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INVITED REVIEW

Epigenetics and its Implications for Plant Biology. 1. The Epigenetic Network in Plants

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• *Background* Epigenetics has rapidly evolved in the past decade to form an exciting new branch of biology. In modern terms, 'epigenetics' studies molecular pathways regulating how the genes are packaged in the chromosome and expressed, with effects that are heritable between cell divisions and even across generations.
• *Context* Epigenetic mechanisms often conflict with Mendelian models of genetics, and many components of the epigenetic systems in plants appeared anomalous. However, it is now clear that these systems govern how the entire genome operates and evolves.
• *Scope* In the first part of a two-part review, how epigenetic systems in plants were elucidated is addressed. Also there is a discussion on how the different components of the epigenetic system—regulating DNA methylation, histones and their post-translational modification, and pathways recognizing aberrant transcripts—may work together.

Key words: Epigenetics, DNA methylation, histones, chromatin, RNA, paramutation, transgenes, silencing, gene expression.

INTRODUCTION

In the past decade, there has been a remarkable revolution in the field of molecular genetics. This revolution has been founded on many experimental observations that indicate that the DNA sequence alone does not carry all of the information required to determine the phenotype of the organism. It has long been known that there are many awkward exceptions to 'normal' Mendelian genetics that have defied explanation by conventional theory, for instance, paramutation that was first identified in the 1950s. Now, these have been joined by the discovery of new phenomena of the same non-Mendelian nature, most significantly 'transgene silencing' in plants where new DNA sequences have been added to the genome. These observations challenged what was the contemporary view of the phenotype as a system dictated entirely, and in a linear fashion, by the sequence of DNA nucleotides coding for proteins. The intensive efforts to determine why these apparent 'exceptions' to Mendelian rules exist, and to discover the molecular mechanics and rules of behaviour governing these non-Mendelian phenomena, has generated a profoundly important and exciting set of data. This review intends to briefly and simply introduce and review this new field, named 'epigenetics' (i.e. 'upon genetics'), in plants for the benefit of an audience unfamiliar with such developments, although we are profoundly aware that substantial and detailed reviews on this subject are now ubiquitous. These will be referenced throughout the review; indeed, a particularly interesting and relevant introduction to the history and changing concept of epigenetics can be found in Jablonka and Lamb (2002). However, the main thrust of this two-part review is to address a hitherto little explored ramification of these discoveries, i.e. the relationship of epigenetics to the evolution, morphology and taxonomy of plants. We believe this new field of epigenetics will be an 'epiphany' in these conventional fields of botany when its exceptional significance becomes known more widely.

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IDENTIFYING AND DEFINING THE 'EXCEPTIONS' TO MENDELIAN GENETICS

It seems surprising that it has taken such a long time for the scientific community to take an interest in non-Mendelian phenomena, given that they are often highly noticeable and appear to be quite widespread in plants. Evidence for the existence of these effects can be found in many different circumstances and these are listed below with brief descriptive details of the phenomena.

Interploidy crosses and imprinting

In intraspecific crosses between plants of different ploidies, it has been observed in many cases that the development such as seed size (endosperm size) of the resulting seeds differs dramatically between the direction of the interploidy cross (reviewed in Haig and Westoby, 1991, Spielman *et al*., 2001, 2003; Table 1).

As the switching of the direction of the cross is not accompanied by any inherent change in the nucleotide sequence or gene copy numbers of the parental nuclear genomes contributing to the offspring, this implies that the effect is derived either from differences in the inherited cytoplasmic genomes or whether the DNA is passed through the maternal or the paternal meiotic–gametophytic programmes. Cytoplasmic effects can be ruled out simply because the directional effect is only seen when the nuclear

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TABLE 1. Arabidopsis thaliana *seed size resulting from combinations of different parental ploidies (adapted from Scott* et al.*, 1998)*

Interploidy cross (maternal × paternal)	$6x:2x$	$4x:2x$	$2x:2x$	$2x:4x$	$2x:6x$
Maternal : paternal ratio in endosperm	6 : 1	4 : 1	2 : 1	2 : 2	2 : 3
Seed size (indicated by number of dots)	●	●●	●●●●	●●●●●●	X
Viability	Lethal	Viable	Viable	Viable	Lethal

ploidies are different. This leaves as the only logical explanation that a 'tag' or 'mark' is added to (or removed from) the DNA during meiotic-gametophytic processes, and this labelling system not only differs dramatically between the male and female but also has a profound effect on the developmental programme of the triploid endosperm. Often, interploidy crosses fail to even initiate any substantial post-fertilization development, with early death of the developing embryo and endosperm. This is also problematic to conventional ideas, when parents of both ploidies are normal, viable and genetically identical or compatible, as it suggests that a system is in place that somehow detects ploidy imbalance at this stage of the life cycle.

Studies of gene expression patterns in endosperm in normal fertilization also highlighted that something was quite amiss. The same alleles at some loci, for example, the endosperm-expressed zeins and the anthocyanin-regulating *R* locus (reviewed in Alleman and Doctor, 2000), were not equivalently expressed from the maternally contributed copies ($2n$, from the two polar nuclei) and the paternally contributed copy (n, from a sperm nucleus) to the triploid ($3n$) endosperm tissue. Due to the unusual ploidy of endosperm tissue, these observations were originally attributed to the somewhat nebulous explanation of 'gene dosage'. As these observations of parent-of-origin expression effects were all restricted to endosperm tissue so this dosage explanation appeared inviting. However, this was not an adequate mechanistic explanation for these observations and an alternative but complementary hypothesis developed and eventually became far more supported by evidence (for a historical perspective, see Alleman and Doctor, 2000). This hypothesis centred on the DNA sequence at such loci carrying extra information. Although the DNA sequence was the same whether transmitted through the maternal or paternal gametophyte, some form of 'imprint' made on the sequence during gametophytic development that marks its parental origin also distinguished the expression of the sequence. It is now clear that in imprinted genes of plant endosperm it is typically the paternal copies that are 'silenced' in normal development, although paternally expressed and maternally silenced exceptions are known (Gutierrez-Marcos *et al.*, 2003). Indeed, theoretical predictions of imprinting and parent-of-origin effects at important loci were at first far in advance of empirical work (Haig and Westoby, 1989, 1991). This hypothesis has been applied to, and supported by, data from mammalian reproduction as well, although a major difference is that plants develop a triploid endosperm as the nutritive source, linked to the maternal sporophyte, for the developing embryo (for comparative reviews, see Alleman and Doctor, 2000; Spielman *et al.*, 2001). However, these original ideas advanced by Haig and Westoby have now been modified; for instance, this work focused on imprinting as a binary system where the allele transmitted by one parent was completely silenced and therefore contributed no gene product. Dilkes and Comai (2004) propose the 'differential dosage' hypothesis that relaxes the requirement for 'binary' imprinting in favour of differential gene expression from the alleles contributed by the two parents, a pattern that is seen at many loci. Indeed, Dilkes and Comai point out that *gametophytically* derived gene products in differing quantities from the different parents may produce the same effect as 'imprinting' (Tables 2 and 3).

TABLE 2. *Hypothetical gene expression patterns from imprinted loci showing binary imprinting and differential expression patterns*

Mature plant $2n$	Male microspore/ gametophyte n	Female gametophyte n	Endosperm $3n$	Embryo $2n$
Binary imprinting				
–/–	–	–	F +++. M –	–/–
Differential expression				
–/–	–	+	F ++++. M +	+/+

M, Male genome; F, female genome. Expression level from each genome is indicated by + and −.

Interspecific hybrids

Hybrids between different plant species (and the rarer cases of hybrids between different genera) frequently produce complex or unpredictable outcomes both in the wild and under experimental conditions (for many examples, see Grant, 1981). These phenotypic outcomes could be easily dismissed by the explanation that they are derived simply from the interactions of different proteins. These would be encoded by the divergent coding nucleotide sequences from the two (or more) previously isolated parental genomes. Their interactions during development of a hybrid would have the potential to generate intermediate or new phenotypic states. However, this simple explanation cannot even cover all of the outcomes that are possible in plant hybrids. An indication that this explanation is not powerful enough comes from rare hybrids where the phenotype of the hybrid is always skewed disproportionately towards one of the parents, which has been named uniparental phenotypic dominance (see Bennett, 1988; Heslop-Harrison, 1990).

At a microscopic scale, and far more ubiquitous in plant hybrid systems, the phenomenon of nucleolar dominance has also been perplexing. This is a consistent, reproducible event in many hybrids where the enormous rDNA repeat

TABLE 3. *Developmental stage and mechanism of imprinting of the* FWA *locus*

Mature plant 2*n*	Male microspore/ gametophyte *n*	Female gametophyte *n*	Endosperm 3*n*	Embryo 2*n*
–/–	–	– except specifically activated in polar nuclei	F ++. M –	–/–
Silenced by MET1		Maternal polar nuclei copies activated by DEMETER	Female copies active, male copy silenced	Silenced by MET1

M, male genome; F, female genome. Expression level from each genome is indicated by+and –.

region, which is the core of the nucleolus in the nucleus, is no longer transcribed from the genome of one of the parents (for reviews, see Pikaard, 1999, 2000). This loss of transcription results in the disappearance of an active nucleolus or nucleoli from one of the genomes; consistently, the same genome is affected every time the same hybrid combination is made. Nucleolar dominance therefore represents a situation where a previously active, multigene repeat locus from one species is stably switched off when displaced into a hybrid environment. It represents a situation where one gene set from one species is 'dominant' over the gene set of another species, even though these sequences have never been co-existent in the same genome. In fact, in taxa where a series of hybrids between different species can be produced, a complete dominance series of rRNA sequences have been discovered—although this 'absolute' nature of nucleolar dominance has been dispelled recently (Chen and Pikaard, 1997*b*; Chen *et al*., 1998; Hasterok and Maluszynskya, 2000; Pontes *et al*., 2003). Even if this phenomenon appears to be inconsequential, it represents an exceptional example of gene expression in a hybrid that may not conform to conventional Mendelian rules.

Another observation in hybrid systems that indicates conservative explanations are inadequate is the unusual frequency of quite dramatic phenotypic changes and reversions seen in some hybrids (e.g. those reported by Comai *et al*., 2000). These spontaneous changes in phenotype are akin to somaclonal variation (see discussion below) and must be the result of a 'metastable' shift of gene expression.

Somaclonal variation

When plant cells and tissues are cultured *in vitro*, regenerated plants can show a higher than expected frequency of phenotypic changes that can be stable or unstable (for review, see Kaeppler *et al*., 2000). Whereas clonal variation that is stable could be attributed to a higher than normal rate of nucleotide mutations in artificial culture (and evidence for such changes is considerable; for review, see D'Amato, 1991), unstable clonal variation is less easily explained as it has to result from a shift of gene expression between two different states.

