Review

Special Issue: Noncoding and small RNAs

Sensing the epigenome

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Recent studies of plant development and environmental stress responses have converged on the roles of RNA and its metabolism as primary regulators of gene action. This RNA-based system appears to represent a versatile platform both for maintaining epigenetic memory and for reprogramming gene control in response to external signals. The fast-paced research reviewed here highlights exciting new trends in plant research relating to mechanisms and roles of the RNA-dependent epigenome in both development and evolution.

Emerging regulatory roles of RNA

Barbara McClintock was the first to surmise that the activation of previously silenced transposons was related to the visible changes in cytological heterochromatin brought about by stressful events [1]. From these observations, she proposed that coordinated changes in the heterochromatin were responsible for cellular differentiation during development [1]. Subsequently, the genetic activities of other transposons [2], transgenes (reviewed in [3]), and endogenous genes [4,5] were shown to be heritably altered by specific environmental conditions. Forward genetic screens in both *Arabidopsis* (*A. thaliana*) and maize (*Zea mays*) have begun to identify the nuclear

Glossary

AGO4: *Arabidopsis* gene *ARGONAUTE4*, encoding an argonaute-type protein found associated with ~24-nt RNA species representative of mostly transposon sequences.
ARGONAUTE: class of eukaryotic proteins having single-stranded RNase action guided by bound siRNA effectors.
ATGB2: *Arabidopsis* gene (At4g35860), encoding a Rab2-like small GTP-binding protein.
AtRAP: *Arabidopsis* gene (At2g31890), encoding a protein with a putative RNA-binding domain abundant in apicomplexans.
ATSWI3B: *Arabidopsis* gene *ARABIDOPSIS THALIANA SWITCHING PROTEIN 3B*, encoding a SWI-type chromatin remodeling protein.
AvrRpt2: avirulance gene specific to *Pseudomonas syringae pathovar tomato*.
B1-Intense: allele of the maize *plant color1* (*b1*) locus expressing a basic helix-loop-helix type transcriptional activator of anthocyanin biosynthetic pathway genes.
DCL: (Dicer-like) Class of RNaseIII-type endonucleases targeting dsRNA species.
DCL1: *Arabidopsis* gene *DICER-LIKE1*, encoding a DCL involved in primary miRNA processing.
DCL2: *Arabidopsis* gene *DICER-LIKE2*, encoding a DCL involved in the production of miRNAs and siRNAs.
DCL3: *Arabidopsis* gene *DICER-LIKE3*, encoding a DCL primarily involved in siRNA processing from transposon-type sequences.
DCL4: *Arabidopsis* gene *DICER-LIKE4*, encoding a DCL primarily involved in production of tasiRNAs
DDM1: *Arabidopsis* gene *DECREASED DNA METHYLATION1*, encoding an ATPase capable of chromatin remodeling; necessary for the maintenance of cytosine methylation patterns.
DRD1: *Arabidopsis* gene *DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1*, encoding a Rad54-type ATPase necessary for RNA-directed DNA methylation.
En/Spm: a well-characterized autonomous TIR CACTA-type DNA transposon, *Enhancer/Suppressor-mutator*, described in maize.
FCA: *Arabidopsis* gene *FLOWERING TIME CONTROL PROTEIN*, encoding a RNA-binding protein regulating flower development.
FLC: *Arabidopsis* gene *FLOWERING LOCUS C*, encoding a MADS-type protein that acts as a repressor of flowering.
