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Long non-coding RNAs and their functions in plants

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Eukaryotic genomes encode thousands of long noncoding RNAs (lncRNAs), which play important roles in essential biological processes. Although lncRNAs function in the nuclear and cytoplasmic compartments, most of them occur in the nucleus, often in association with chromatin. Indeed, many lncRNAs have emerged as key regulators of gene expression and genome stability. Emerging evidence also suggests that lncRNAs may contribute to the organization of nuclear domains. This review briefly summarizes the major types of eukaryotic lncRNAs and provides examples of their mechanisms of action, with focus on plant lncRNAs, mainly in *Arabidopsis thaliana*, and describes current advances in our understanding of the mechanisms of lncRNA action and the roles of lncRNAs in RNA-dependent DNA methylation and in the regulation of flowering time.

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Introduction

Transcriptome studies in fungi, plants, and animals have revealed that pervasive transcription from over 90% of the genome generates a multitude of non-coding RNAs (ncRNAs) [1,2], including tens of thousands of plant lncRNAs, broadly defined as ncRNAs longer than 200 nt in length that do not have discernable coding potential [3•,4••,5]. Early studies questioned the importance of lncRNAs because of their low expression and sequence conservation compared with mRNAs, and attributed their existence to transcriptional noise. However, emerging evidence indicates that many lncRNAs play key roles in diverse biological processes across eukaryotes, ranging from the regulation of mating type in yeast [6,7] to the pluripotency of embryonic stem cells in mammals [8]. Plant lncRNAs play key roles in flowering time, gene silencing, root organogenesis, seedling photomorphogenesis, and reproduction [5,9•,10,11•,12••,13••].

Types of lncRNAs

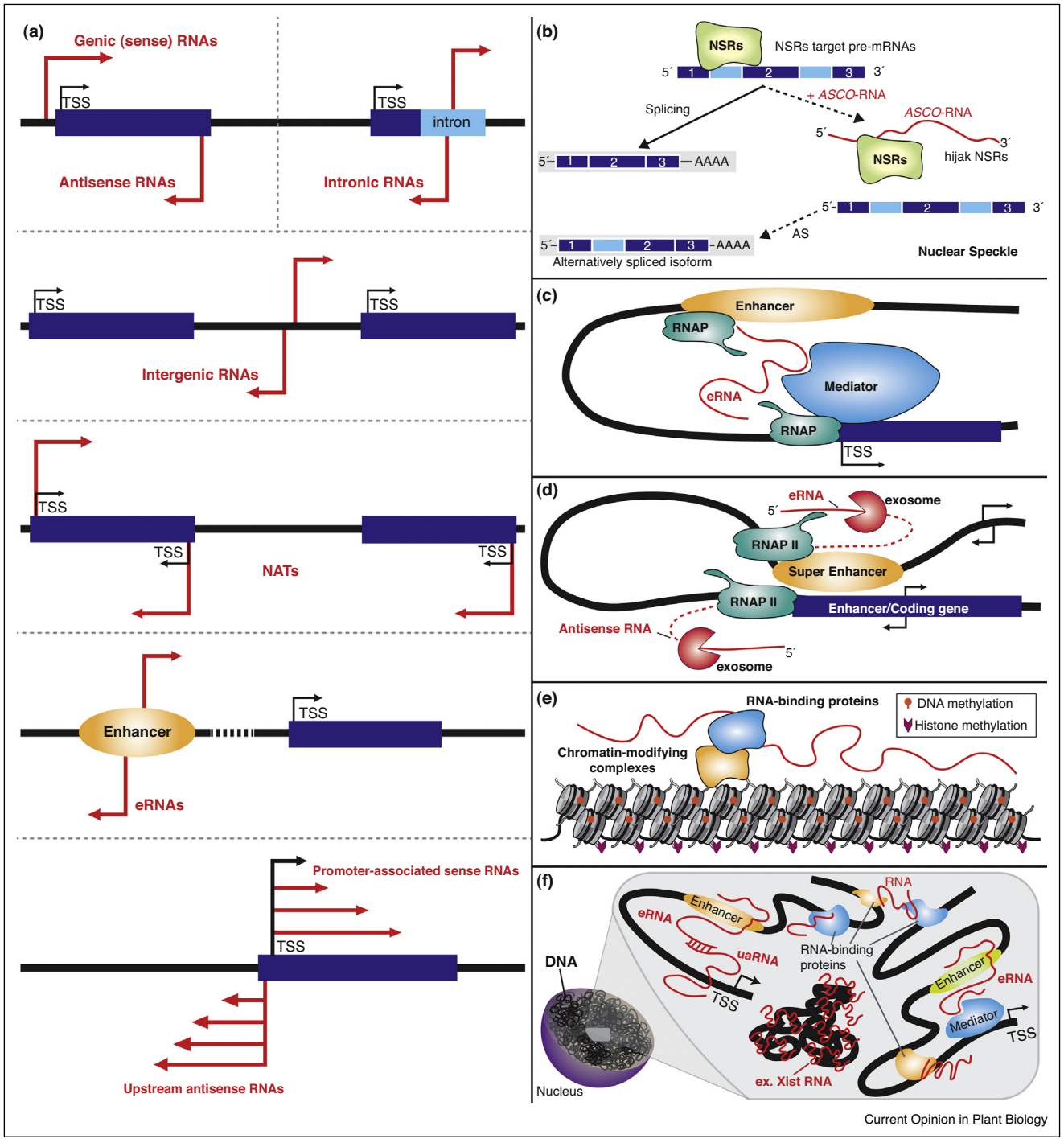
Our emerging understanding of the importance of lncRNAs has only begun to come to terms with their remarkable variety of types and origins. LncRNAs arise from intergenic, intronic, or coding regions in the sense and antisense directions. On the basis of their genomic origins, lncRNAs can be broadly classified as: first, long intergenic ncRNAs (lincRNAs); second, intronic ncRNAs (incRNAs); and third, natural antisense transcripts (NATs) transcribed from the complementary DNA strand of the associated genes (Figure 1a) [14•].