Paramutation

The phenomenon of paramutation (Fig. 1) was first discovered and extensively analysed in maize in the 1950s by Alexander Brink (for historical perspective, see Chandler and Stam, 2004). Its significance is that it provides a clear and unambiguous example of Mendelian rules for the behaviour of genes being completely overturned. A violation of one of Mendelian genetics' central rules—that in a heterozygote, one allele does not alter in any way or influence the expression of the other allele—is seen in paramutation. In maize, several loci that encode regulators of the anthocyanin synthesis pathway [*red1* (*r1*), *booster1* (*b1*) and *purple plant1* (*pl1*)] show paramutation interactions between their different respective alleles (reviewed in Hollick *et al*., 1997; Alleman and Doctor, 2000; Chandler *et al*., 2000). Three classes of alleles occur in paramutation: paramutagenic, paramutable and neutral. In a heterozygote, paramutagenic alleles are able to convert paramutable alleles to a paramutagenic allele, which is seen as a huge drop in expression from the converted allele. Not only does this conversion persist in the heterozygote and result in highly reduced expression of the locus, but the alteration to the paramutable allele is heritable. Hence, the modified, silenced allele can be segregated away from the original paramutagenic allele, and it can then persist and behave as a paramutagenic allele itself and convert other naïve paramutable alleles (Fig. 1). Typically, this new state is stable but it can convert back spontaneously to the naïve paramutable state (Chandler *et al*., 2000). Of course, this indicates that the alteration is not at the nucleotide level and indeed this has been

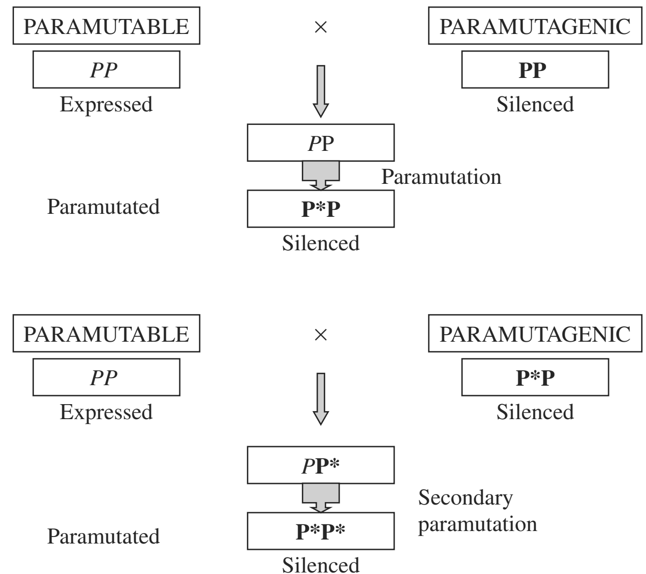

FIG. 1. The process of paramutation (for further explanation see text).

demonstrated to be the case in paramutation. Although paramutation was originally discovered in maize, several examples of paramutation in other plants have been discovered, overlapping with transgene silencing (see below) in some cases (see examples reviewed in Hollick *et al.*, 1997, Chandler and Stam, 2004, see also Sidorenko and Peterson, 2001), although it remains rare and exceptional.

Transgene silencing

The advent of the technology to transform plants with DNA sequences, i.e. the generation of transgenic or GM plants, allowed an entirely new direction for the exploration of genetics. However, the development of this technology, in the early years in particular, served to dramatically underline how the traditional Mendelian view of genetics and genomes was inadequate and poorly descriptive of reality. Anomalous, unexpected results abounded and initially were perplexing and highly challenging to the contemporary dogmas. These 'anomalies' could be visually dramatic and iconoclastic. Appropriately and not unexpectedly with hindsight, the most significant event was the discovery in *Petunia* that the addition of extra copies of the endogenous chalcone synthase gene, encoding the enzyme catalysing an essential step in the pathway leading to anthocyanin pigment biosynthesis, did not lead to expected results (van der Kroll *et al.*, 1990; Napoli *et al.*, 1990). The predicted result was that extra active copies of the endogenous gene would lead to a significant increase in the amount of chalcone synthase transcripts and protein, with the downstream effect of increasing the amount of anthocyanin pigment. The reality was that extra copies could have the *reverse* effect—rather than increase the overall level of transcripts and protein, these extra copies decreased them. This decrease could be so dramatic that it fell far below levels seen in normal untransformed plants, leading to the pigmentation of tissues being decreased. Moreover, this 'silencing' effect appeared stochastic in nature, with patterns and variegation appearing in the tissues of the transformed plants, much like previous observations of somaclonal variation and paramutation. This 'co-suppression' of an endogenous gene by extra copies of the same gene was also accompanied by observations that insertion of entirely foreign coding gene sequences did not necessarily lead to their expression.

Other experimentation with transgenic technology led to the discovery that this gene silencing effect on endogenous genes by inserted DNA was more consistent when the inserted gene copy was in reverse or 'antisense' orientation (e.g. van der Kroll *et al.*, 1988; Jorgensen *et al.*, 1996). Perhaps more remarkable was the realization that a whole copy of the gene was not even required to elicit this effect. Clearly, adding new fragments of DNA to the genome not only resulted in the silencing of their own expression, but also the specific silencing of endogenous genomic sequences to which they were homologous. An intact copy with no nucleotide mutation was being 'silenced' by the presence of a foreign sequence at another site in the same genome. Exactly how this extra DNA was delivered, e.g. via *Agrobacterium* or biolistic integration, did not matter. Still more surprising was the discovery that even DNA was dispensable as engineered RNA viruses, which never form DNA or integrate into the host genome, could also elicit the same effect in an infected plant (e.g. Jones *et al.*, 1999; Dalmay *et al.*, 2000; Gosselé *et al.*, 2002). This was a key observation, as it definitively showed that RNA sequences had to be centrally involved in this type of phenomenon in a form of 'epigenetic communication' in the cell. Indeed, it was implicit from early antisense experiments that duplex RNAs may be a key part of silencing (van der Kroll *et al.*, 1988).

Recently, evidence has been presented that silencing may also be triggered by the level of transgene transcripts exceeding a threshold level, with this threshold being set by the nature of the coding region for each sequence (Schubert *et al.*, 2004). There is also evidence that there is a balance between the exonuclease-mediated mRNA turnover and the silencing pathways (Gazzani *et al.*, 2004). Evidently, such quantitative effects and flux through different pathways are of prime importance in the generation of silencing in plants.

THE MOLECULAR EXPLORATION OF EPIGENETICS

The range of different 'anomalous' phenomena described above has provided a smorgasbord for many different researchers with access to molecular analysis. All these attempts to unravel each fascinating and infuriating 'exception' to the accepted model are now providing a clearer picture of how an epigenetic system operates in plants. Importantly, the huge body of work is beginning to show these are not separate 'anomalies' but rather the different faces of the same underlying system. In this section, we hope to provide a whistle-stop tour of the many different avenues which have been explored in much detail in the past 15 years or so. There is no doubt that this will appear superficial and trite to those already steeped in the field of epigenetics. However, the purpose of this discussion is to initiate those less familiar with these developments, before discussing the ramifications of these molecular studies in the wider context. This section has been deliberately split into four sections. The first three describe how the molecular studies of these diverse phenomena have all come to converge on three different ways in which non-DNA-sequence based (epigenetic) information can be encoded. These are DNA (cytosine base) methylation, chromatin (histone proteins and their post-translational modifications) and RNA and represent the 'three pillars' of epigenetics. The fourth section discusses how these may all interact to build an epigenetic code, structure the genome and provide a defence system against foreign nucleic acid sequences.

THE THREE PILLARS OF EPIGENETICS

DNA methylation

Patterns of DNA methylation in plants. Cytosine DNA methylation, the chemical modification of cytosine bases with a methyl group, has long been known in plant genomic DNA sequences. There is a substantial amount known about

basic patterns of cytosine methylation in their genomes (reviewed in Ruffini Castiglione *et al*., 2002), although these studies have been largely descriptive (e.g. discovery of unmethylated gene-rich CpG islands in plants; e.g. Antequera and Bird, 1988; Ashikawa *et al*., 2001). More significantly, it has been shown that these patterns of cytosine methylation are not static and immutable in the same plant genome. There is evidence that these methylation patterns across the genome can change substantially with both the developmental state and the environmental conditions (e.g. Burn *et al*., 1993). However, their actual relevance to the organism remained rather mysterious.

In plants, DNA methylation would appear to be more complex than in other organisms (indeed, not every eukaryotic group even shows detectable cystosine methylation) (Gruenbaum *et al*., 1981) as the sequence context of the cytosine methylation can encompass several variations. DNA methylation can be split into cytosines methylated at 'symmetric' and 'asymmetric' sites. CpG and CpNpG methylation (where N is any nucleotide) are the symmetric methylation sites, the most common, as the sequence is self-complementary with methylatable cytosines in pairs on opposite stands, whilst an asymmetric site is a cytosine in any other sequence context. Asymmetric methylation sites are found in plants though not as frequently as their symmetric counterparts.

Whilst cytosine methylation seemed initially somewhat unremarkable, the fact that in many cases of genetic loci with 'anomalous' behaviour there was a strong connection with variable states of methylation pointed to this modification being important in some way. Simple pharmacological studies utilizing azacytidine treatment of plant material, an agent that inhibits cytosine methylation, have been shown to temporarily derepress the silenced rRNA gene repeats in nucleolar dominance (e.g. Chen and Pikaard, 1997*a*) and the silencing of transgenes (e.g. Murfett *et al*., 2001). For more precise analysis, it is fortunate for researchers that restriction enzymes exist which are sensitive to whether the DNA sequence at the restricton site is methylated or not. Enzymes which cut at the same site but have differential sensitivity to methylation are known as isoschizomers and these pairs of enzymes have become a major tool in the identification of methylated DNA loci (e.g. Cubas *et al*., 1999). A more recent yet equally significant development has been 'bisulphite sequencing' which allows the site-specific identification of methylated cytosines on an amplified sequence (Frommer *et al*., 1992). Although not absolutely consistent, increased DNA methylation (hypermethylation) at a locus was often found to correlate with a reduction in expression of locus or its complete silencing. Some suspected loss-of-function mutations have turned out to be apparently spontaneous silencing events where the locus has become hypermethylated. The most famous examples are at the *SUPERMAN* locus in *Arabidopsis thaliana* (where loss of expression results in formation of extra numbers of functional stamens and carpels) (Jacobsen and Meyerowitz, 1997) and the *Lcycloidea* locus in *Linaria vulgaris* (where loss of expression results in 'peloria', with ventralization of flower development and a more radially symmetrical rather than strictly bilateral flower) (Cubas *et al*., 1999). Studies of 'imprinted' loci have shown with predictable consistency that the allelic copy with reduced or eliminated expression had higher levels of DNA methylation in endosperm (reviewed in Grossnikalus *et al*., 2001; Gehring *et al*., 2004). Either this must be acquired at some point in gametophytic development of the parental genomic contribution in which the locus is silenced, or selectively acquired post-fertilization, or the sequence is constitutively methylated and 'silent' only to be selectively demethylated and activated in the copy or copies from one parent. In hybrid systems, the methylation patterns of many loci have been shown to be altered in hybrid offspring compared with their state in the parental genomes (e.g. Comai *et al*., 2000). These alterations in the methylation state are often accompanied with substantial changes to the expression of the affected locus. In paramutation, it has been found that the paramutable alleles were distinguishable by their sequence in repetitive DNA structures (reviewed in Chandler *et al*., 2000) and these repetitive regions can be hugely distant from the coding region as shown by Stam *et al*. (2002). Most importantly, these repeat regions were susceptible to altered DNA methylation that correlated very strongly with changes in expression and acquisition of the paramutagenic state. Methylation patterns have also been associated with other more specialized phenomena such as sex determination in plants (reviewed in Ruffini Castiglione *et al*., 2002).