FLD: *Arabidopsis* gene *FLOWERING LOCUS D*, encoding a histone deacetylase that acts to promote flower development.
FPA: *Arabidopsis* gene (At2g43410), encoding a RNA-binding protein regulating flower development.
Imprinted module: a set of neighboring genes showing differential action depending on parent-of-origin transmission.
lbl1: maize locus *leafbladeless1*, expressing a SGS3-type protein required for tasiRNA production.
LINE: eukaryotic retrotransposon of the long-interspersed nuclear element class.
LTR: long-terminal repeat, a structural motif distinguishing a broad class of eukaryotic retrotransposons.
MET1: *Arabidopsis* gene *DNA METHYLTRANSFERASE*, encoding a cytosine methyltransferase protein required to maintain cytosine methylation patterns in CG dinucleotide pairs.
mop1: maize locus *mediator of paramutation1*, expressing a RDR2-like protein required for paramutation.
Morphogen: a dosage-dependent molecular elicitor of a developmental response.
Mutator: a well-characterized class of TIR-type DNA transposons.
NRPD1a: *Arabidopsis* gene *NUCLEAR RNA POLYMERASE D1a*, encoding the large subunit of a Pol IVa holoenzyme.
nrpd1b: *Arabidopsis* locus *nuclear rna polymerase d1b*, expressing the large subunit of a Pol IVb holoenzyme.
P5CDH: *Arabidopsis* gene (At5g6230), encoding a mitochondrial 1-pyrroline-5-carboxylate dehydrogenase.
Paramutation: a meiotically-heritable change in gene regulation influenced by allelic interactions.
pl1: maize locus *purple plant1*, expressing a Myb-type transcriptional activator of anthocyanin biosynthetic pathway genes.
PPRL: *Arabidopsis* gene (At4g35850), encoding a pentatricopeptide repeat protein.
r1: maize locus *colored1*, expressing a basic helix-loop-helix type transcriptional activator of anthocyanin biosynthetic pathway genes; paralog of *b1*.
RDR2: *Arabidopsis* gene *RNA-DEPENDENT RNA POLYMERASE2*, encoding an RNA-directed RNA polymerase necessary for accumulation of siRNAs primarily representing transposon sequences.
RDR6: *Arabidopsis* gene *RNA-DEPENDENT RNA POLYMERASE6*, encoding an RNA polymerase necessary for accumulation of tasiRNAs and other siRNAs.
rld1: maize locus *rolled1*, expressing a class III homeodomain leucine zipper (HD-ZIPIII) transcription factor required for adaxial leaf fate specification.
rmr1: maize locus *required to maintain repression1*, expressing a Rad54-type ATPase required for co-transcriptional repression of RNA produced from repressed epigenetic states of the *Pl1-Rhoades* allele.
rmr6: maize locus *required to maintain repression6*, defined by recessive mutations as necessary for maize paramutation and proper plant development.
RPS2: *Arabidopsis* gene *RESISTANT TO PSEUDOMONAS SYRINGAE2*, encoding a plasma membrane protein that confers disease resistance to *Pseudomonas syringae*.
SGS3: *Arabidopsis* gene *SUPPRESSOR OF GENE SILENCING3*, encoding a protein of unknown function required for post-transcriptional gene silencing.
SLRRK: *Arabidopsis* gene (At2g31880), encoding a putative leucine-rich receptor-like protein kinase.
SRO5: *Arabidopsis* gene (At5g62520), encoding a protein with NAD+ ADP-ribosyltransferase activity.
SUL: *Arabidopsis* gene At4g18480 (*CHLORATA*), annotated as a magnesium chelatase required for chlorophyll biosynthesis.