A comprehensive analysis of over 200 *Arabidopsis thaliana* transcriptome data sets identified ~40,000 putative lncRNAs, including over 30,000 NATs and over 6000 lincRNAs [3•,4••,15]. Most of these lncRNAs are not associated with smRNAs and their transcript levels are 30-fold to 60-fold lower than those of mRNA, similar to mammalian lncRNAs. NAT pairs, lncRNAs generated from the opposite strands of coding or noncoding genes, are surprisingly widespread in Arabidopsis with ~70% of Arabidopsis protein-coding loci encoding potential NAT pairs of 200–12,370 nt [4••]. NAT pairs can either overlap completely (~60%) or have complementary sequences in their 5′ or 3′ regions (Figure 1a). NAT expression is also highly tissue-specific and many NATs respond to biotic or abiotic stresses. Recent analysis of expression of sense–antisense NAT pairs in response to light uncovered about 1400 light-responsive NATs, with about equal proportions regulated either concordantly or discordantly relative to the sense transcript. Genes encoding many light-responsive NATs also showed high levels of histone acetylation, which dynamically correlated with NAT expression changes [4••].

The above classification does not do justice to the rich variety of plant lncRNAs. For example, the diverse group of lincRNAs includes lncRNAs that serve as precursors and/or scaffolds for smRNAs in RNA-dependent DNA methylation (RdDM) silencing pathway (described below), and these lincRNAs likely differ in function from other lincRNAs transcribed by RNA Pol II. Also, ncRNAs produced from PHAS loci serve as precursors to generate 21-nt and 24-nt secondary phased phasiRNAs in many plant genomes [16–18]. Moreover, many additional types of plant lncRNAs likely remain to be discovered.

