}]. The two states of *FWA* thus represent true Mendelian traits, the basis of which is a difference in the methylation of DNA and not in the sequence. In addition to *FWA*, methylated and silenced epialleles of the *SUPERMAN* and *PAI* genes have been described [10,11]. Although not as persistent as *FWA*, the existence of these alleles might indicate that epigenetic inheritance is fairly common in plants, especially considering that these alleles were discovered fortuitously by researchers attempting to resolve genetic conundrums.

Taming transposable elements

Why are the *FWA* repeats subject to DNA methylation and silencing? The majority of sequences known to be methylated in *Arabidopsis* are present in more than one copy, and most repeats are derived from transposable elements [12,13]. It has long been thought that methylation and silencing of such sequences represents a defense system aimed at suppressing transposition. The duplication in *FWA* has thus allowed the gene to ‘borrow’ the transposon-silencing machinery to control transcription. A similar situation can occur when a transposon integrates close to or within a gene sequence. An interesting example of this is another *Arabidopsis* flowering-time gene, *FLC* (*FLOWERING LOCUS C*). In some ecotypes of *Arabidopsis*, a high level of *FLC* expression significantly delays flowering unless the plants are exposed to a prolonged period of cold [14]. Other *Arabidopsis* ecotypes do not express *FLC* at high levels and, thus, do not need cold exposure for rapid flowering. In two such ecotypes, *Ler* and *Da* (1)-12, transposon insertions in the first intron of *FLC* prevent the gene from being upregulated to levels that repress flowering [15,16,17^{*}]. The transposons in the two ecotypes belong to different families, indicating that these were independent adaptive events. Thus, for both *FWA* and *FLC*, the transposon-silencing machinery has been fortuitously co-opted to regulate *Arabidopsis* development.

DNA methylation and histone modifications

Three types of DNA methylation are found at *FWA*. Most of the methylation at this locus, as is generally the case throughout the genome, is found in symmetric CG dinucleotides [5,12,13,18]. This type of methylation is maintained by the DNA methyltransferase MET1 by filling in hemi-methylated sites following DNA replication. Small amounts of CNG and asymmetric (not CG or CNG) methylation are also found at *FWA* [18]. The CNG methylation is maintained primarily by CHROMOMETHYLASE3 (CMT3), the enzyme that maintains this type of methylation throughout the genome [18–20]. CNG and asymmetric methylation at *FWA* is also dependent on a third methyltransferase, DOMAINS REARRANGED METHYLASE2 (DRM2) [18]. DRM2 is primarily a *de novo* methyltransferase, required to establish DNA methylation on naïve templates, including *FWA* [8].

Genetic and biochemical evidence indicates that CMT3 is guided at least in part by histone methylation. CMT3 contains a chromodomain within its catalytic C-terminus [19,20]. Chromodomains are found in several chromatin-related proteins — hence the name — and have been found to specifically interact with the tail of histone H3 when it is methylated at either lysine 9 (H3K9) or 27 (H3K27) [21]. The chromodomain of CMT3 is, to date, unique in that it requires both lysines to be methylated for binding *in vitro* [22]. Loss-of-function mutations in the H3K9-specific methyltransferase KRYPTONITE (also known as SUVH4) abolish most, but not all, CMT3-dependent DNA methylation [23,24]. Histone methylation, however, is not the whole story, because some loci are associated with both methylated H3K9 and methylated H3K27, but not with DNA methylation [25,26]. Additionally, CNG and asymmetric methylation at *FWA* and several other loci is dependent on DRM2 [18,27]. One possibility is that in addition to histone methylation CMT3 also requires pre-existing DNA methylation, thus leading to a dependence on DRM2 for establishment and, in some cases, maintenance of CNG methylation.