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machinery responsible for maintaining small interfering RNA (siRNA)-type heterochromatin. Mutant analyses confirm that this RNA-based repression mechanism operates on diverse transposons [6,7] and on certain known developmental regulators [8–10]. Understanding how this nuclear system integrates environmental perception and developmental programs presents an emergent grand challenge for plant genetics research.

Several recent studies have highlighted apparent functional roles for antisense RNAs and RNA processing in triggering or maintaining small RNA-based repression [10–15], and still others point to regulatory crosstalk between distinct small RNA pathways [16,17]. Recognition that small RNAs can be trafficked throughout the plant [18] has suggested roles for these molecules as potential morphogens (i.e. dose-dependent elicitors of developmental response) [17] and as environmental response regulators [11–13]. These studies point to largely undiscovered and poorly understood roles for antisense transcription and for non-coding RNA (ncRNA) metabolism in epigenetic mechanisms underlying both development and adaptive responses to extracellular cues. The reader is referred to other contributions in this issue for updates regarding the biogenesis of the various small RNAs. Here, I highlight recent work in both *Arabidopsis* and maize that implicates novel roles for small RNA pathways both in responses to environmental stress and in plant development.

Coordinated regulation by *cis*-natural antisense transcripts

Cis-natural antisense transcripts (*cis*-NATs), which are RNAs with overlapping 3′ complementarity that are produced from convergently transcribed genes, have recently emerged as environmentally sensitive entry points into small-RNA-type regulatory pathways. On the basis of our understanding of eukaryotic RNA interference (RNAi), we know that complementary RNA transcripts can act as triggers of a small RNA cascade that leads to the maintenance of small RNA populations through repeated actions of RNA-dependent RNA polymerases (RDR) and dicer-like (DCL) RNA endonucleases (see discussion in [19]). This concept is supported in *Arabidopsis* by three examples [11–13] in which RNA from the overlapping 3′ ends of convergent gene pairs facilitates the accumulation of specific small RNA molecules that represent a region of these overlaps. These studies show that small RNAs can accumulate following the induction of one of the *cis*-NAT pair in response to environmental stress (Figure 1).

The founding example of a *cis*-NAT gene pair consists of two adjacent genes (*SRO5* and *P5CDH*) that have overlapping 3′ untranslated regions [11]. *SRO5* transcription is induced by increases in reactive oxygen species (ROS) brought about by osmotic stress [11]. Coincident with *SRO5* induction, a single *SRO5* 24-nt RNA from the larger overlap between *SRO5* and *P5CDH* 3′ RNAs accumulates and establishes a register for the production of consecutive 21-nt *SRO5* RNAs. Both small RNA species have the potential to downregulate *P5CDH* RNA levels [11]. Mutational analyses show that both size classes of *SRO5* small RNA species require the concerted actions of a plant-specific putative DNA-dependent RNA polymerase large

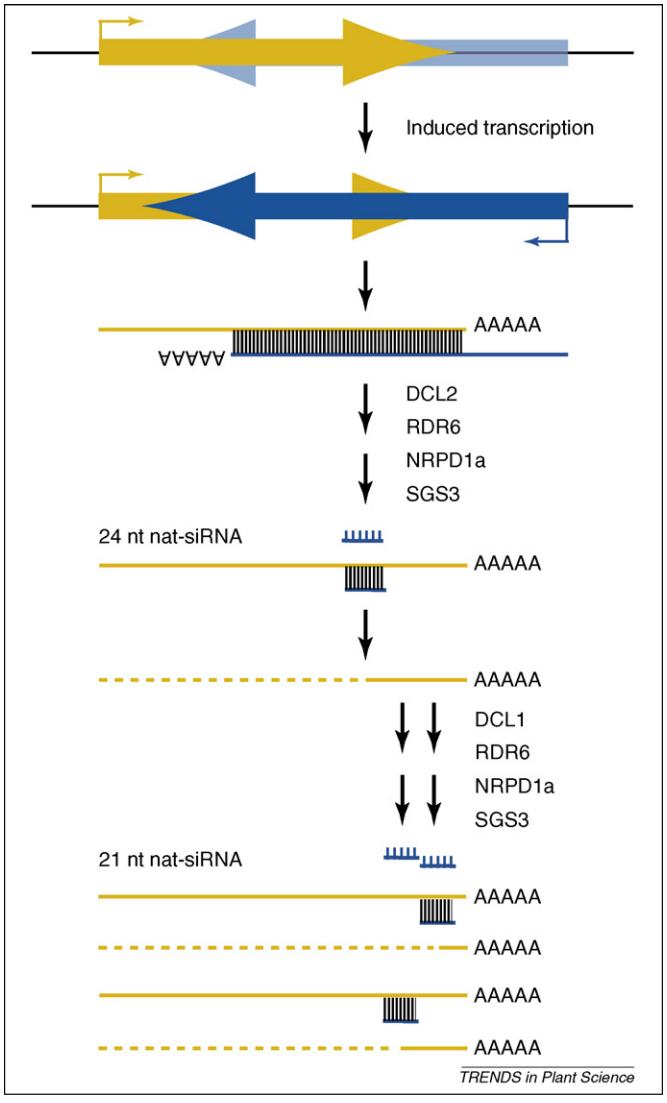

Figure 1. Conceptual model for *cis*-NAT RNA-based regulation. Transcription (small colored arrows) from overlapping transcription units (large colored arrows) produces a pair of RNA species (solid colored lines) with overlapping sequence complementarity. These presumably anneal (black hatching) to form a double-stranded substrate for entry into a pathway requiring DCL2, RDR6, NRPD1a and SGS3 functions for accumulation of a single 24-nt siRNA species. The 24-nt siRNA species guides the targeted cleavage of complementary sequences found in mature polyadenylated RNA and probably provides a reference point for the production of consecutive 21-nt siRNAs. Accumulation of the 21-nt siRNAs requires DCL1, RDR6, NRPD1a and SGS3 functions [11]. Both 21-nt and 24-nt siRNA species are hypothesized to facilitate mRNA degradation (gold hatched lines) through an exosome-based pathway.