Mutational screens for altered methylation. However, by far the most dramatic and compelling evidence for the involvement of DNA methylation in establishing 'anomalous' behaviour at genetic loci comes from transgenic work (for review of early literature, see Fagard and Vaucheret, 2000). Not only has methylation been correlated on many different occasions with gene silencing, it has been possible to dissect the silencing phenomena and patterns of methylation. Where the coding sequence of a gene is expressed in a transgenic construct and elicits silencing of the endogenous copies and the transgenic copy, what is always seen is a drop in transcript levels. However, the production of transcripts continues, which would suggest that there is induction of a targeted destruction system. This type of silencing has been called PTGS or 'post-transcriptional gene silencing'. What has also been seen on several occasions in PTGS events is that the silenced DNA coding sequence, both transgenic and endogenous, acquires increased DNA methylation (for a review, see Wassenegger, 2000). Most intriguingly, where the inducing transgenic sequence is shorter than the endogenous copy or copies, not only is the region of homology to the inducing sequence methylated in the endogenous copy but the methylation spreads out beyond it (e.g. in viral silencing experiments by Jones *et al*., 1999). However, in PTGS the methylation is restricted to the DNA sequence that forms the mRNA transcript and does not normally spread into adjacent untranscribed regions such as the promoter (Paskowski and Whitham, 2001). The reverse situation is true when an untranscribed region of a gene controlling its expression, for example the promoter, is expressed in a transgenic construct and induces silencing of the previously expressed locus. [For clarification, these

experiments have involved using both transgenic constructs as 'inducers' (either integrated transgene locus or recombinant virus) and the target 'endogenous' locus, e.g. those described in Jones *et al*. (1999), Sijen *et al*. (2001) and Matzke *et al*. (2004).] Here, the transcript was not shown to be transcribed at normal levels from the start and hence this has been called TGS or 'transcriptional gene silencing' to indicate that transcription itself was suppressed (reviewed in Vaucheret and Fagard, 2001; Matzke *et al*., 2004). As might be predicted, DNA methylation was restricted to the untranscribed region and did not spread out beyond the region homologous to the inducing expressed sequence. This DNA methylation was induced whether the transgenic construct was DNA based or RNA based.

Screenings for genetic mutations that affected these various phenomena have also been a popular route for exploration and dissection of their genesis. Not surprisingly, some of the first loci that appeared from this work turned out to be genes encoding plant DNA methyltransferases (reviewed in Finnegan *et al*., 1998). In *Arabidopsis*, the *MET1* DNA methyltransferase has repeatedly emerged as critical for normal methylation patterns (e.g. Vongs *et al*., 1993). In *met1* mutant and antisense *MET1* lines there is a progressive loss of genomic DNA methylation from generation to generation (reviewed in Finnegan *et al*., 1998) although paradoxically there is a corresponding increase in methylation and gene silencing at certain loci such as *SUPERMAN* and *AGAMOUS*, with corresponding phenotypes (Finnegan *et al*., 1996; Jacobsen *et al*., 2000; Kishimoto *et al*., 2001). *met1* mutants have been shown to have the power to demethylate and release silencing at previously methylated, silenced loci, e.g. transgene loci (e.g. Furner *et al*., 1998). Loss of function of *MET1* in one parent can also dramatically affect the phenotypic outcome of imprinting (e.g. Vinkenoog *et al*., 2000; Kinsoshita *et al*., 2004), intraspecific interploidy crosses (Adams *et al*., 2000) and the outcome of allotetraploid hybrid crosses (Bushell *et al*., 2003). However, continual selfing of *met1* mutants also leads to an array of deleterious phenotypes, such as reduction in fertility and altered apical dominance (reviewed in Finnegan *et al*., 1998). Clearly, widespread demethylation can affect the plant profoundly. However, loss of MET1 protein affects predominantly CpG methylation sites—this enzyme is a member of the DNMT1 subfamily of 'maintenance methyltransferases' which maintain CpG methylation during DNA replication as they have a high affinity for the hemimethylated CG pairs, the asymmetric structure produced after DNA replication. *MET1* is but one of four members of the *DNMT1* subfamily in *Arabidopsis thaliana* (Genger *et al*., 1999) but from current evidence it appears to be the most important for maintaining existing methylation. In its absence, replicating DNA remains hemimethylated and further rounds of replication leaves DNA strands without a methylation mark.

By contrast to loss of function in *met1*, mutants in the CpNpG methyltransferase *CMT3* show no developmental or physiological aberrations (Lindroth *et al*., 2001). Even after several generations of inbreeding no visible changes were apparent, although decreased CpNpG methylation throughout the genome was observed. In the same way, two loci *DRM1* and *DRM2* encoding methyltransferases that methylate at asymmetric sites have been discovered and these also do not show mutant phenotypes (Cao and Jacobsen, 2002*a*, *b*). However, these enzymes appear to fulfil the role of *de novo* methyltransferases rather than maintenance methyltransferases, by acting to establish a methylation pattern rather than preserve an existing one. Their loss of activity does not reverse previously established 'silencing' at transgene loci, but their absence does prevent establishment (Cao and Jacobsen, 2002*a*, *b*).

In contrast to active mechanisms to establish and maintain methylation, there appears to be less evidence for active mechanisms of demethylation in plants. No demethylases have yet been uncovered in plants and there is no evidence for a system to wipe out established methylation marks even in reproduction. Evidence for active demethylation in plants comes from demethylation mediated by the TnpA protein of the *Suppressor-mutator* transposon (Cui and Fedoroff, 2002). This is in contrast to mammals where evidence, albeit limited, for methyl-dCpdG demethylases exists (Bhattacharya *et al*., 1999; Cervoni *et al*., 1999) and a high level of genome-wide demethylation in gamete formation is known (reviewed in Reik *et al*., 2001). However, there is recent evidence that there exists a system for 'editing' methylated cytosine, at least in imprinting systems in the endosperm. *DEMETER*, encoding a nuclear-localized DNA glycosylase, is essential for expression of the maternally transmitted allele of the imprinted *MEDEA* gene in endosperm (Choi *et al*., 2002). It may act to selectively excise a methylcytosine by inducing a single-stranded break by base excision, followed by action of an endonuclease to create a nick in the DNA. Indeed, it has been shown that DME and MET1 act antagonistically on *MEDEA* (Xiao *et al*., 2003).

However, interesting twists emerged from the focus on methylation. Firstly, early on it became apparent that methyltransferases were not the entire story. One mutant, repeatedly isolated in screens of *Arabidopsis*, shared many features of the *met1* mutant—releasing transgene silencing, depletion of global methylation levels and a range of phenotypic effects (e.g. Vongs *et al*., 1993; Furner *et al*., 1998; Jacobsen *et al*., 2000). However, subsequent analysis revealed that this locus, *ddm1*, did not encode a methyltransferase enzyme but was a member of a family of DNA-dependent ATPases involved in defining chromatin structure (Kakutani *et al*., 1995; Jeddeloh *et al*., 1999). A further shock for those wishing to make a simple link between methylation and the behaviour of loci came from the isolation of the *mom1* mutant, which released silencing of a methylated transgene without altering any kind of methylation (Amedeo *et al*., 2000). Both these loci will be discussed in more detail later in this review. Far from being a simple outcome of DNA methyltransferase action, the control of DNA methylation patterns and the presumed effects on gene expression are more complex than was previously anticipated. These complexities will be discussed in the next two parts on chromatin/histones and RNA.

A further revelation from these studies was that transposable elements and related sequences, including inverted repeats, were often reactivated in loss-of-function lines

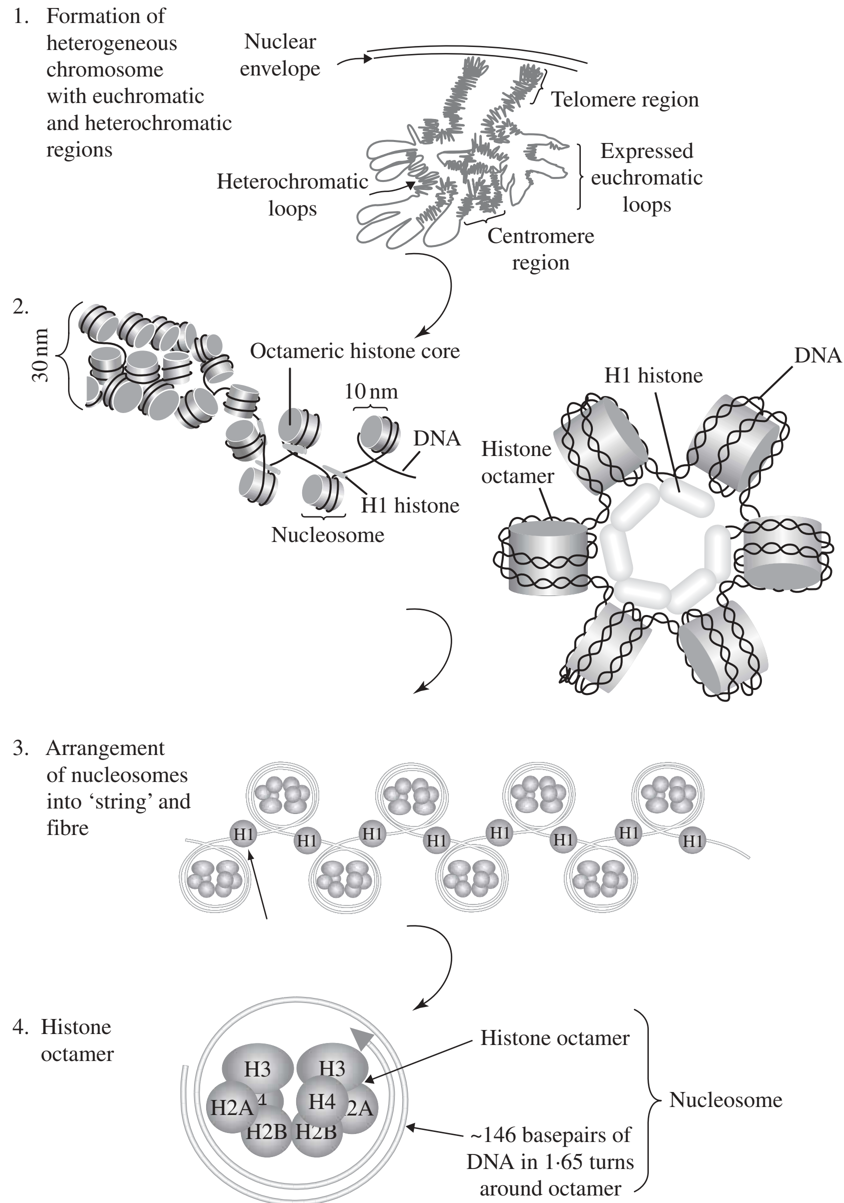

FIG. 2. Chromatin structure; from chromosome to nucleosome to histones.

(e.g. Steimer *et al*., 2000; Bartee and Bender, 2001; Tompa *et al*., 2002). This was compelling evidence that the activity of endogenous mobile elements could in some way be quelled by DNA methylation. An observation of particular interest is that many of these sequences were derived from the heterochromatic pericentromeric region (Steimer *et al*., 2000). Joining this is evidence from maize where screens for mutants capable of suppression of paramutation have revealed several loci that modify paramutation (Dorweiler *et al*., 2000; Hollick and Chandler, 2001). At least one suppressor of paramutation, the *mop1-1* mutant, also had the effect of reactivating previously dormant, silenced transposons and release transgene silencing without affecting global methylation (Lisch *et al*., 2002; Chandler and Stam, 2004).