In addition to lincRNAs, incRNAs, and NATs, work in other organisms has identified various types of lncRNAs transcribed from the regions around transcription start sites (TSSs), enhancer regions, intron splicing sites, and transcription termination sites. The lncRNAs expressed

Figure 1



LncRNA types and functions. **(a)** Classification of lncRNA relative to protein-coding genes. Blue boxes indicate protein-coding genes and red lines indicate lncRNAs. Arrows show the direction of transcription. **(b)** ASCO-RNA competes with the binding of nuclear speckle RNA-binding proteins (NSRs) to their targets and changes the splicing patterns of NSR-regulated mRNA targets resulting in production of alternative isoforms and alteration of developmental fates in plant roots. **(c)** eRNAs act as nascent transcripts that function in *cis* as scaffolds for the recruitment of co-activator complexes mediating chromosome looping between enhancer and promoter regions. **(d)** The exosome-regulated divergently transcribed seRNAs expressed from super-enhancers can interact with other ncRNAs arising from divergently transcribed enhancer/s or promoter/s of protein coding genes, engaging in long-distance interactions and affecting DNA topology and gene expression. **(e)** LncRNAs serve as scaffolds in the recruitment of chromatin-modifying factors. **(f)** LncRNAs modulating intra-chromosomal and inter-chromosomal nuclear architecture. Various individual lncRNAs interacting with multiple chromatin proteins and different chromatin domains could act together in establishing and maintaining higher-order structure in the nucleus.
(d) Adapted from [36].

from around TSSs include exosome-sensitive yeast CUTs (cryptic unstable transcripts) and SUTs (stable unannotated transcripts) [19], mammalian PROMPTs, and uaRNAs (upstream antisense RNAs) [20], Xrn1-sensitive XUTs [21], Nrd1-dependent NUTs [22], and others. A large proportion of mammalian non-polyadenylated lncRNAs also correspond to divergently transcribed, exosome-sensitive eRNAs mapped to enhancer regions [23], although plant eRNAs have not yet been reported.

Recent work in Arabidopsis and rice also uncovered a group of intermediate-sized ncRNAs (im-ncRNAs) of 50-300 nt in length [24,25]. Classification of im-ncRNAs on the basis of their proximity to protein-coding genes identified 299 im-ncRNAs originating from 5′ UTRs, coding, and intronic regions. The presence of 5′ UTR im-ncRNAs correlated with higher expression of the associated genes and with positive histone marks, such as H3K4me3 and H3K9ac, but not with negative marks. Down-regulation of some im-ncRNAs caused molecular or developmental alterations [24].

Expression of lncRNAs

Most lncRNAs are transcribed by RNA Pol II. Two additional plant-specific RNA polymerases, Pol IV and Pol V, also produce lncRNAs [26,27]. Most lncRNAs are polyadenylated; however, many yeast and mammalian lncRNAs are non-polyadenylated [23]. Some key plant lncRNAs are also non-polyadenylated [28,29] and recent work in Arabidopsis identified hundreds of non-polyadenylated lncRNAs induced by specific abiotic stresses [30].

Many plant lncRNAs are developmentally and environmentally regulated and likely represent functional components of the transcriptome. For example, many lincRNAs show significant changes in different organs or during stress, suggesting that they are dynamically regulated and might function in development and stress responses [15]. However, the regulation of lncRNAs in plants remains poorly understood.

Like all transcripts, lncRNA expression is regulated at the transcriptional level and by the machineries involved in their biogenesis, 3′ end processing and degradation. One of these factors is the exosome complex, the main 3′–5′ exoribonuclease machinery conserved in eukaryotes, which comprises a nine-subunit core associated with two additional subunits, Rrp44 and the nuclear-specific Rrp6, which provide the enzymatic activity. Indeed, various groups of polyadenylated ncRNAs were originally identified in Arabidopsis exosome mutants [1]. One of the distinct subclasses of these ncRNAs comprises upstream noncoding transcripts, which we termed UNTs, originating from TSSs of protein-coding genes and resembling CUTs and PROMPTs. UNTs are collinear with the 5′ ends of protein-coding transcripts and frequently extend into the first intron of their respective overlapping genes [1].