subunit encoded by *NUCLEAR RNA POLYMERASE 1Da* (*NRPD1a*), a specific RDR encoded by *RDR6*, and a protein of unknown function encoded by the *SUPPRESSOR OF GENE SILENCING3* (*SGS3*) locus [11]. At least two separate DCL proteins are utilized in this pathway: mutations in *DCL2* abolish the accumulation of 24-nt *SRO5* RNA and mutations in *DCL1* affect production of the phased 21-nt *SRO5* RNAs [11]. The autoregulatory induction of *SRO5*, which is thought to mitigate the ROS levels that induce it, combined with its siRNA-type repression of *P5CDH*, which results in higher proline levels that offset osmotic stress and increased ROS, represents an elegant and integrated use of small RNA pathways to provide a rapid response to environmental stress. Considerable attention has therefore been given to understanding the extent to which

functional *cis*-NAT pairs are utilized in plant growth, development, and responses to the environment.

Two recent studies have shown that pathogenesis can also be perceived by specific *cis*-NATs [12,13]. In these investigations, *cis*-NAT siRNAs (nat-siRNA) were identified that are induced by infecting *Pseudomonas syringae* bacteria carrying the avrRpt2 effector. This effector is recognized by the host resistance factor encoded by *RPS2* initiating immunity responses that are mounted following a signal transduction cascade. In the first of the two studies, a specific NRPD1a/RDR6/SGS3/DCL1-dependent 22-nt RNA (nat-siRNAATGB2) induced by this pathogenesis was associated with downregulation of an apparent negative regulator of *RPS2* expression (*PPRL*). The *PPRL* transcription unit encodes a pentatricopeptide repeat protein and overlaps in antisense orientation with a small GTP-binding protein gene (*ATGB2*) that is induced in response to *AvrRpt2*. Thus, the *ATGB2–PPRL cis*-NAT pair acts to enhance host resistance to the pathogen by seeding the production of nat-siRNAATGB2, which targets *PPRL* transcripts for presumed RNA catabolism. In the second, more recent example, longer nat-siRNAs (~34 nt; referred to as long siRNAs or lsiRNAs) were identified from the *SRRLK–AtRAP* pair [13]. The suggestion was made that these lsiRNAs are derived from an ARGONAUTE-dependent slicing action, not unlike the mode of generation for *piwi*-type or repeat-associated siRNAs described in metazoans (reviewed in [20]). Here, as in the previous studies, T-DNA insertions were used to show that accumulation of the nat-siRNA is dependent on the presence of both antisense transcripts. These new studies provided independent confirmation of the *cis*-NAT regulatory model and extended its potential scope of action to both abiotic and biotic stress responses.

Despite these tantalizing examples, recent bioinformatic analyses of available transcriptomes and small RNA profiles from *Arabidopsis* have failed to provide compelling signatures of widespread *cis*-NAT regulatory pairs [21,22]. Only a slight bias of anticorrelated expression for the estimated 1000 *cis*-NAT pairs in *Arabidopsis* has been detected, and relatively few pairs provide this expression pattern any statistical support. Consistent with the hypothesis that overlapping transcripts would serve as DCL targets, however, it has been noted that a greater density of small RNAs is represented in the overlapping regions than in the non-overlapping regions of the same pair [21]. Absence of differential representation of these *cis*-NAT members in the profiles of siRNA-defective mutants indicates that any observed anticorrelated patterns of expression are unlikely to be due to siRNA-mediated repression [21]. The archetypical *SRO5–P5CDH* pair did not exhibit anticorrelated expression in any of the microarray-based transcriptome datasets [21], consistent with the suggestion that most *cis*-NAT pairs are responsive to specific biotic and abiotic stresses. This idea was recently supported by the finding of a set of novel nat-siRNAs in stress-induced libraries interrogated by 454 sequencing technology [22]. Given that some of these new nat-siRNAs have the potential to pair with more than one target [22], these *cis*-NAT pairs could have even broader regulatory capabilities. With roughly 1300 *trans*-NATs identified in the *Arabidopsis* genome, many of these also identified as members of *cis*-NATs [23], the possibilities for rapidly coordinated RNA-based regulation are staggering. Moreover, improved annotation of the gene models promise to expand the list of potential *cis*-NAT pairs. Comparisons of detailed small RNA profiles for materials that are exposed to specific stress conditions is needed to help clarify the scope and prevalence of these gene-based regulatory modules.