Histones, histone modifications and chromatin structures

From DNA to chromatin fibre to chromosome (Fig. 2). It would not be an exaggeration to state that the general trend in genetics and molecular biology has been to see only the DNA sequence as the most significant structure of the chromosome and the nucleus. This over-simplified view is likely to lead to the impression that the DNA of the chromosomes is naked and relatively unstructured when nothing could be further from the truth. Whilst preparation of DNA for experimental manipulation such as amplification and sequencing requires purification and stripping away of all the associated proteins, it now appears that removing these proteins can be undesirable for full knowledge of how the sequence operates. Whilst the DNA sequence provides a linear information resource, how this polymer is structurally

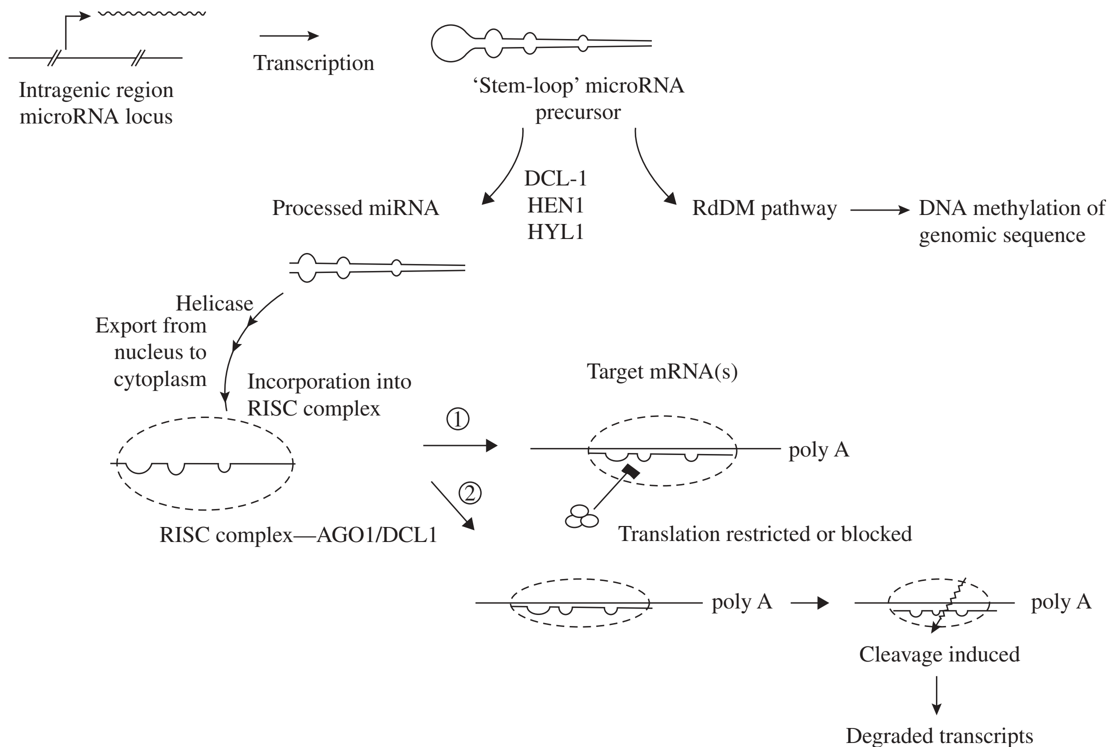

FIG. 3. The microRNA processing pathway.

organized and packaged into functional chromosomes depends largely on the histone family of proteins. These proteins wrap up DNA by the formation of an octamer of 'core' histone proteins—pairs of H3, H4, H2A and H2B proteins—that wraps typically approx. 140–150 base pairs (bp) of DNA in two turns around it (Fig. 3; for a review, see Aalfs and Kingston, 2000). This unit has been called the nucleosome. A length of DNA is wrapped into these nucleosomes, which are then spaced apart by linker sequences of around 20–35 bp in length. These linker sequences are associated with other histones, called 'linker' histones such as H1. By providing protein–protein contacts to the core histones, linker histones promote the formation of arrays of nucleosomes bonded by tight protein–protein interactions. In this way, arrays of nucleosomes form the next level of structural organization, the chromatin fibre (Horn and Peterson, 2002). As might be expected from a higher-order structure generated by protein–protein interactions, the structures of nucleosome arrays and chromatin are not static but highly dynamic. This is hardly surprising given the alteration in chromosome structure during the cell cycle, but even in interphase their structure has been shown to be in flux (Fransz *et al*., 2002; Kamakaka, 2003). Even those generally unfamiliar with this field will recognize the terms 'euchromatin' and 'heterochromatin' to distinguish the different ends of the spectrum in chromatin formation. Heterochromatic regions display particularly dense, highly condensed chromatin fibres, more readily identified through microscopy than euchromatic regions. They have been shown to be generally poor in transcribed coding genes and unusually rich in repetitive DNA sequences. These heterochromatic regions are not only impoverished in coding genes but they also show highly suppressed recombination rates (Anderson and Stack, 2002). Heterochromatic compartments of the chromosome are associated with stretches of repetitive DNA and can be of tremendous functional significance, most notably the repeat regions that comprise the centromere (Murata, 2002). The proteinaceous centromere of each chromsosome, where it links the two sister chromatids, is the key site for interaction between the chromosome and the nuclear cytosketetal array in both meiotic and mitotic division (for review, see Murata, 2002). By contrast, euchromatic regions are typically richer in coding gene sequences and have high rates of recombination, and are associated with far less repetitive DNA. In arabidopsis, Haupt *et al*. (2001) have charted the dramatic (53-fold) decrease in recombination frequency from the euchromatic regions to the heterochromatic centromere. Nevertheless, distinction between euchromatic and heterochromatic regions is not always clear-cut in plants; for instance, sequencing of the centromere of chromosome 8 of rice revealed not only the expected highly repetitive DNA sequences but also a number of active genes (Nagaki *et al*., 2004).

Chromatin remodelling and post-translational histone modifications. Although heterochromatic and euchromatic regions can be readily distinguished by morphology on the same chromosome where the regions comprise many kilobases to megabases of DNA, the dynamic activity at the level of the nucleosome and individual section of chromatin need more sophisticated molecular techniques for their

study. However, evidence for these interactions being of relevance to the 'anomalies' in genetic behaviour at some loci had already started to accumulate. The discovery that the *ddm1* locus (see above) is homologous to the SW1/SNF1 ATPases involved in 'remodelling' chromatin (Jeddeloh *et al*., 1999) was compelling evidence that alterations at the nucleosome level could affect DNA methylation and gene expression. *ddm1* mutants also show substantial disintegration of centromeric heterochromatin which fits with a central role in regulation of chromatin (Mittelsten Scheid *et al*., 2002). Exactly what is meant by 'chromatin remodelling' and what is its functional relevance to the sequence associated with the chromatin? Simply, the position of histones and nucleosomes on a piece of DNA can act as a spatial block that suppresses the access of transcription factors to the promoter for transcription (see Aalfs and Kingston, 2000). 'Remodelling complexes' consisting of heterogeneous assemblies of chromatin-modifying proteins, often in co-operative interactions with transcription factors, can act to modify the local structure of DNA–nucleosome interactions such as sliding the nucleosome along the DNA to an altered position (for extensive discussion, see Aalfs and Kingtson, 2000; Owen-Hughes, 2003). This can unlock previously blocked transcription sites, or effect the reverse. In other organisms, remodelling can take the form of substitution of histones in nucleosomes (e.g. the substitution of one variant of a histone protein for another with different properties; for reviews, see Wolffe and Pruss, 1996; Kamakaka and Biggins, 2005) or the enzymatic modification of existing histones, with post-translational modifications on the tails of the histones such as site-specifc methylation and acetylation (for extensive reviews, see Goodrich and Tweedie, 2002; Loidl, 2004). In plants, both systems of modification have been uncovered. An introduction to plant chromatin and its involvement in controlling gene expression and development, with detailed examples, can be found in Li *et al*. (2002).

Screens for mutants that release the silencing of transgenes has revealed a histone deacetylase enzyme, AtHDA6 (Murfett *et al*., 2001; Probst *et al*., 2004), as being important, whilst screens for release of silencing at two endogenous loci has revealed KRYPTONITE/SUVH4, a histone methyltransferase that possesses specificity for lysine 9 of the histone 3 protein (Jackson *et al*., 2002; Malagnac *et al*., 2002). Reduction or increase of expression of the histone methyltransferase SUVH2 has been shown to affect transgene silencing in a dosage-dependent manner (Naumann *et al*., 2005). Yet these represent just a fraction of a myriad, either known to have activity in post-translational modification of histones through enzymatic assays or genetic studies (for review, see Lusser *et al*., 2001), or putative activity discerned through their homology (see The Plant Chromatin Database, http://www.chromdb.org).

The various modifications of histone tails has lead to the histone code hypothesis: interplay between the different post-translational modifications forms a dynamic code that acts to 'fine-tune' gene expression (Turner, 2000; Jenuwein and Allis, 2001). Histone tails may have a greater degree of structure than previously suspected and *in vivo* may form a highly structured, interlinked complex of nucleic acid–protein interactions. A site modification or substitution in one region of a chromatin fibre could generate a structural change in a relatively distant region. The actual 'histone code' would appear to be read by various proteins with binding specificities to these modifications, with proteins sharing bromodomain motifs likely to bind acetylated lysine residues (Turner, 2000) and those with the chromodomain motif performing the same function for methylated lysine residues (Jenuwein and Allis, 2001). In arabidopsis, use of monoclonal antibodies raised to the different modifications with *in vivo* labelling of chromosomal domains by Soppe *et al*. (2002) has shown that heterochromatic and euchromatic regions have distinct patterns of histone modifications (see Table 4).

TABLE 4. *Patterns of histone modifications in arabidopsis chromosomes as identified by monoclonal antibodies raised to different modified histones (data from Soppe* et al., *2002)*

Chromatin modification	Euchromatin	Nucleoli	Heterochromatin
H4 Ac5	High	Low	Low
H4 Ac8	High	Low	Low
H4Ac12	High	Low	Low
H4Ac16	High	Low	Low–high
Tri-/tetra-Ac-H4	High	Low	Low
H3Ac9	High	Low	Low
H3 methyl K4	High	Low	Low
H3 methyl K9	Low	Low	High

More recent data from Naumann *et al*. (2005) on euchromatic and heterochromatic marks is included in Table 5 for contrast; data from this study on the effects on the loss of function of three SUVH genes on histone modifications are also tabulated. So far, SUVH2 appears to be the most important histone methyltransferase, with a strong effect on all heterochromatic histone methylation marks (see Table 5).

Fully understanding the 'histone code' and its control in plants, where it may be more complex and involve more marks at different sites than in other eukaryotes (Loidl, 2004), is still far off. In plants, understanding the interdependence or independence of the different histone marks will be essential for gaining fluency in the 'histone language' (Loidl, 2004).