The mechanisms underlying the maintenance of histone methylation patterns at *FWA* and throughout the genome are still largely unknown. An intriguing possibility is that different methylation states of histone H3 are the result of deposition of two H3 variants. In most eukaryotes, including plants, replication-coupled chromatin assembly uses an H3 protein with a slightly different sequence from the variant used in replication-independent assembly [28]. In both plants and animals, the two histone variants differ in post-translational modifications, with the replication-coupled H3 enriched in methylated K9 and K27 [29,30]. Good evidence exists that replication-independent assembly is coupled to transcription [31,32], suggesting that differing histone modifications might be as much readouts as they are effectors of transcriptional activity.

siRNA-mediated silencing

In addition to *drm2*, mutations in several other genes eliminate non-CG DNA methylation and prevent establishment of any DNA methylation at *FWA* [9[•]]. These genes encode components of a siRNA-mediated silencing pathway that is conserved in plants, animals and fungi [33]. siRNAs corresponding to the *FWA* repeats have also been detected [34]. siRNAs are small RNA molecules, roughly 25 nucleotides in length, that are produced from longer double-stranded RNA molecules by a ribonuclease called Dicer [33]. In some organisms, including plants, an RNA-dependent RNA polymerase (RdRP) might convert single-stranded RNA species into double-stranded RNA, which then acts as a substrate for Dicer [33]. The siRNAs become incorporated into effector complexes that contain members of the Argonaute family of proteins [33].

siRNA-mediated silencing was first described as a process that degrades mRNA. The effector complex for this process is an Argonaute-containing sequence-specific ribonuclease termed RISC (RNA-induced silencing complex) [33]. The Argonaute protein within RISC is the catalytic subunit, using the incorporated siRNA to specifically cleave mRNAs that match the siRNA sequence. This process represents a potent defense against RNA viruses, many of which encode proteins that suppress siRNA-mediated silencing [35,36]. A variation of this mechanism uses microRNAs (miRNAs), which are identical to siRNAs except that miRNAs are specifically processed from an endogenous hairpin transcript to yield a single ~25 nucleotide RNA species. miRNAs control development either by degradation of target mRNAs or by repression of their translation [33,37]. Considering that endogenous siRNAs can control *Arabidopsis* genes in a similar fashion to miRNA-mediated control, and that at least some miRNA genes have evolved from inverted duplications that originally gave rise to siRNAs [38,39,40[•]], the control of development by miRNAs is almost certainly derived from the antiviral siRNA pathway.

Ten years ago, a study was published [41] showing that infecting a plant with an RNA viroid that does not go through a DNA intermediate in its life cycle causes DNA methylation of sequences in the plant genome that are homologous to the viral RNA. This process, termed RNA-directed DNA methylation, was the first evidence that RNA can induce changes in chromatin. Subsequent studies reproduced the phenomenon, using other viruses and inverted-repeat transgenes designed to produce hairpin RNA [42]. More recently, mutations in genes involved in siRNA-mediated silencing (*Dicer*, *RdRP* and *Argonaute* genes) have been shown to relieve transcriptional gene-silencing in plants, animals and fungi [42]. These mutations lead to loss of H3K9 methylation in several organisms, and to loss of DNA methylation in *Arabidopsis*. siRNA-mediated transcriptional silencing thus appears to be as ancient as post-transcriptional silencing, serving to

protect the genome against transposable elements and retroviruses.