Molecular functions of lncRNAs

LncRNAs can regulate gene expression on multiple levels via a number of complex mechanisms. They can function in either *cis* or in *trans* by sequence complementarity or homology with RNAs or DNA, and/or by structure, forming molecular frames and scaffolds for assembly of macromolecular complexes. Most of the studied lncRNAs function in regulation of gene expression at the transcriptional level; however, some lncRNAs have been reported to regulate gene expression posttranscriptionally in a variety of ways.

On the simplest level, lncRNAs can serve as decoys that prevent the access of regulatory proteins to DNA or RNA by mimicking their targets. Some Arabidopsis lncRNAs interact with microRNAs (miRNAs) as competitors and function as miRNA target mimics, similarly to animal miRNA sponges. For example, the IPS1 lncRNA acts as a non-cleavable competitor for *PHO2* mRNA, as miR399 targets the *PHO2* mRNA for degradation [31]. Many endogenous miRNA target mimics have also been predicted by bioinformatics approaches and the function of some has been experimentally confirmed in Arabidopsis [32]. The decoy Arabidopsis lncRNA ASCO regulates plant root development by binding to the regulators of alternative splicing, nuclear speckle RNA-binding proteins, and hijacking them to change the patterns of alternative splicing to produce alternative splice isoform (Figure 1b) [12••].

The best-known functions of lncRNAs are their roles as regulators of transcription. LncRNAs can directly regulate the Pol II transcription machinery. For example, animal lncRNAs promote the phosphorylation of transcription factors (TFs) and thus regulate their DNA-binding activity [33]. Many eukaryotic lncRNAs play important roles in regulation of transcription initiation and elongation, including control of RNA Pol II pausing, function through transcriptional interference and as scaffolds recruiting chromatin remodelers, which in turn can affect chromatin topology and nuclear organization (reviewed in [34•]). The Arabidopsis *trans*-acting lncRNA HID1 associates with the chromatin of the TF gene *PIF3* and represses its transcription [13••]. The APOLO lincRNA participates in the spatial association and interaction between APOLO and the distant *PID* genomic regions via formation of a dynamic chromatin loop that determines *PID* expression [9•].

Some mammalian enhancer RNAs (eRNAs) act as nascent transcripts and function in *cis* as scaffolds to recruit co-activator complexes that mediate chromosome looping between enhancer and promoter regions, controlling chromatin topology and modulating gene activation

(Figure 1c) [35,36••]. eRNAs also function at superenhancers, elements characterized by high densities of individual enhancers. Recent findings suggest that so-called supereRNAs and divergently transcribed lncRNAs produced from other enhancers or TSSs may act together to form higher-order chromosomal structures that enable control of gene expression. Interestingly, in this case the exosome machinery affects enhancer activity by regulating the antisense lncRNAs via either post-transcriptional RNA degradation or by repression of RNA synthesis via promotion of early termination of transcription [36••]. A remarkable correlation was also found between the presence of genes producing exosome regulated TSS-associated antisense lncRNAs in the vicinity of a superenhancer (within up to 310 kb), suggesting that expression and/or processing of these lncRNAs may control the interaction between the superenhancers and their counterpart genes. Interestingly, the exosome also protects regions expressing eRNAs from genomic instability by resolving deleterious R-loops [36••], stable RNA-DNA triplexes that naturally form during transcription, but persist in divergently transcribed regions [37]. These findings led to the proposal that activity of the exosome can modulate the interaction between regulatory elements that control both gene expression and nuclear organization, via regulation of lncRNAs produced from these elements (Figure 1c–f).

