Review

Small RNAs and heritable epigenetic variation in plants

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Recent studies suggest that inheritance of phenotypes in plants is more likely to involve epigenetics than in mammals. There are two reasons for this difference. First, there is a RNA-based system in plants involving small (s)RNAs that influences *de novo* establishment and maintenance of DNA methylation at many sites in plant genomes. These regions of methylated DNA are epigenetic marks with the potential to affect gene expression that are transmitted between dividing cells of the same generation. Second, unlike mammals, DNA methyltransferases in plants are active during gametogenesis and embryogenesis so that patterns of DNA methylation can persist from parent to progeny and do not need to be reset. We discuss how the effects of stress and genome interactions in hybrid plants are two systems that illustrate how RNA-based mechanisms can influence heritable phenotypes in plants.

RNA silencing, epigenetics, and heritable variation in plants

The standard paradigm of genetics requires that all heritable variation between members of a population corresponds to their DNA sequence. An alternative view recognises that, although genetic differences are the main source of phenotypic variation in a population, there is an additional minor component of heritability linked to epigenetic modification (see Glossary) of DNA or chromatin [1–4]. Evidence is emerging to support this idea that epigenetics and genetics operate as parallel, although unequal, systems to influence heritable variation, adaptation, and evolution in plants. The plausibility of this idea is reinforced by the discovery that regions of plant genomes are epigenetically modified through the action of non-coding sRNAs of 21–24 nucleotides (nt) in length [5].

Epigenetic modification often results in silencing of gene expression and the process involving sRNAs is 'RNA silencing' [6]. The involvement of RNA is central to the potential for heritable epigenetic variation because, unlike DNA sequences that do not vary, the expression of RNA can change under the influence of environmental and other factors. The epigenetic mark in the examples discussed below is DNA methylation (the addition of a methyl group onto the fifth position of the pyrimidine ring of cytosine residues) and so the mechanism involving sRNAs is 'RNA-directed DNA methylation' (RdDM) [6]. We refer to 'establishment' as the phase of RdDM in which the sRNA

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Glossary

Companion cells: cells in the gametophytes of plants that do not contribute genetically to the progeny. The vegetative cell is the companion to the sperm cells in the male gametophyte and the central cell is the companion to the egg cell in the female gametophyte. One sperm cell fertilises the egg cell to produce the embryo and the other sperm cell fertilises the central cell to produce the endosperm that acts as a supportive tissue to the developing embryo.
Epiallele: any two or more genetically identical alleles that are epigenetically distinct from each other due to differences in epigenetic marks such as DNA methylation.
Epigenetics: changes in gene expression or cellular phenotype that are stably transmitted during mitosis and meiosis without a change in the underlying DNA sequence.
Epigenetic marks/modifications: molecular alterations to chromatin such as methylation of cytosine residues of DNA or post-translational modifications of histone proteins. Epigenetic marks are often, but not always, associated with either active or repressive gene expression and therefore underlie most epigenetic phenomena.
Epigenome: comprises chemical compounds that modify, or mark, the genome (DNA or histone proteins) as a way to regulate the activity (expression) of all of the genes and DNA elements within the genome.
Epimutation: an epigenetic change (such as DNA methylation) that causes a silent gene to be activated or an active gene to be silenced without alteration to the underlying DNA sequence.
Germ cells/germ line: unlike animals, plants do not have their germ line set aside in early development. Instead, the germ line is formed from somatic cells in the adult (such as the floral meristem of flowering plants). The germ line is thus defined by a population of somatic cells that differentiate into the gametophytes that includes the germ cells. The male germ line is the sperm cell and the female germ line is the egg cell.
Heritable phenotypic variation: the heritability of a trait within a population is the proportion of observable differences in the trait between individuals within the population.
Paramutation: the interaction between two alleles, or homologous sequences, whereby one variant allele (paramutagenic) induces a heritable epigenetic change at the other allele (paramutatable) whenever the two alleles are present in the same nucleus.
Priming: an implicit memory effect in which exposure to a stimulus influences a response to a later stimulus, be it the same or similar.
RNA-directed DNA methylation: guidance of the *de novo* DNA methylation machinery to DNA by sRNAs complementary to the target locus.
Small RNA (sRNA): non-coding sRNAs of 21–24 nt in length confer specificity to a set of pathways collectively termed 'RNA silencing' that are involved in controlling and regulating gene expression.
Transgenerational epigenetic inheritance: the effects on phenotype or patterns of gene expression that are transmitted from one generation to the next via the germ line that cannot be explained by changes in the underlying DNA sequence. Also referred to here as heritable epigenetic variation.
Transposon/transposable element (TE): also known as 'jumping genes'; DNA sequences that move from one location in the genome to another.
Virus-induced gene silencing (VIGS): a RNA-based technology that exploits the sRNA-mediated antiviral defence mechanism in plants. In plants infected with unmodified viruses, the mechanism is specifically targeted against the viral genome. However, with viral vectors carrying inserts derived from host genes the process can be additionally targeted against the corresponding endogenous RNAs.

pathway is active for the first time and a DNA methylation mark is introduced to the genome. 'Maintenance' is the phase of RdDM when the methylated state is copied from one genome to another during cell division or between generations.