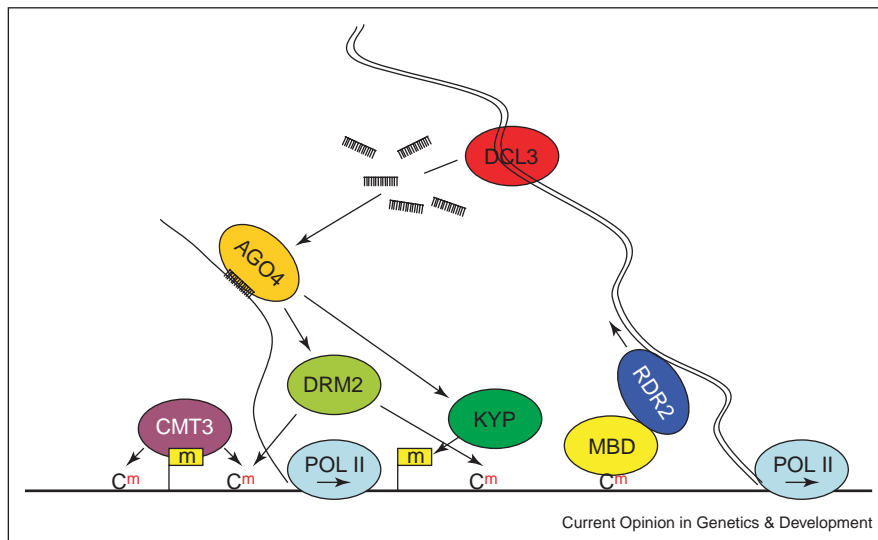
siRNA-mediated silencing *in cis*

In light of the involvement of siRNAs in *FWA*-silencing, an intriguing feature of active *FWA* alleles is that they remain active even in the presence of a silent *FWA* allele [5,8]. In other words, despite the presence of siRNAs corresponding to the *FWA* repeats, an active copy of *FWA* does not become silenced when introduced by a cross. The *SUPERMAN* gene exhibits similar *cis*-limited behavior [8,10]. This phenomenon is recapitulated genome-wide in *met1* and *ddm1* mutants, which lose most DNA methylation. When the mutants are crossed with wild type plants, normally methylated sequences remain largely hypomethylated on the chromosomes derived from the mutant parents, despite the presence of corresponding siRNAs [34,43,44].

A potential mechanism for the *cis* preference of siRNA-mediated transcriptional silencing was glimpsed recently by researchers studying the *Arabidopsis* *PHABULOSA* (*PHB*) gene [45^{••}]. In wild type plants, *PHB* is regulated by a miRNA. The miRNA recognition site is present only in the spliced *PHB* transcript; it is split in half by an intron in the DNA sequence [46]. *phb-1d*, a dominant mutation in *PHB*, disrupts the regulation of *PHB* by the miRNA by destroying a splice junction and creating a mis-spliced transcript in which the miRNA recognition site is disrupted. In wild type plants, a region of the *PHB* gene, downstream of the miRNA binding site, is densely methylated, and this methylation is largely lost in *phb-1d* mutants [45^{••}]. Given that there is no miRNA-recognition site in the DNA sequence, the methylation must be caused by the interaction of the miRNA with the *PHB* transcript. In heterozygous *phb-1d* plants, DNA methylation is lost primarily from the mutant chromosome [45^{••}]. Methylation must, therefore, be preferentially triggered by miRNA interaction with the nascent transcript, because it is the only time at which the methylation machinery can distinguish the chromosome from which the transcript was made from its homolog.

Recent evidence indicates that siRNA-mediated transcriptional silencing in the fission yeast, *Schizosaccharomyces pombe*, also exhibits *cis* preference. The *S. pombe* siRNA-mediated transcriptional silencing effector complex, RITS (RNA-induced initiation of transcriptional gene silencing), contains an Argonaute protein, a chromodomain protein (Chp1) and siRNAs [47]. In wild type cells, RITS associates with silent chromatin, as does the *S. pombe* RdRP, Rdp1 [48[•],49[•]]. Loss of Clr4, the *S. pombe* H3K9 methyltransferase, or a mutation in the Chp1 chromodomain that prevents Chp1 from binding methylated H3K9 disrupts the association of RITS with chromatin and leads to loss of siRNAs [48[•],50]. A mutation that abolishes the catalytic activity of Rdp1 has the same

Figure 1



Model for *cis*-limited siRNA-mediated chromatin silencing in *Arabidopsis*. A nascent transcript is recognized by a RITS-like siRNA-mediated transcriptional silencing complex containing AGO4. This recognition triggers DNA methylation (C^m) by DRM2, and histone H3K9 methylation (yellow flags) by KYP. H3K9 methylation is recognized by CMT3, resulting in further DNA methylation. Methylated DNA is recognized by a methyl-DNA binding protein (MBD), which recruits RDR2 to the locus. RDR2 converts nascent transcripts into double stranded RNA, which is processed by a Dicer-like enzyme (DCL3) into siRNAs. The siRNAs are incorporated into the RITS-like complex, completing the cycle. Abbreviations: AGO4, ARGONAUTE4; CMT3, CHROMOMETHYLASE3; DCL3, DICER-LIKE3; DRM2, DOMAINS REARRANGED METHYLASE2; KYP, KRYPTONITE; RDR2, RNA-DEPENDENT RNA POLYMERASE2; RITS, RNA-INDUCED INITIATION OF TRANSCRIPTIONAL GENE SILENCING.