Unannotated cis-NATs and aberrant RNA

A clear example of a previously unannotated *cis*-NAT pair comes from recent work on *FLOWERING LOCUS C* (*FLC*) regulation. *FLC* has emerged as a paradigm of epigenetic regulatory control in development because of its central role as a MADS-type protein regulating the time to flowering in *Arabidopsis* (see review [24]). Using tiled hybridizations across the *FLC* transcription unit, specific NRPD1a/RDR2/DCL3-dependent 24-nt antisense RNAs and 30-nt antisense RNAs can be found that apparently derive from a previously unknown spliced antisense transcript overlapping the *FLC* 3′ region [15]. Interestingly, the abundance of this antisense transcript is reduced in an *NRPD1a* mutant, suggesting this previously unannotated transcription unit represents an as-yet-elusive Pol IVa (a putative DNA-dependent RNA polymerase holoenzyme assembled with the NRPD1a large subunit) template [15]. A follow-up study [10] identified a second, differentially spliced antisense RNA whose relative prevalence was influenced genetically by functions encoded by the *FLOWERING TIME CONTROL PROTEIN* (*FCA*) and *FLOWERING LOCUS D* (*FLD*) genes. FLD was recently identified as a putative H3K4 lysine demethylase [10,25]. FCA contains RNA-recognition motifs (RRM), and genetic data suggest that it might be involved with RNA 3′ end processing [26,27]. Interactions between FCA and a putative chromatin-remodeling ATPase (ATSWI3B) have been cited as evidence that FCA might act directly on nascent RNA templates [10]. Mutant analyses suggest that *FLC* RNA levels are anticorrelated with those of the antisense isoforms favored by FCA and FLD action [10]. Although the relationships and functional connections identified in these studies between RNA processing, stabilized small RNAs, and chromatin modifications remain to be elaborated, it is increasingly suggested that co-transcriptional processing of nascent RNAs, similar to that seen in *Schizosaccharomyces pombe*, can have epigenetic consequences (see [28]). Other components likely to be involved with RNA processing and 3′ end formation were also identified in genetic screens for enhanced silencing mutants of a reporter transgene, hinting that improperly processed RNA transcripts might feed into RNAi-type pathways [14]. Mutant analyses show that both FCA and FPA (another RRM protein) act in parallel to the RNA-dependent DNA methylation (RdDM) pathway to repress the expression of several known targets of RdDM [29]. Baurle *et al*. [29] suggest that FCA and FPA flag aberrant RNAs that are produced from RdDM targets and act as adaptors for effector proteins, such as FLD, which respond by modulating chromatin structures. The extent to which cryptic *cis*-NATs such as that found at *FLC* constitute important regulatory modules remains to be seen.