These antibodies targeting specific types of chromatin are being increasingly used in the chromatin immunoprecipitation assay (ChIP) where pull-down of chromatin bound to DNA, using antibodies specific to histones with particular marks, has allowed identification of the DNA sequences (through PCR) associated with known chromatin states in both euchromatic (e.g. Chua *et al*., 2004) and heterochromatic (e.g. Lippman *et al*., 2004) contexts. This technique is now supplanting older tools to study chromatin structure at specific loci such as nuclease assays (different chromatin techniques are described in detail by Bowler *et al*., 2004). However, traditional nuclease assays (where sensitivity of chromatin-bound DNA to digestion by nuclease enzymes is analysed) has revealed that the paramutable *B-I* allele of maize has a more open chromatin state than the paramutagenic *B*' allele (Stam *et al*., 2002). Furthermore,

TABLE 5. *Patterns of histone modifications in arabidopsis heterochromatin and euchromatin in wild-type and SUVH mutants (data from Naumann* et al., *2005)*

Chromatin modification	Euchromatic or heterochromatic in wild type?	Loss of SUVH1	Loss of SUVH4	Loss of SUVH2
Mono-methyl H3 K9	Heterochromatic	Weakly reduced	Weakly reduced	Strongly reduced
Di-methyl H3 K9	Heterochromatic	Weakly reduced	Weakly reduced	Strongly reduced
Mono-methyl H3 K27	Heterochromatic	No change	No change	Strongly reduced
Di-methyl H3 K27	Heterochromatic	No change	No change	Strongly reduced
Mono-methyl H4 K20	Heterochromatic	No change	No change	Strongly reduced
Tri-methyl H3 K9	Euchromatic	No change	No change	No change
Tri-methyl H3 K27	Euchromatic	No change	No change	No change
Di-methyl H3 K36	Euchromatic	No change	No change	No change
Tri-methyl H3 K36	Euchromatic	No change	No change	No change
Di-methyl H4 K20	Euchromatic	No change	No change	No change
Tri-methyl H4 K20	Euchromatic	No change	No change	No change

spontaneous reversion of *B-I* to *B*' has been studied and the new spontaneous revertant alleles show a distinct chromatin structure intermediate between the two even *before* there is a change of DNA methylation. The establishment of a new chromatin state before a DNA methylation change is imposed is further evidence that DNA methylation is just one component of a larger and more complex epigenetic system. Nuclease assays have also been able to show that silenced transgenes can have a different chromatin state, with more heterochromatic features as expected from their silenced state (reviewed in Muskens *et al*., 2000).

Histone substitution and histone variants. The idea of a 'histone code' becomes more complex with the realization that histones themselves can be substituted for variants with different properties. For example, the replication of the genome during mitosis allows a window for substitution of histones. A total of 40 histone genes have been identified in the Arabidopsis genome (13 H2A, 11 H2B, 13 H3 and eight H4, with five H1) (see The Plant Chromatin Database, www.chromdb.org). The combinatorial possibilities available to form nucleosome assemblies with these various core and linker histones are enormous even before the various post-translational modifications are considered. In plants, little is known but what has been published so far is exciting and tantalizing. H1 variants (linker histones) have been explored in plants more than any other organism. Spatial variation in H1 variants has been explored using immunological techniques: a variant specific to the nucleolar region has been uncovered which excludes other H1 forms (Tanaka *et al*., 1999) whilst the distribution of three variants (H1-1, H1-2 and H1-3) in arabidopsis has shown that the former two have a wide distribution across the chromosomes, whilst the latter is absent from regions with repetitive sequences such as 5S rRNA genes (Ascenzi and Gantt, 1999). Temporal, developmentally controlled variation in H1 variants was uncovered in lily, where a novel H1 (meiotin-1/PMCP1) has been identified that appears only at the time of the switch from the mitotic to the meiotic programme (Sasaki *et al*., 1990; Riggs, 1994). It appears to be a centromerically localized histone and possibly critical for chromatin condensation in meiosis (Riggs, 1994; Suzuki *et al*., 1997). Although meiotic-specific variants have not been identified in other plant species, the importance of linker histone variants to the meiotic programme has been highlighted by antisense work. Down-regulation of H1A and H1B levels in tobacco by antisense resulted in altered stoichiometry of the various linker histones (H1A–H1F) (Prymakowska-Bosak *et al*., 1999). This changed gross chromatin morphology to a less condensed state and resulted in various phenotypic effects (increased branching, reduced plant size, and aberrant floral development) but most dramatically affected male meiosis, greatly reducing pollen fertility.

Core histones have been investigated, to a lesser extent. A mutation in a H2A gene in arabidopsis, *rat5*, increased the resistance of the genome to T-DNA integration which suggests a role for histones in regulating access of the genome to invasive or foreign DNA (Nam *et al*., 1999; Mysore *et al*., 2000). In plants, as in other eukaryotes, the centromeres and centromeric repeats are defined by their unique centromere-specific variants and one H3 variant of this kind has been described from *Arabidopsis thaliana* (Talbert *et al*., 2002). This H3 variant has been shown to bind specifically to the 180-bp repeat of arabidopsis centromeres in ChIP experiments (Nagaki *et al*., 2003). Spatio-temporal patterns of serine phosphorylation of CENH3 may be important for centromere function in both mitosis and meiosis (Zhang *et al*., 2005).

One newly discovered histone variant, H2A.Z, has been shown to have a profound effect on structuring chromatin at a higher level (Fan *et al*., 2002) and functional homologues are likely to exist in plants. It has the unique property of being able to promote stable intramolecular folding of nucleosome arrays whilst preventing formation of a highly condensed and compacted chromatin structure by oligmerization. In this way, it may enable formation of chromatin domains that are 'open' and poised for transcription.

The histones and the structures they form again show that typical Mendelian behaviour and loci that 'break' these rules are defined far more by their chromatin context than by their actual base sequence. Furthermore, the two seem inseparable when the unifying properties of chromatin are considered. The structural and architectural properties of

DNA sequences on a chromosome are created by their histone–nucleosome–chromatin associations. The same is true of their transcriptional properties or transcriptional potential. What is most significant is that there is a potential for great lability in the structure of chromatin—it is not a static entity as it has been previously considered—and tremendous variation. One of the great challenges for biology is to identify to what extent the DNA sequence can constrain and control this variation. Certainly, where mutations in machinery that regulate chromatin are combined there is the potential for substantial reorganization accompanied by dramatic phenotypes (e.g. Mittelsten Scheid *et al.*, 2002). This suggests that nucleotide sequences may just be a framework for a more complex code—encoded within proteins—that has a major regulatory effect on the underlying framework at both a local and chromosome-wide scale.

The RNA world

Until recently, the central dogma—that information passes in a linear fashion from DNA to protein via an RNA intermediate (messenger RNA, aided by tRNAs and rRNAs)—was accepted almost without question. The major role for RNA was as an intermediate to protein formation, although exceptions such as ribozymes were documented. It was the advent of plant transformation and the strange behaviour of some transgenes that, in part, initiated a new appreciation of the dynamic role of RNA in the genome. In plants with PTGS-silenced transgenes, both endogenous and non-endogenous, Hamilton and Baulcombe (1999) identified small RNA species of around 25 nt size that corresponded to the transgene transcript. However, these short RNA sequences, now known as short interfering RNAs (siRNAs) were also in the antisense orientation whereas the transgene was in the sense orientation. This indicated that these antisense RNAs were derived not directly from the transcripts but from active processing of the sense precursor.

In this section, an attempt is made to introduce this now complex and fast-moving field in the RNA world; more extensive reviews can be found in Baulcombe (2004), Dugas and Bartel (2004), Kidner and Martienssen (2005) and Matzke and Birchler (2005).

Constituents of RNA processing pathways. The role of RNA in silencing was made abundantly clear in experimental systems where engineered viral infections, comprising only of RNA, were capable of inducing both PTGS and TGS of endogenous expressed sequences (either a truly endogenous gene, or a stably integrated expressed transgene) (e.g. Jones *et al.*, 1999, 2001). By development of such systems in arabidopsis, mutant screens for plants defective in RNA-triggered silencing were possible (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). These strategies have yielded a number of mutants and the identification of some of the proteins involved in recognition and processing of the types of RNA that triggers silencing. *sde1* proved to be an RNA-dependent RNA polymerase (RdRp), whose cellular function would appear to be to synthesize double-stranded RNA from transcripts recognized as 'aberrant' in some way (Dalmay *et al.*, 2000). *sde3* would appear to be an RNA helicase, again fitting with a role in processing RNA (Dalmay *et al.*, 2001).

Perhaps the most surprising discovery by Fagard *et al.* (2000) was that some of the silencing-defective mutants from these screens were phenocopies of previously described *argonaute1* mutants, which have various developmental defects such as narrow leaves and abnormal stem and inflorescence structures. *AGO1* turned out to be homologous to loci from other organisms that are involved in RNA-based silencing. Proteins of this ARGONAUTE (AGO) family show a conserved PAZ (**P**IWI–**A**RGONAUTE–**Z**WILLE) domain (Carmell *et al.*, 2002). It therefore appears that the system of RNA-based silencing is well conserved across the disparate parts of the eukaryotic phylogenetic tree. The role of AGO1 protein would appear to be conserved in plants; as in other eukaryotes it physically associates with other proteins to form the RNA-induced silencing complex (RISC). This complex is involved in catalysing the degradation of mRNA transcripts when bound by homologous small interfering RNAs. The highly conserved PAZ domain of AGO proteins appears to be the receptor site for siRNAs and aligns them with their homologous mRNA transcript for the subsequent cleavage event. Modelling work based on an archaeal PIWI protein also suggests that the PIWI domain of AGO proteins may possess nuclease activity and cleave the target mRNA (Parker *et al.*, 2004). AGO proteins are therefore integral to the function of RISC complexes. In contrast to some other organisms, multiple AGO genes are found in *Arabidopsis* and this suggests considerable functional diversification of this family in plants (see discussion of AGO4 function below). Baulcombe (2004) speculates that some may have developmentally specialized roles in silencing, and some may not even be involved in silencing complexes at all.

The conservation of AGO proteins and the RISC complex in plants necessitates a system for the production of siRNA species from longer double-stranded RNA species. The SDE1 RdRP would catalyse the synthesis of dsRNA from 'aberrant' transcripts that are not already double-stranded; for instance a lack of a 5′ cap or 3′ poly (A) tail would mark out a transcript as being aberrant and allow access to RdRP machinery for synthesis of a complementary RNA strand. However, formation of siRNAs from longer RNAs that are completely or partially double-stranded requires the action of another enzyme. In other eukaryotes, this function is carried out by the RNase III Dicer proteins that specifically recognize and cleave dsRNA. As would seem fitting with the evidence for conservation of RNA silencing mechanisms, plants also possess functional equivalents of Dicer.