Most work on lncRNAs has focused on their roles in the recruitment of chromatin regulatory proteins to genomic DNA locations. Different classes of chromatin-bound lncRNAs function as scaffolds for the cooperative assembly of chromatin-modifying complexes, recruiting them in either smRNA-dependent or smRNA-independent manners. The most-studied RNAi-dependent pathway is plant-specific RdDM, as described below [11•]. Other lncRNA scaffolds recruit chromatin-modifying complexes independently of smRNAs, although how protein complexes recognize lncRNAs to jointly target genes remains unclear. Mammalian lncRNAs can positively regulate transcription via interacting with Trithorax group proteins to trimethylate histone H3K4 [38], while other lncRNAs negatively regulate transcription via targeting repressive histone-modifying activities, for example by interacting with Polycomb-Repressive Complex 2 (PRC2) to methylate histone H3K27 [39].

Thus, lncRNAs regulate gene expression at the transcriptional and post-transcriptional levels, by multiple, complex mechanisms, which we are just beginning to understand. The sections below provide more detail on two of the best-studied functions of lncRNAs, in RdDM and the regulation of flowering time.

LncRNAs in RdDM

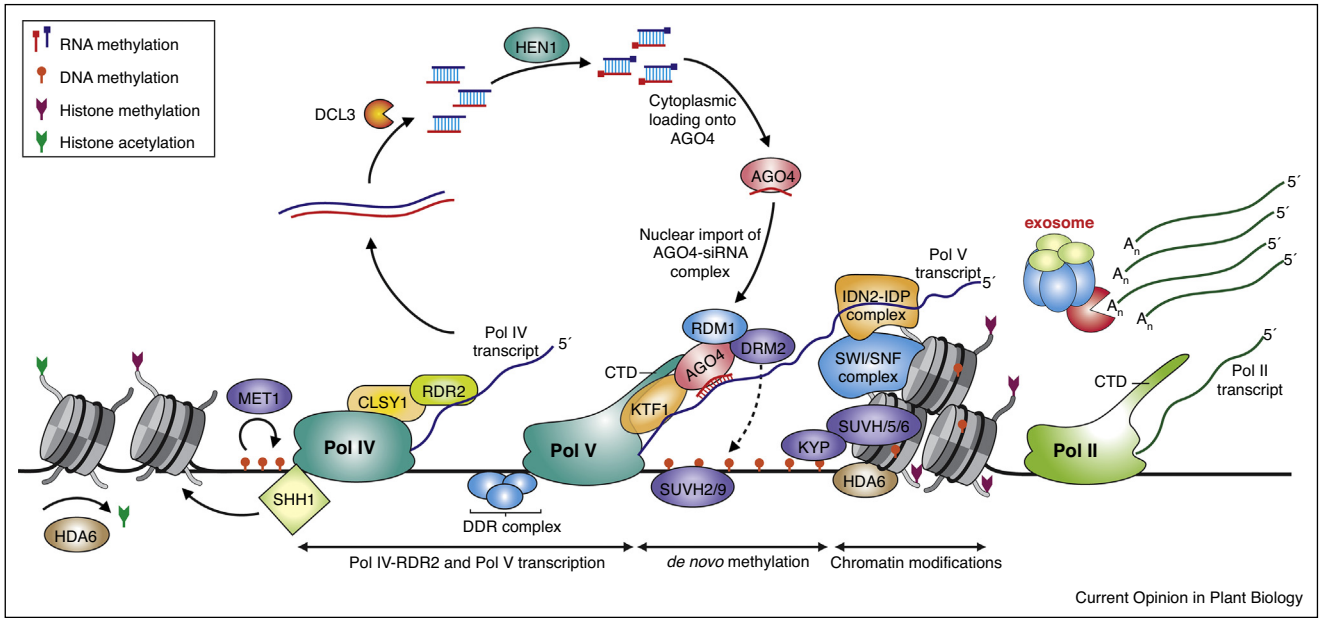
Plant lncRNAs can contribute to epigenetic silencing via RdDM, which primarily silences repetitive sequences and requires the plant-specific RNA polymerases Pol IV and Pol V [26], with some involvement of RNA Pol II (see Figure 2) [40]. A group of lncRNAs transcribed by Pol IV produces 24-nt small interfering RNAs (siRNAs), and lncRNAs produced by Pol V function as scaffold RNAs recognized by the siRNA-Ago complex through sequence complementarity (reviewed in [11•]).