Non-coding cis-NATs and paramutation

Knowledge regarding the various regulatory roles of ncRNA has increased dramatically over the past few years (reviewed in [28]). In many cases, ncRNAs are derived from convergently transcribed regions or from a gene that constitutes half of an imprinted module (i.e. one of a gene-pair manifesting different anticorrelated expression patterns based on parent-of-origin transmission). One recent example of a non-coding *cis*-NAT in maize was described for a region of directly repetitive sequences, located about 100 kb 5′ of the *B1-Intense* (*B1-I*) allele, that are required to mediate an interhomolog *trans*-sensing regulatory interaction known as paramutation [30]. Paramutation interactions have been described for specific alleles of several maize loci that are involved in regulating anthocyanin pathways (Figure 2). Epigenetically repressed expression states of *B1-I* (referred to as paramutant states) facilitate the acquisition of a repressed state by a highly expressed *B1-I* allele found on the opposite homolog. *In vitro* transcription reactions using isolated nuclei revealed evidence of anti-parallel transcription from the repeated region upstream of *B1-I* [31], and small RNAs from this repeat are rare but detectable [32]. Although the functional significance of these small RNAs is still unknown, the *trans*-sensing interaction of paramutation is prevented in plants that are homozygous for mutations in the *mop1* (*mediator of paramutation1*) locus [33], which encodes an RDR2-like protein presumed necessary for the production of small RNA molecules [31,34]. Epigenetic silencing of *Mutator* transposons is also maintained by MOP1 action [35]. Another gene, *rmr1* (*required to maintain repression1*), encoding a DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (DRD1)-type ATPase is required to maintain repressed paramutant states at the *purple plant1* (*pl1*) locus [36]. In parallel with *Arabidopsis* models, these results imply roles for a heterochromatin-type siRNA pathway in mediating paramutation and for RdDM in maintaining paramutant states. The functionally important repeated sequences upstream of *B1-I* are thought to serve as a type of chromosomal boundary element [30], and hence non-coding *cis*-NAT pairs might act to organize higher-order chromosome architecture.

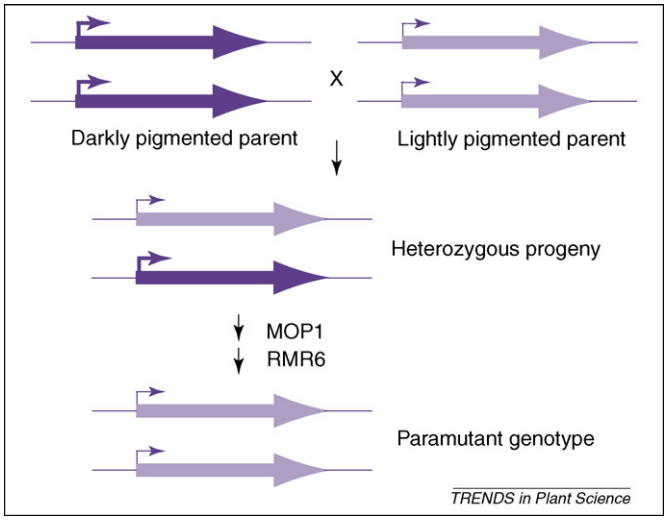

Figure 2. Conceptual model for paramutation. An allele that is susceptible to paramutation (large colored arrows) can be maintained in either a high-expression reference state (large dark colored arrow with large arrow above) or a relatively repressed paramutant state (large lightly shaded arrow with small arrow above). Paramutation occurs in specific heterozygous combinations through *trans*-interactions requiring MOP1 and RMR6 functions [33,57]; high-expression reference states are invariably changed to meiotically heritable paramutant states.

The emerging connections between paramutation and a presumed heterochromatin siRNA pathway are intriguing given that, first, the process of paramutation has been shown to be sensitive to environmental stress [4], and second, some mutations found in genetic screens for components that are required for paramutation cause developmental defects that resemble those resulting from dysregulation of miRNA targets [33,37]. The extent of paramutation occurring between alleles of the *colored1* (*r1*) pigment locus could be influenced by unorthodox temperature and light treatments applied during the period of apical inflorescence determination [4]. Remarkably, the differential regulatory states of the *r1* allele resulting from paramutations that are induced by contrasting environments are meiotically heritable [4]. These results, in light of the emerging connections between paramutation, RDR2-type function and RdDM, raise the possibility that these RNA-type regulatory pathways act, in some respect, to integrate developmental genetic programs with the perception of extracellular cues.

Transposons as controlling elements

Work by Barbara McClintock [38] and Nina Fedoroff [39] showed that maize transposons such as *En/Spm* can exist in different levels of epigenetic repression. Recent *Arabidopsis* studies paint a molecular picture in which the most constitutive repression of transposons is associated with a maintenance-type cytosine methylation pathway involving *DECREASED DNA METHYLATION1* (*DDM1*) [40] and *DNA METHYLTRANSFERASE* (*MET1*) [41]. A more facultative-like repression primarily involves the RdDM pathway [42]. Mutant analyses and recent deep-sequencing profiles of *rdr2* [43,44], *nrpd1a/nrpd1b* [45,46] mutants and ARGONAUTE4-associated small RNAs [47] support this association between transposons and the 24-nt siRNA pathway required for RdDM.