The first Dicer to be identified in plants, *DICER-LIKE 1* (*DCL-1*), had been isolated multiple occasions as its mutant alleles have pleiotropic effects on many parts of plant development (Schauer *et al.*, 2002). Along with the phenotypes of *ago1* mutant alleles, these effects in *dcl1* mutants on endogenous development implicate not only *endogenous* production of dsRNA species but also their processing via Dicer and RISC complexes as essential for normal plant development. Silencing and degradation of target transcripts is not just a defence mechanism against non-self

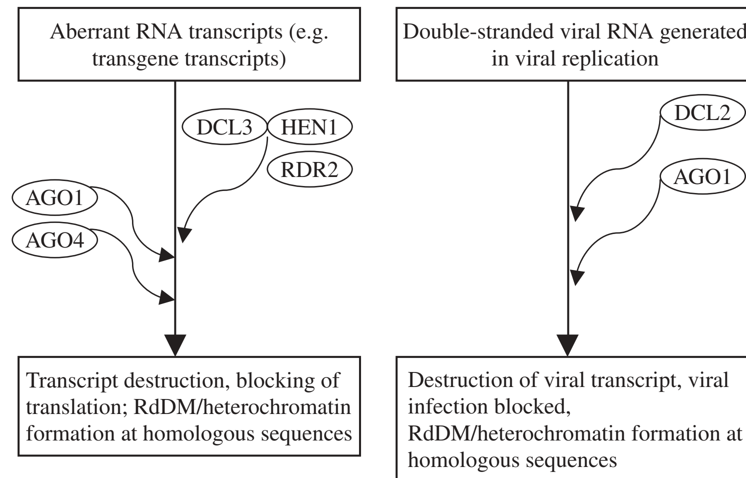

FIG. 4. Other siRNA-generating pathways in plants.

sequences but appears to be an integral part of controlling endogenous gene expression. Importantly, the fact that the known *DCL-1* mutant alleles *do not* compromise PTGS or TGS of transgenes indicates that it does not process all the triggering dsRNA species and that there is a separate pathway unique for the control of endogenous development. The endogenous targets for processing by AGO1 and DCL1 in plants turned out to be genes encoding microRNAs (miRNAs), non-coding sequences first identified in nematodes. These sequences as transcribed naturally form hairpin double-stranded RNA, which are then enzymatically processed by DCL1 in the nucleus to form corresponding smaller RNAs of around 21 nucleotides. These processed small miRNA species are then incorporated into RISC complexes to catalyse degradation of homologous mRNAs. In plants where *ago1* function has been lost, the coding mRNA transcripts that are the targets of endogenous miRNA species accumulate to abnormally high levels (Vaucheret *et al*., 2004). Further proteins involved in processing these miRNAs have now been identified, for instance the *HUA ENHANCER1* (*HEN1*) (Boutet *et al*., 2003) and *HYPONASTIC LEAVES1* (*HYL1*) (Han *et al*., 2004) proteins. Export of processed miRNA sequences from the nucleus to the cytoplasm where they are active against target mRNAs may also require HASTY, a plant exportin homologue (Bollman *et al*., 2003). RdRp proteins are not implicated as the dsRNA is already present and does not require *de novo* synthesis from a single-stranded template (Xie *et al*., 2004). The role of these non-coding microRNA genes in controlling endogenous gene expression at a post-transcriptional level will be discussed later.

Unlike in other organisms, there are several Dicer-like genes in the plant genome. This correlates with evidence for distinct size classes of small RNAs from plants produced by enzymatically distinct DICER proteins (Hamilton *et al*., 2002; Tang *et al*., 2003). Recent work using mutants in *DICER-LIKE 2* (*DCL2*) and *DICER-LIKE 3* (*DCL3*) show that these have separate functions. DCL2 has been implicated in anti-viral RNA processing and defence (Xie *et al*., 2004). It would seem likely that AGO1 is again a partner in this pathway as *ago1* mutants are hypersensitive to viral infection (Morel *et al*., 2002). The DCL3 protein would appear to be the core component of a processing system for 'aberrant' RNA transcripts that do not form perfect dsRNA templates such as from many transgene transcripts, transposable elements and inverted repeats. DCL3 appears to operate in conjunction with HEN1 and the RdRp RDR2 in generating corresponding small RNAs (Xie *et al*., 2004). Again, AGO1 is a part of this pathway as *ago1* mutants release transgene silencing (Fagard *et al*., 2000). In parallel to the finding that multiple DICER-LIKE enzymes function in plants, the AGO family also shows more than one member with different functions. The discovery of *ARGONAUTE 4* (*AGO4*) shows the complexity of silencing pathways. Loss of *ago4* function affects the methylation differentially at inverted repeats and direct repeats; it would appear that there may be different pathways that control different repetitive sequences (Zilberman *et al*., 2004).

In addition to pathways that promote PTGS, there appear to be repressors of RNA processing pathways. A calmodulin-like protein from *Nicotiana* that represses PTGS has been described (Anandalakshmi *et al*., 2000).

Target RNAs and downstream effects. As already described above, at least three pathways exist with different target RNAs processed in each (Figs 3 and 4). The microRNA pathway processes microRNA precursor transcripts, a pathway largely restricted to the nucleus (Kidner and Martienssen, 2005). In plants, the number of identified microRNA genes is growing rapidly (for comprehensive review, see Dugas and Bartel, 2004). This has been achieved by different methods of identification, from direct cloning of miRNAs to genomic analysis to identify putative miRNA precursor genes with their distinct stem–loop structure. How does the miRNA pathway operate to bring about a phenotype? The precursor miRNA transcripts are cleaved at the dsRNA hairpin structure by DCL1 to form 21 nt dsRNAs. The antisense strand incorporated into the RISC complex then targets complementary nucleotide regions on mRNAs. Binding of the small RNA either induces the block of translation or, more commonly, triggers destruction of the

transcript by the Dicer RNase activity. In this way, an expressed miRNA gene can bring down homologous transcripts and prevent translation and accumulation of proteins. It is interesting to note that several mutants isolated in the past have now been shown to map their nucleotide changes to regions of complementarity to their miRNA (e.g. dominant gain-of-function alleles of *PHABULOSA* and *PHAVOLUTA*; Rhoades *et al*., 2002), resulting in reduced binding and abnormally increased levels of the transcript and protein. However, the mechanism for miRNA action may be more complex following the discovery that the miRNA also interacts with the genomic DNA and is likely to generate transcriptional changes (Bao *et al*., 2004). There is now evidence (see Dunoyer *et al*., 2004) that miRNA-precursor transcripts may be cleaved to form 21-nt and 24-nt populations and these different sizes may participate in different activities. The 21-nt miRNA may be directing mRNA target cleavage whilst 24-nt miRNA may interact with the target genomic DNA sequence.

The viral silencing pathway also has the benefit of a dsRNA template as when ssRNA viruses replicate they form a dsRNA intermediate. This is recognized and viral transcripts are degraded in the DCL2 pathway (Xie *et al*., 2004). However, in the DCL3 pathway the triggering RNAs recognized are not necessarily double-stranded hairpin structures. This pathway may use single-stranded RNA recognized as 'aberrant' by virtue of disrupted splice sites and aberrant open reading frames or dsRNA formed by separately transcribed sense and antisense single transcripts (Bender, 2004). As yet the exact properties which make them recognized and processed are not fully known. Interestingly, recent work in arabidopsis indicates that uncapped mRNAs not involved the translation machinery and RISC-cleaved mRNA can be either routed into RNA interference, or degraded by the XRN4 ribonuclease and hence stopped from having silencing activity (Gazzani *et al*., 2004). These aberrant RNAs are the transcripts most likely to be formed from transposable elements and repetitive structures in the genome, e.g. inverted repeats from transgene integration events. It is important to note that finding the triggering RNA species and derived small RNA species from endogenous sequences by experimental means has proved to be difficult compared with miRNAs and transgene-derived RNAs but this has now been achieved. This is probably due to the rarity of transcripts and the efficiency of their processing.

A particularly important facet of RNA-based silencing is that it can act in a self-propagating positive feedback loop. Therefore a small amount of initial RNA can, if there is a target transcript, generate large populations of small RNAs (Klahre *et al*., 2002). This is by virtue of a system where the initial siRNA may act as a primer for copying the target mRNA into a dsRNA by 'transitive' RNA-dependent RNA polymerase activity in a 5′ to 3′ direction (Vaistij *et al*., 2002). These newly synthesized dsRNAs then feed back into the system by producing more siRNAs, corresponding to the entire length of the target mRNA, through Dicer action. These new secondary siRNAs can then target other genes to which they are homologous (Van Houdt *et al*., 2003). The RdDM systems also use these secondary siRNAs to direct genomic epigenetic changes. Equally, spreading in a 3′ to 5′ direction has been observed (e.g. Braunstein *et al*., 2002; Vaistij *et al*., 2002) that cannot be explained by a priming mechanism. It is possible that both directions of spreading can be explained by RdRp action without a primer, from the 3′ ends of cleaved mRNAs from the RISC complex, forming dsRNAs that are then substrates for Dicer action (Tang *et al*., 2003). It appears that miRNA pathway does not show this self-amplifying 'transitive' system (Tang *et al*., 2003). This may be due to methylation of miRNAs by the methyltransferase HEN1, an exclusive part of the miRNA pathway, which may act to inhibit RdRP activity (Yu *et al*., 2005).

Small RNA species are now known to be systemic agents that can spread from cell to cell (Klahre *et al*., 2002) and strength and propagation of the systemic signal is aided by the positive feedback loop described above (García-Pérez *et al*., 2004). Systemic signalling appeared to be most important for the DCL2 defence system controlling viral pathogens. However, it has now been demonstrated that all classes of small RNAs produced by the three DICER pathways are chaperoned through the phloem by small RNA binding proteins that specifically bind small single-stranded RNAs (Yoo *et al*., 2004). Similar proteins are likely to chaperone small RNA species from cell to cell as cell–cell transmission via plasmodesmata of naked small RNAs has not been demonstrated.

How many RNA processing pathways exist? Given that plants have multiple DICER, RdRP and ARGONAUTE family genes, how many pathways exist, or at least overlap, in the processing of RNA sequences? At least one new pathway has been identified by Vazquez *et al*. (2004), Peragine *et al*. (2004) and Williams *et al*. (2005). A new class of endogenous non-coding RNA sequences was discovered that, like miRNAs, targets endogenous mRNAs. As with miRNAs, they require AGO1, DCL1, HEN1 and HYL1 for their operation, and are transcribed from loci that are different from their target in both location and sequence. However, these non-coding RNA sequences differ from conventional miRNAs in that they do not form perfect stem–loop structures but instead are transcribed from both strands and also require the RdRP RDR6 and SGS3 proteins for their biogenesis. Therefore their biogenesis and activity requires components from two pathways that previously appeared distinct. As a consequence, these endogenous RNA loci have been christened trans-acting short interfering RNAs (tasiRNAs). Intriguingly, tasiRNAs may be hugely abundant in the genome (Vazquez *et al*., 2004) and the conservation of one tasiRNA between monocots and dicots also suggests that they have a major role in regulating expression of endogenous genes at a post-transcriptional level (Williams *et al*., 2005).

FITTING TOGETHER THE EPIGENETIC JIGSAW

What should be immediately apparent from the above discussions is that there are several different molecular components that appear to be involved in determining how gene

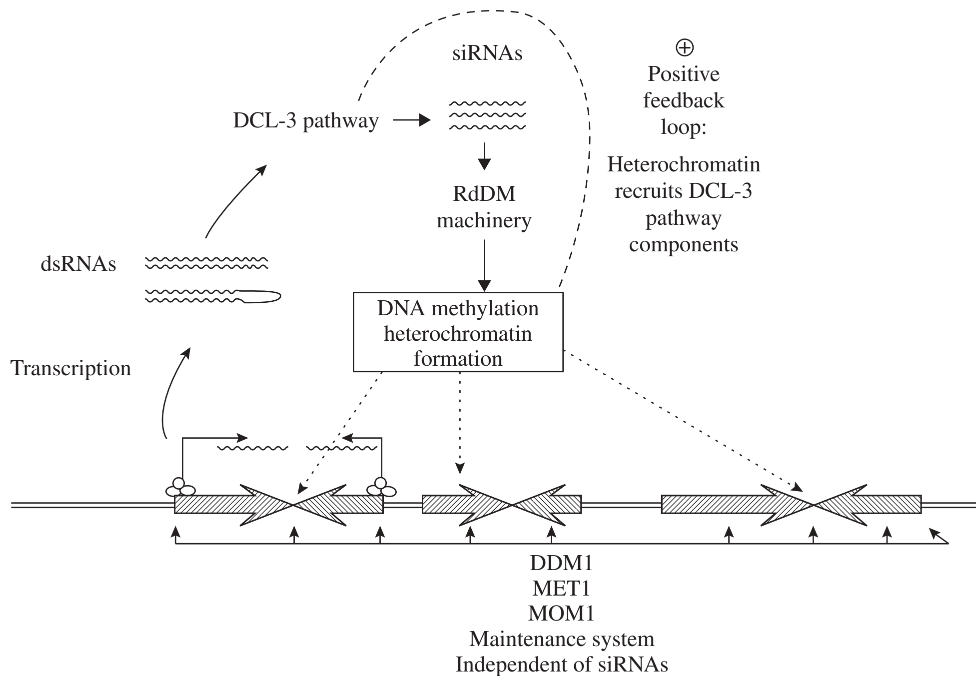

FIG. 5. Heterochromatin formation and maintenance pathway.

sequences on the chromosome actually operate and behave, whether in a typical Mendelian sense or in a manner deviating from this simplistic model. In some way, these molecular components interact to form an 'epigenetic' regulatory system. This system also has branches involved in genomic defence. The idea of reticulate relationships in epigenetic control is certainly nothing new. Clearly, this set of molecular machinery that is spanning not only the nucleus but the entire organism cannot be reduced to a simple universal 'code' in the same way that the DNA and RNA codes were 'cracked'. So, the great challenge now is to fit these pieces of the jigsaw together into a coherent picture of how the epigenetic system operates at a molecular level. What is most important to bear in mind at all times is the question—is the system maintaining an existing state or is there an induction event where the system is being geared to make a change in an existing state?