phenotype [49^{*}]. It therefore appears that once a *trans*-acting siRNA-mediated signal sets up silent chromatin, chromatin serves to recruit the siRNA-mediated silencing machinery to maintain and reinforce silencing. An analogous situation might exist in *Arabidopsis*, wherein mutations in the DNA methyltransferases DRM2 and MET1 result in the loss of some endogenous siRNAs [51,52]. RDR2, the RdRP required for endogenous siRNA production in *Arabidopsis* [53] might be recruited to methylated DNA, perhaps by one of the methyl-DNA-binding proteins encoded in the genome (Figure 1) [54,55]. RDR2 would then convert any transcripts from the locus into double-stranded RNA, thus turning even very low level transcription into a potent silencing signal.

What could be the reason for a *cis* preference of siRNA-mediated silencing? Some insight might be gained by considering siRNA-mediated silencing as a genomic immune system that is designed to identify and suppress invasive sequences such as transposons. As with our own immune system, auto-immunity is a major concern: generating siRNAs to and silencing endogenous genes would be disastrous for the organism. To avoid this, a two-tier system could be in place. A strong source of siRNAs, such as an actively replicating transposon or a transgene designed to express double-stranded RNA, would elicit siRNA-mediated silencing *in trans*. Once recognized as deleterious, it would be advantageous to reduce transcription of such sequences to the lowest level possible without

losing the ability to recognize them as targets for silencing. Here, siRNA-mediated silencing would operate *in cis*, ensuring that any remaining transcription only reinforces silencing.

Recent evidence suggests that *cis*-limited siRNA-mediated silencing might be involved in mammalian genomic imprinting. Imprinted genes are mono-allelically expressed from either the paternal or the maternal chromosome [56]. Several imprinted loci have been found to overlap with antisense non-coding RNAs [56]. One of the best-studied examples is the mouse *Igf2r* gene cluster, in which the maternal cluster expresses *Igf2r* and two other genes, *Slc22a2* and *Slc22a3*. The paternal cluster expresses *Air*, a non-coding, unspliced RNA that is antisense to and overlaps with the *Igf2r* gene [56]. Expression of *Air* and *Igf2r/Slc22a2/Slc22a3* is mutually exclusive [56]. The *Air* RNA itself, not just transcription from the *Air* promoter, is required [57]. It is possible that transcription of an overlapping unspliced antisense RNA triggers siRNA-mediated transcriptional silencing [58]. If the process is largely *cis*-limited, silencing would be confined only to the locus making the antisense transcript, resulting in imprinted expression.

Conclusions

Perhaps the most exciting consequence of recent work on epigenetic inheritance in plants is that we now have molecularly defined components of siRNA-mediated

silencing and chromatin-based inheritance that can be studied without the constant need to redefine epigenetics. In the next few years, much effort is likely to be focused on unraveling the mechanisms underlying these processes. For instance, although we know that siRNAs can target DNA and H3K9 methylation, virtually nothing is known about how this happens. The recent discovery that a putative plant-specific DNA-dependent RNA polymerase is required for this process in *Arabidopsis* indicates that a previously unanticipated aspect of the mechanism involving specialized transcription might exist [59,60]. We are also just beginning to appreciate the extent to which siRNA-mediated silencing influences development [61]. In addition to the many miRNA genes identified in *Arabidopsis*, about 30% of all genes have been found to generate antisense transcripts [62], providing a potentially vast source of endogenous siRNAs. A large fraction of *Arabidopsis* genes have also been found to contain dense clusters of CG methylation within the transcribed region, possibly arising as a consequence of bidirectional transcription [63]. It might indeed turn out that many, if not most, *Arabidopsis* genes are influenced by miRNAs or siRNAs, indicating that the ancestral immune system has been deeply integrated into plant development.

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