It is curious that, to date, the *Arabidopsis* RdDM pathway appears to be non-essential. Numerous examples (see review in [48]) support the proposal that the expression of certain genes is influenced, and perhaps regulated, by epigenetic changes taking place at proximate transposons. A recent profile of outward reading transcripts from a recently expanded set of ~1000 *Dasheng* long terminal repeat (LTR) elements in rice (*Oryza sativa*) documented tissue-specific differences [49]. In a more detailed analysis, Kashkush and Khasdan found that many of the outward reading transcripts were antisense to, and anticorrelated with, transcripts from annotated gene space. Similarly, differential cDNA display profiles of *drd1* mutants highlighted many genes affected by the RdDM pathway that are associated with flanking transposons. In one example, the *drd1* mutant produced a transcript from a unique LTR element of the *Copia* class (LTRCO), which was antisense to a transcript made from an adjacent LINE transposon. This 'non-coding' *cis*-NAT is 5′ of a gene model for a ribosomal protein (RPL18C). The fact that both the

LTRCO and *RPL18C* transcripts were upregulated in the various RdDM mutants [42], coupled with the absence of general developmental dysfunction in the various RdDM mutants, led Huettel *et al*. [50] to speculate that the RdDM pathway serves regulatory roles in responses to the environment. The nature of the evolutionary selection pressure(s) acting to maintain the RdDM system could thus prove challenging to understand in culture alone.

Given our nascent molecular understanding of the RdDM pathway and of RNAi pathways, investigations relating transposon activity to development and environmental stress represent an exciting new area of plant genetics research. Because in the grasses nearly all genic regions are flanked by transposons, it will be particularly interesting to see the extent to which transposons play significant regulatory roles in this plant family. As mentioned earlier, maize *mop1* mutants are defective in paramutation [33] and *Mutator* transposon silencing [6], and display developmental defects [33]. Function from the *rmr6* locus is also required for paramutation and normal maize development [37]. RMR1, a DRD1-related protein, targets a CACTA-type transposon fragment found 5′ of a *pl1* gene for RdDM-like cytosine modifications, yet *rmr1* mutant plants are phenotypically normal [36]. These recent examples of RdDM-type effects on transposons in both maize and *Arabidopsis* nonetheless provide a molecular framework in which to consider Barbara McClintock's controlling element hypothesis [1,7,48]. Readout transcription from transposons can influence the expression of adjacent genes or the use of regulatory features, and thus epigenetic control of these elements can be integrated in a regulatory capacity. Continued research related to the RdDM pathway promises to highlight additional examples over the next few years. Our challenge is to understand exactly how the nuclear system in question perceives and reacts to specific environmental stresses. By treating *Arabidopsis* with short-wave radiation or preparations of bacterial flagellin, genome-level changes that affect rates of homologous recombination within a GUS-type reporter construct can be meiotically heritable [51]. This result is consistent with earlier studies [1,2] showing that environmental stresses (breakage-fusion-bridge cycles or UV treatment) can have widespread epigenomic consequences that are manifested by changes in transposon behaviors.

Regulatory crosstalk between small RNA pathways

Genetic screens and transcriptome profiling have served to compartmentalize our understanding of the various small RNA pathways. Yet several recent studies indicate that the flow of small RNA metabolism can be dynamic and perhaps subject to regulatory control. For example, the bulk of RDR2-derived products are processed by DCL3 to a 24-nt size class, but in a *dcl3* mutant, these sequences are represented in size classes typical of those resulting from DCL1, DCL2, and DCL4 action [44]. This observation highlights the potential for sequences, say of transposon origin, to crossover to other small RNA pathways. A similar concept was illustrated by a recent study [16] in which various *dcl* mutant combinations were assayed for inter- and intra-cellular silencing of an endogenous gene (*SUL*) triggered from phloem-specific expression of a hairpin-type double-stranded RNA (dsRNA) transgene. Because this tissue-specific transgene is expressed at levels around 50-fold lower than a comparable CaMV 35S construct, the authors discovered a 'hierarchical' action of the various DCLs not previously seen. At elevated levels of any given siRNA, regardless of the DCL involved, any of the size classes (21 nt, 22 nt or 24 nt) could be associated with inter-cellular silencing. Even more remarkable were the findings that mutations in *nrpd1a* and *rdr2* prohibit this spread of *SUL* silencing [16]. Because neither DCL3 nor AGO4 are required for this inter-cellular silencing, these results identify distinctive roles for fundamental components of the heterochromatic siRNA pathway in mediating intercellular communication of small-RNA-based information.