In Arabidopsis, Pol IV generates most siRNAs, although, Pol V and to a lesser extent Pol II produce the templates for siRNAs, indicating the complexity of siRNA biogenesis [41–44]. The lncRNAs produced by Pol IV and Pol V have been difficult to identify, possibly due to their very low abundance or stability. For example, only several Pol V-transcribed scaffold lncRNAs, which are non-polyadenylated and either tri-phosphorylated or capped at the 5′ ends, have been reported to date [26]. Recent work identified Pol IV/RDR2-dependent transcripts, P4RNAs, derived from thousands of loci in Arabidopsis, mainly at intergenic regions, and 65% of them overlapped with annotated transposons or repeats, but only 9% of them overlapped with genes [45••]. These Pol IV/RDR2-dependent transcripts are non-polyadenylated and, intriguingly, correspond to both DNA strands. A surprising finding was that the 5′ ends of P4RNAs bear a monophosphate instead of a 5′ triphosphate, or a cap structure [45••].

Pol V transcripts may also have additional functions outside of the RdDM pathway, as indicated by a genome-wide study to identify Pol V-associated loci [46,47]. About 75% of genomic sites occupied by Pol V correspond to transposons and repeats that are also associated with 24-nt siRNAs and DNA methylation, indicating that Pol V mediates RdDM at these sites. By contrast, the remaining 25% of the sites occupied by Pol V lack these features and are biased towards genes, suggesting that Pol V also participates in different silencing pathways [46]. Pol II also produces scaffold transcripts that recruit AGO4-bound siRNAs to elicit RdDM and transcriptional gene silencing at some loci [40], suggesting an intricate collaboration between Pol II and Pol V. However, the characteristics that attract Pol II to some intergenic loci and the requirements for Pol II interaction with Pol IV and Pol V remain unknown.

Our previous genome-wide studies of exosome targets revealed a large number of polyadenylated exosome substrates corresponding to ncRNAs that originate from centromeric regions, repetitive elements and other loci that produce siRNAs and are silenced through RdDM [1]. However, we found that the loss of the Arabidopsis core exosome subunits had a minor effect on global smRNA populations [1,48], by contrast to the fission yeast exosome, which controls the spurious entry of RNAs into smRNA pathways [49]. Instead, it resulted in decreased histone H3K9me2 at several examined RdDM-controlled loci.

Figure 2



LncRNAs in RdDM. Pol IV transcripts serve as precursors for 24 nt siRNAs and Pol V transcripts act as targets of siRNAs. The H3K9me reader SHH1 recruits Pol IV to its genomic loci and the chromatin remodeler CLSY1 facilitates the passage of Pol IV [74]. Pol IV transcripts are made double-stranded by RDR2, processed by DCL3 into 24-nt siRNAs, stabilized by methylation at the 3′ end, and reimported into the nucleus in the AGO-siRNA complex to guide the targeting of nascent Pol V scaffold transcripts. The DDR complex facilitates Pol V transcription [47]. DNA methylation readers SUVH2 or SUVH9 aid Pol V recruitment to its genomic loci [75] and the IDN2–IDP complex bound to Pol V scaffold RNAs interacts with SWI/SNF complex, which adjusts nucleosome positioning [76]. AGO4 interacts with Pol V and with a putative transcriptional elongation factor KTF1 recruiting AGO4-siRNA to nascent Pol V transcripts. The siRNA base pairs with the nascent Pol V transcript and together with RDM1 (RNA-DIRECTED DNA METHYLATION 1) recruits DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE 2) to catalyze *de novo* methylation at the homologous genomic sites. H3K9 methylation, deposited by KYP, SUVH5, and SUVH6, amplifies DNA methylation-mediated silencing (reviewed in [11]). Adapted from [11]. In silencing of the solo LTR region, the exosome does not act in siRNA metabolism and DNA methylation. The exosome associates with transcripts emanating from the adjacent scaffold-producing region, and participates in silencing by affecting H3K9 histone methylation to maintain or establish chromatin structure, in parallel to RdDM, which affects siRNAs and DNA methylation.