Maintaining the epigenetic status quo

In the maintenance of an existing state, there is now some evidence that there is a self-reinforcing loop that exists between DNA methylation and marks on the histones in plants (Fig. 5). The CMT3 protein carries a chromodomain and this has attracted speculation that methyated histones are bound by CMT3 which brings about CpNpG methylation. This binding mediated by the chromodomain only comes about when both H3 lysine 9 and 27 are methylated. Direct interactions are possible, although it would appear that other histone marks are necessary for its recruitment (Lindroth *et al*., 2004). However, the MET1 protein does not have a chromodomain and it is possible that MET1 either does not require a histone methylation mark to reinforce its methylation patterns, or it may utilize histone acetylation patterning instead (Bender, 2004). In this way, a histone modification on the chromatin can reinforce underlying patterns of DNA methylation. Equally, information and reinforcement flows in the opposite direction. In animal epigenetic systems, methyl-cytosine binding proteins recruit histone modification machinery. In plants, methyl-cytosine-binding proteins are abundant (Nan *et al*., 1998) but their role in the overall scheme has yet to be fully characterized. Several studies in arabidopsis have identified proteins with conserved methyl CpG-binding domains (MBD proteins); of 12 MBD genes, ten are expressed and three (AtMBD5, AtMBD6 and AtMBD7) have been shown to bind symmetrically methylated CpG sites (Berg *et al*., 2003; Zemach and Grafi, 2003). Zemach *et al*. (2005) have recently shown that different MBD proteins show different sub-nuclear localization patterns, and confirmed that, as expected, AtMBDs 5–7 are associated with methylated CpG-rich heterochromatin. Little can be inferred from the sequences as to how these MBD proteins may operate, as plant MBD proteins appear to have evolved separately from their mammalian counterparts and do not share other conserved regions outside of the methyl CpG-binding domain (Berg *et al*., 2003; Scebba *et al*., 2003). However, Zemach and Grafi (2003) have shown that at least one protein of this kind (AtMBD6) from arabidopsis interacts with protein complexes with histone deacetylase activity. This supports earlier pharmacology-based evidence for silencing by histone deacetylases being mediated in part by DNA methylation states. Self-reinforcement cycles of DNA methylation and histone deacetylation also appear to operate in plants: there is evidence from the rRNA repeats where the HDT1 and HDA6 histone deacetylases operate in concert with methyltransferases to form a repressive state (Lawrence *et al*., 2004; Probst *et al*., 2004).

The global effect of DDM1 on chromatin and DNA methylation has been fitted into the scheme; it has been hypothesized that its role is to 'open up' the chromatin structure to methyltransferases and to histone modifying enzymes. Equally, DDM1 may operate as an assembly platform for MBD proteins and guide them to their binding sites, and by facilitating such interactions protects the DNA from demethylating activities (Zemach *et al*., 2005). This view is engendered by evidence that DDM1, the methyl-CpG-binding AtMBD5 and AtMBD6 and as yet-unidentified HDAC enzyme(s) are likely to form a complex that binds methylated DNA (Zemach *et al*., 2005).

The role of MOM1 is even more mysterious as it can affect gene silencing without any DNA methylation changes. *mom1* mutants have no appreciable affect on genomic DNA methylation levels nor on the global organization of heterochromatin, hinting at a novel level of epigenetic control (Probst *et al*., 2003). As a protein its sequence holds few clues as to how it may operate. The fact that in *mom1 ddm1* double mutants, the effects on release of silencing, plant morphology and chromatin changes are synergistic indicate that DDM1 and MOM1 do not act in the same linear pathway (Mittelsten Scheid *et al*., 2002). MOM1 may comprise part of a separate regulatory mechanism independent of DNA methylation, ensuring that rapid epigenetic deregulation does not occur in plants with methylation deficiencies.

If histone marks and the interactions of nucleosomes in generating chromatin structures are so important for gene transcription, what preserves the state when the genome replicates during cell division? Clearly, during the formation of a tissue, a derived population of cells must retain extraordinary fidelity of these states to retain a co-ordinated programme of development. Certainly the feedback loop between DNA methylation and histone modifications must play a role. Symmetric methylation can be restored by the enzymes which recognize hemimethylated sites after DNA replication. However, it is harder to see how absolute fidelity of an 'information state' in chromatin is retained during DNA replication when this information is randomly distributed between the nucleosomes of the daughter strands and diluted by incorporation of new nucleosomes. The role of preserving chromatin states in this way may be the domain of another two groups of proteins, the Polycomb-Group (Pc-G) and the Trithorax-Group (TrxG). Originally identified in *Drosophila*, their functional conservation in plants has been repeatedly demonstrated. In particular, Pc-G mutants in plants have been connected to disturbances in phenomena with proven epigenetic basis (e.g. imprinting) and they frequently display significant developmental aberrations (Hsieh *et al*., 2003). Pc-G proteins appear to operate by maintaining a repressive chromatin state on target genes where this 'off' state has been previously established. Conversely, the Trx-G proteins appear to operate by maintaining an active chromatin state. However, this may be a gross oversimplification of how the two groups control states of gene expression. Although at the molecular level the plant Pc-G and Trx-G proteins are hardly understood compared with their counterparts in *Drosophila*, it does appear that they operate in a similar manner. This means that they do not act as singular entities but in multiprotein complexes with various proteins with DNA-binding, histone-modifying and chromatin remodelling properties. However, in plants it is not yet known which DNA elements are bound (in *Drosophila*, conserved elements are well described). Current evidence points to the plant PcG genes as being important in establishing the H3K27 mark but only in euchromatic contexts (Lindroth *et al*., 2004). Their exact mode of operation is still enigmatic; notably, the mechanism by which they stably maintain the fate of chromatin through mitosis is not understood as they have been shown to dissociate in mitosis. Goodrich and Tweedie (2002) hypothesize that either not all become dissociated and act as recruitment points for further complexes after mitosis is completed, or marks on histones are generated and when the nucleosomes are distributed these marks act in a self-propagating way by recruiting further complexes to mark adjacent newly incorporated nucleosomes after mitosis.

Maintenance of epigenetic states also appears to require the chromatin assembly complex, which acts just behind the replication fork to chaperone histones to the new DNA. Mutations in three genes identified in arabidopsis that form proteins in this complex (FAS1, FAS2 and MSI1) generate mitotically heritable changes to expression of key developmental genes and phenotypic defects (reviewed in van Nocker, 2003). Some of these phenotypes appear to be phenocopies of PcG mutants where AtMSI1 is lost and heterochromatin is also lost; therefore both euchromatic and heterochromatic regions are affected. This suggests that chromatin-assembly complexes and chromatin-modifying complexes interact intimately after DNA replication to preserve marks on the chromatin. Recently, evidence has been presented that the MSI1 protein is also found in MEA/FIS PcG complexes (Köhler *et al*., 2003). However, both Korber and Hörz (2004) and Henikoff *et al*. (2004) have pointed out that distinct histone variants not only demonstrate different properties in the nucleosome context but also show different nucleosome assembly pathways. Henikoff *et al*. (2004) argue that mechanisms for conservative deposition of histone variants may be more important in preserving chromatin states and epigenetic information through cell division than marks on histones, but in line with convention exclude the possibility that distribution of histones from nucleosomes to daughter strands is itself even and conservative. Korber and Hörz (2004) take a different view, citing evidence for disassembly of histone octamers in the absence of replication. Additional evidence for chaperones for the histones, which are specific for each histone variant, indicate that they carry dimers and not tetramers, opening up the possibility that nucleosome disassembly into dimers could occur and allow even distribution onto daughter strands during replication. Clearly, with such significant implications, this will become a hotly debated and much investigated area of epigenetics.

Eliciting epigenetic change

How are the self-reinforcing cycles described above broken? How can one self-perpetuating steady state be shifted to a different one? If studies of shifts from euchromatic

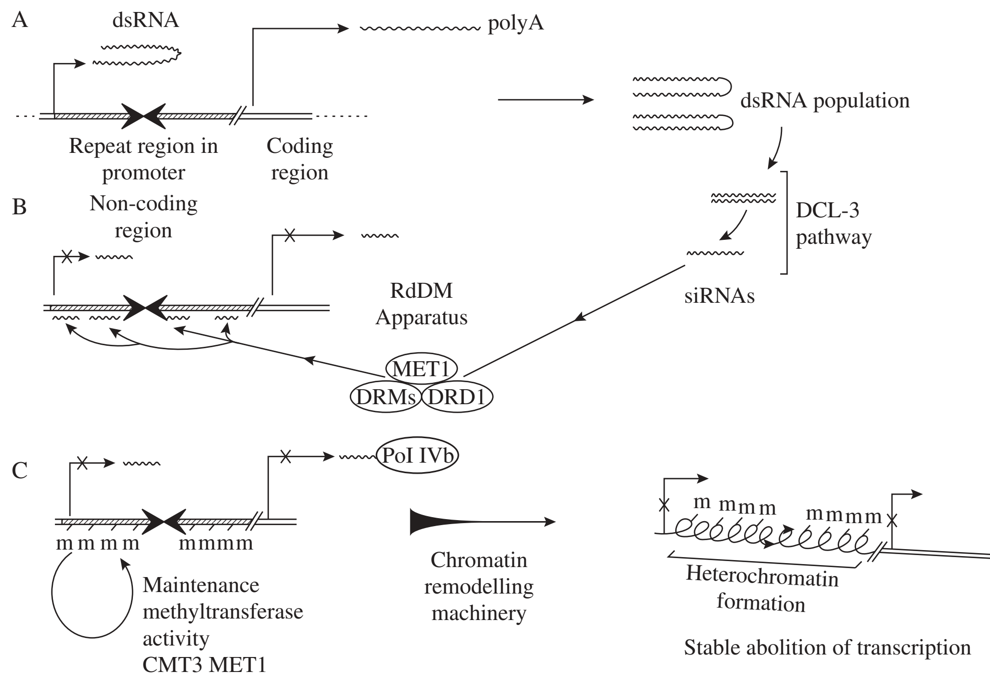

FIG. 6. RNA-dependent DNA methylation (RdDM) pathway in transcriptional gene silencing (TGS).