In terms of regulating development, microRNAs (miRNAs) have emerged as primary determinants (see reviews by John Bowman and Michael Axtell and by Alison Mallory this volume). One of the most intriguing developments regarding miRNA functions was instigated by a study showing that leaf polarity depends on opposing adaxial and abaxial patterns of an auxin response factor tasiRNA (tasiR-ARF) and a miRNA (miR166), respectively, in incipient maize leaves [17]. Adaxial leaf fates in maize are promoted by the class III HD-ZIP transcription factor encoded by *rolled1* (*rld1*), and earlier work had shown that miR166 directs cleavage of *rld1* RNA [52]. In the latest study, Nogueira *et al*. [17] showed that adaxial expression of an SGS3-type protein encoded by *leafbladeless1* (*lbl1*) led to the production of tasiR-ARF and was responsible, either directly or indirectly, for delineating the abaxial expression domain of miR166. The *in situ* hybridization patterns of *lbl1* RNA, mature tasiR-ARF products, and miRNA166 targets have been interpreted as supporting the inter-cellular trafficking of the siRNAs [17,52]. Mathematical modeling of this opposing set of potentially diffusible siRNAs was then used to describe how non-catalytic use of such siRNA in mediating mRNA target degradation would lead to boundary sharpening or the refinement of spatial expression patterns [53]. This computational analysis, also applied to *ultrabithorax* RNA expression boundaries in *Drosophila melanogaster* embryos, argues that sharpening occurs as siRNAs diffuse into cells that have relatively low levels of mRNA expression and target these for degradation. If this concept of opposing siRNA domains controlling boundary formation turns out to be a general principal of plant development, then it is possible to imagine that stress-induced alterations in the flux of siRNAs through inter-cellular pathways could provide one way of integrating environmental perception with developmental programs.

Perspectives, promises and challenges

The research highlighted here suggests that much work is needed to allow us to understand the diverse roles played by RNA and its metabolism in specifying epigenetic landscapes. Part of the experimental challenge is that we are limited, in most cases, to assaying the end points of RNA degradation, processing, and/or stability. Inducible knockdowns of the *Arabidopsis* exosome (the primary 3′ to 5′ RNA exonuclease) uncovered a hereto unseen diversity of

RNA molecules frozen in the act of degradation [54]. Specific studies highlighted in this review [10,15,29] suggest that more basic knowledge regarding normal and abnormal RNA processing and catabolism is needed. Also yet to be identified is the nature of Pol IV templates (see review by Craig Pikaard this volume). Could Pol IV RNAs derive from DNA templates that are either decorated by specific cytosine methylation or chromatin modifications or simply competed away from normal Pol II association [36]? How does Pol IVa act to mediate intercellular communication of siRNA silencing [16]? How does crosstalk occur between the various small RNA pathways and how important are these relationships to normal development [17,33,37,53]? Answers to these questions promise to identify both metabolic and molecular entry points for translating environmental stresses into epigenomic responses.

Significant attention is given in this review to studies highlighting the roles of antisense transcription in controlling gene regulation and in responses to the environment. Given the relatively few known studies, the current state of genome annotations, and the vast number of different cell-type and environmentally responsive transcriptomes, the full extent to which genomes can be transcribed in antiparallel fashion is unknown. Transposons clearly play a role in mediating the production of sense RNAs [48,49] and these recent reports show that promoter-type activities of transposons can be regulated by the RdDM pathway [36,42]. A recent computational analysis of the *Arabidopsis*, poplar (*Populus trichocarpa*), and rice genomes suggests there could be at least five times more miRNA genes than previously discovered [55], leading the authors to hypothesize that some of these have adaptive uses in response to environmental challenge. If induced readout transcription from transposons is integrated into an environmentally sensitive response, then the possible reprogramming events affecting plant development, especially in genomes littered with transposons, might be of considerable evolutionary significance [56].

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