A hallmark of RdDM is the presence of cytosine methylation in all DNA sequence contexts (CG, CHG, and CHH, where H can be C, A, or T) [7]. DNA methylation that is independent of RNA, by contrast and for reasons discussed below, is generally confined to CG and CHG contexts. Most sites with this RdDM hallmark are within or close to DNA elements that have the ability to transpose from one location in the genome to another. This observation reflects a primary role of RdDM in genome defence [8] via the suppression of these transposable elements (TEs) or transposons from one generation to the next.

However, the influence of RdDM is not restricted to TEs. Transposon-associated DNA methylation can also affect adjacent genes so that the silencing of TEs can influence patterns of gene expression and phenotypes of the plant [9,10] and account for at least some examples of transgenerational epigenetic inheritance in plants [8].

Epigenetic marks can be lost as well as gained and an affected gene can therefore have three variants (epialleles): an expressed primary allele with no DNA methylation, a silenced epiallele with DNA methylation, or an expressed epiallele derived from a previously silenced allele.

In this review, we discuss the role of sRNAs in heritable epigenetic variation of plants. We describe first how sRNAs influence the establishment of DNA methylation. Second, we discuss the role of sRNAs in the maintenance and transmission of DNA methylation from mother to daughter cell and from one generation to the next. Lastly, we discuss the potential for transgenerational epigenetic inheritance due to loss, rather than establishment, of DNA methylation. We describe how RNA-dependent mechanisms may be particularly vulnerable to such loss of silencing if the epigenetic sRNA is suppressed.

Establishment and maintenance of DNA methylation in plants

The initiating phase of RdDM (Figure 1) requires the correct positioning of a plant-specific DNA-dependent RNA polymerase, Polymerase IV (Pol IV) [11], at the appropriate regions of the genome. Chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-seq) experiments revealed that Pol IV occupies $\geq$1000 genomic regions, including TEs and intergenic regions [12]. Recruitment of Pol IV to most of the target loci involves the interaction of SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1), a homeodomain protein that recognises chromatin enriched with unmethylated Lys4 and methylated Lys9 residues of histone H3, with the chromatin remodelling protein CLASSY 1 (CLSY1) [12–14], suggesting that histone modifications are an important local feature for the establishment of RdDM. Both SHH1 and CLSY1 associate with Pol IV [14,15], which physically interacts with RNA DEPENDENT RNA POLYMERASE 2 (RDR2) [15,16]. RDR2 converts the noncoding, single-stranded RNA transcribed by Pol IV into double-stranded RNA (dsRNA) that is cleaved into 24-nt sRNA duplexes by Dicer-like 3 ribonuclease III enzyme (DCL3) [17]. Single-stranded 24-nt sRNAs are loaded into an AGONAUTE effector protein (AGO4, AGO6, or AGO9) [18] and provide additional target specificity for RdDM. AGO4, for example, interacts with members of the DDR complex comprising RDM1, DMS3, and DRD1, which assists in the transcription of nascent scaffold RNAs complementary to the loaded sRNAs produced by another plant-specific DNA-dependent RNA polymerase, Pol V [11,19–22]. Although only a few Pol V transcripts have been identified [19], Pol V occupies $\geq$1000 genomic regions that correlate with Pol IV-binding sites [12,23,24]. The AGO-loaded sRNAs base pair with the Pol V scaffold RNAs [19,20], stimulating recruitment of the *de novo* DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to catalyse *de novo* DNA methylation at the target locus [7]. There may also be variations of this establishment pathway, in which the initiator sRNA is independent of Pol IV [25,26] and is 21 nt in length rather than 24 nt [27]. Alternatively, CHH methylation could be established by CHROMOMETHYLASE 2 (CMT2) independent of RdDM [28].

Maintenance of methylated cytosine residues in a symmetrical context is mediated by maintenance DNA methyltransferases that copy the methylation onto the opposite cytosine residue of the daughter strand [7]. CHG methylation, for example, is maintained by the plant-specific DNA methyltransferase CHROMOMETHYLASE 3 (CMT3). CMT3 contains a chromodomain that binds dimethylated Lys9 of histone H3 (H3K9me2), a repressive histone modification catalysed by the histone methyltransferase KRYPTONITE (KYP), which is often associated with methylated DNA. By contrast, CG methylation patterns are faithfully maintained by DNA METHYLTRANSFERASE 1 (MET1), a homologue of the mammalian maintenance methyltransferase Dnmt1. CMT3 and MET1 act independently of sRNAs but, by contrast, maintenance of CHH methylation is thought to be a continuous operation dependent on the sRNA initiation mechanism. Similar to the establishment phase, DNA methylation is targeted by DRM2 during the maintenance phase, which is guided to the Pol V scaffold transcripts by AGO-bound sRNAs [19,20].

These maintenance mechanisms can operate as interdependent feedforward loops (Figure 2A). In loop 1, the marks established in a sRNA-dependent manner by DRM2 are replicated on the newly synthesised DNA by CMT3 or MET1. In loop 2, the sRNAs that guide DRM2 are stimulated by DNA methylation, perhaps because Pol IV prefers methylated DNA as a template [21,23,29] at chromatin harbouring the correct histone modifications defined by SHH1 and CLSY1 [12,14]. The concerted action of these mechanisms means that RdDM, once established, is a robust, self-reinforcing process. This feature, as discussed below, is central to the potential for sRNA-mediated transgenerational effects in plants.

Maintenance of transgenerational epigenetic inheritance: wipe the slate clean or pass on the memory?

Transgenerational epigenetic inheritance requires transmission of an epigenetic state at a genomic locus from