to heterochromatic states in yeast are also applicable to plants, change from one stable epigenetic state to another may not be instant but require several mitotic divisions (Katan-Khaykovich and Struhl, 2005). Erasing existing DNA methylation patterns can be done in two ways—either through passive demethylation, where the absence of DNA methyltransferase activity through successive cell cycles dilutes the semi-conservative mark, or through active demethylation. In plants any events of this latter kind are likely to be via base excision/DNA glycosylase pathways, as DNA demethylases have not been discovered. The genome-wide demethylation seen in mammalian reproductive programmes does not exist in plants where programmed, active demethylation appears less significant (but see below). In the same way, reduced maintenance of marks on histones or the reduced presence or loading of a histone variant during multiple replication cycles will dilute the specific marks and the specific histone variant. Even without cell division, turnover in histones can occur. Transcription itself has been shown to initiate nucleosome turnover and deplete marked histones site-specifically. Active systems for removal of histones, such as via ubiquitination pathways, appear to be another mode of control. In the case of histone methylation marks, these pathways were hypothesized to play a significant role in deleting existing marks of this kind as histone demethylase counterparts that remove the mark had not been identified until very recently. The discovery of a histone demethylase (LSD1) in fungal and human cells (Shi *et al*., 2004) that is specific to demethylation of H3 K9 opens up the possibility that other hitherto undetected enzymes of this kind may play an essential role in epigenetic regulation. Already, another putative demethylase that may operate by a hydroxylation mechanism has been reported (Trewick *et al*., 2005), and may be conserved in plants. In both these papers, seemingly irreversible histone methylation can be rendered reversible. The situation is different for histone acetylation where the antagonistic, reversible effects of acetylases and deacetylases are well known. This is best understood from rRNA repeats (see Lawrence *et al*., 2004; Probst *et al*., 2004). However, the likely interdependence of different marks on histones means that understanding change from one state to another has proved difficult and awaits further exploration.

The most important factor forcing change in epigenetic systems, as evident over and over again from the discussion above, is the non-coding RNA sequence. As seen repeatedly, there is a clear correlation between RNA triggering silencing and methylation, and chromatin changes at homologous genomic DNA sequences, in both TGS and PTGS. What is the molecular mechanism that transduces this information? In both the TGS and PTGS situations, methylation would appear to be directed by the short interfering RNAs (siRNAs) produced from processing of aberrant or dsRNA sequences. How an siRNA interacts with its homologous genomic DNA counterpart is not fully understood yet. The specificity of RdDM indicates that direct DNA–RNA binding occurs, likely mediated by protein complexes (Fig. 6). The identity of these proteins or complexes remains mysterious, although screens for RdDM-defective mutants have discovered DRD1, a novel plant-specific chromatin remodelling protein of the SW1/SNF2 type is necessary for this process (Kanno *et al*., 2004). Whether it is necessary for actual establishment or maintenance of *de novo* methylation is not known. What is clear is that the complexes formed with homologous DNA attract *de novo* methyltransferases as the DRM genes are required for establishment of silencing (Cao and Jacobsen, 2002*a*, *b*). However, recent work has implicated MET1 in this *de novo*

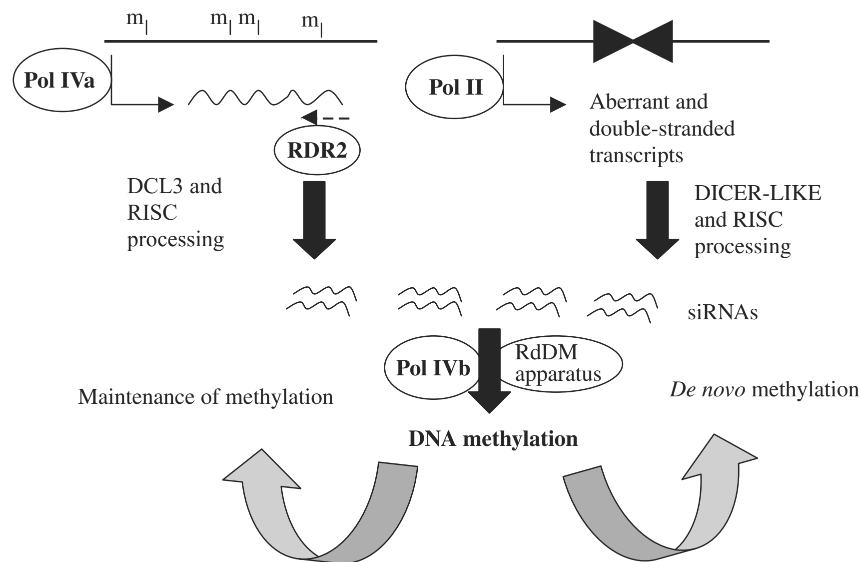

FIG. 7. Two Pol IV complexes act at different points in setting up and maintaining methylation patterns. Pol IVa, comprising NPRD1a and NPRD2a, may act to preferentially transcribe methylated regions of the genome. The transcripts produced by its action are converted to dsRNA species via the RdRP. RDR2 and siRNAs are generated via DCL3 and RISC action. These siRNAs are incorporated into RdDM apparatus which includes the Pol IVb complex, comprising NPRD1b and NPRD2a, and acts to maintain the methylation at the Pol IVa transcribed loci. In contrast, an inverted repeat transcribed by Pol II to form a hairpin RNA or otherwise aberrant RNA structure does not require Pol IVa, but instead becomes processed to siRNAs; again, RdDM requires Pol IVb. In this case, *de novo* methylation patterns are set up. (Adapted from Kanno *et al*. 2005.)

process too (Matzke *et al*., 2004). The two DRM methyltransferases and MET1 therefore act in conjunction to set up the methylation pattern from the RNA template, which is then maintained even if the RNA signal dies out. What is less clear is how chromatin states are set up: either RNA-guiding complexes sets up chromatin changes directly by recruiting modifying enzymes, or there is a strong feedback system from the methylation mark itself that sets up chromatin changes, or both.

It does appear that this RdDM process can be antagonized, with ROS1 likely acting as a demethylating agent to excise 5-methylcytosine from DNA targeted by small RNAs. *ros1* mutants show TGS-type hypermethylation and increased silencing of otherwise expressed transgene structures (Gong *et al*., 2002).

Change and stasis: two sides of the same coin?

On reflection, the two systems that regulate stasis of epigenetic marks and the change of epigenetic marks appear rather different. However, there is recent evidence that dispels this view. In arabidopsis, there are genes encoding sub-units for an RNA polymerase different from the other three typical RNA polymerases, and a mutation in one of these genes, *sde4*, had been identified previously in screens for mutants releasing silencing. This locus is now known as *NRPD1a*. Extensive work by Onodera *et al*. (2005) and Herr *et al*. (2005) has shown that this RNA polymerase IV does indeed operate in a novel way. *SDE4/NRPD1a* is the largest sub-unit of the Pol IV complex. The smaller sub-unit, *NRPD2a*, was discovered by Onodera *et al*. (2005). Mutations in both these catalytic sub-units lead to loss of genomic methylation and loss of heterochromatin in regions of repetitive DNA. However, solely the regions forming facultative heterochromatin (such as transposon and pericentromeric sequences) rather than constitutive heterochromatin (e.g. centromeric repeats) appear to be affected. As the siRNAs corresponding to the affected regions are essentially eliminated in the mutants, this implicates RNA polymerase IV in the production of RNA sequences processed to form siRNAs that direct DNA methylation and heterochromatin formation in the mechanism described above. However, Herr *et al*. (2005) report that there are developmental effects as the *nrpd1-a1* mutant under investigation showed dramatic developmental patterns in its silencing release. The exact mode of operation of Pol IV remains unknown; it is possible that this enzyme complex may preferentially recognize and transcribe methylated regions and aberrant RNAs may simply form due to the transcript properties. Alternatively, Pol IV may operate downstream on existing aberrant transcripts to generate local production of high levels of heterochromatin-inducing RNAs (Onodera *et al*., 2005). The most recent work by Kanno *et al*. (2005) has added new dimensions to this problem. Screens for mutants defective in RdDM revealed another version of the largest catalytic sub-unit, named *NRPD1b*. *nrpd1b* mutants did not show any loss of dsRNA transcripts or derived siRNAs from either a transgene construct or an endogenous transposon, but did still show loss of methylation and release of silencing of both. This points to a different non-redundant role for *NPRD1b* and the formation of two, functionally diverse Pol IV complexes. NRPD1a–NRPD2a complexes (Pol IVa) act to transcribe from methylated heterochromatic regions whilst NRPD1b–NRPD2a complexes (Pol IVb) act downstream of the siRNAs derived from both Pol IVa- and Pol II-derived aberrant transcripts. Pol IVb complexes act in the pathway that guides siRNAs to RdDM of homologous DNA sequences (see Fig. 7).

At least in facultative heterochromatic regions, a self-reinforcing cycle may exist in which methylated DNA itself induces the formation of aberrant transcripts which maintain both DNA methylation and heterochromatin. Whether the same system exists in constitutive plant heterochromatin remains to be seen, but something of a similar nature would seem probable. The discovery of Pol IV in plants is resolving the paradox of why heterochromatic regions are maintained when their state would normally suppress continual production of an RNA trigger.

Other factors in the epigenetic system

What other factors need to be considered in this overall scheme? For simplicity, and to reflect the trend in current approaches to epigenetics, a discussion is not included on the possibility that in plants there is a communication *in trans* between alleles of homologous chromosomes mediated by physical pairing effects (rather than via diffusible RNA signals). Pairing of homologous regions and interactions between the different epigenetic landscapes, which may allow transfer of information without RNA intermediates (e.g. in paramutation) may be an important yet neglected facet of the epigenetic system. This area has been reviewed in detail elsewhere (Grant-Downton and Dickinson, 2004).

The term 'epigenetic' is now becoming even more difficult to define as self-propagating protein entities, folded variants of the same protein sequence, termed 'prions' also come within this field. Although to date prions have yet to be found in plants, a recent study has shown that inhibition of Hsp90, which has an important role in folding of proteins *in vivo*, can generate phenotypic effects in plants (Queitsch *et al*., 2002).

Perhaps not surprisingly, evidence has come to the fore that there may be a strong link between metabolics and epigenetics (Fig. 7). The size of the pool of metabolic precursors required for DNA methylation can have a dramatic effect on DNA methylation. Mutations in the *HOMOLOGY-DEPENDENT GENE SILENCING* (*HOG1*) locus have been shown to release silencing of transgenes and generate a level of genomic demethylation (Furner *et al*., 1998). Rocha *et al*. (2005) have shown that *HOG1* encodes the *S*-adenosyl-L-homocysteine hydrolase (SAH) enzyme, and *hog1* mutants show an altered ratio of *S*-adensoyl methionine to *S*-adenosyl homocysteine (SAM : SAH) which affects the availability of precursors for methylation of DNA and proteins. Indeed, this ratio is so critical that null *hog1* mutants are homozygous lethal. The quantitative effects of availability of precursors on functioning of epigenetic systems probably have yet to be fully realized, for example, under conditions of environmental stress. Physical factors such as temperature are also likely to play a much greater role in regulating epigenetic systems than their sparse representation in the literature would suggest. The genome-wide changes in methylation produced after arabidopsis plants were exposed to a reduction in temperature are interesting (Burn *et al*., 1993) and it has been considered that methyltransferase activity could show substantial temperature-sensitivity. Temperature-sensitivity has been shown to definitively occur in the operation of RNA pathways (Szittya *et al*., 2003). This work would warrant further investigation.

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