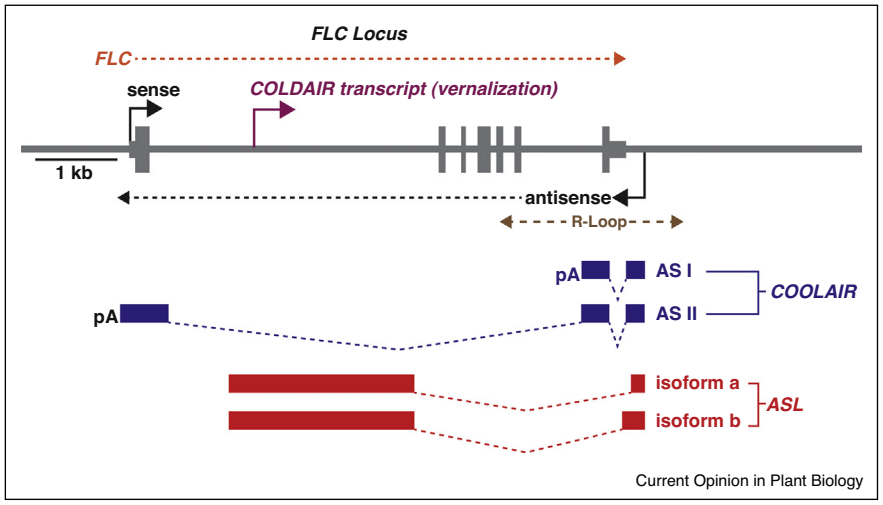
The exosome also exhibits a strong genetic interaction with RNA Pol V, but not Pol IV at these loci, and physically associates with polyadenylated Pol II transcripts arising from the regions generating Pol V scaffold RNAs (Figure 2) [48]. Together, these data suggest that the Arabidopsis exosome participates in the metabolism or processing of lncRNAs produced from scaffold-generating regions and may contribute to coordination of the transcriptional interplay between Pol II, Pol V, and Pol IV RNA polymerases, to achieve the appropriate level of transcriptional repression. How the Arabidopsis exosome or the exosome-controlled lncRNAs facilitate recruitment of chromatin modifiers to enforce silencing through repressive histone modifications remains an open and interesting question.

Arabidopsis exosome complex subunits are functionally diverse [1]. In addition, one of the nuclear catalytic subunits of the exosome complex, RRP6L1, which has exosome core complex-dependent and also complex-independent functions, affects the metabolism of siRNAs and DNA methylation [50] as well, suggesting that exosome subunits functions are essential in regulating various ncRNAs in RdDM, including siRNAs.

LncRNAs in the regulation of flowering

Epigenetic regulation by lncRNAs plays a key role in the regulation of flowering by controlling the expression of Arabidopsis *FLC* (*FLOWERING LOCUS C*). The transcription factor FLC represses flowering in a dosage-dependent manner, blocking the expression of genes required for flowering. *FLC* participates in the vernalization pathway, which regulates flowering time in response to prolonged cold, and in the autonomous pathway, which regulates flowering independently of environmental signals [51]. Epigenetic silencing of *FLC* plays central roles in both of these pathways and mainly involves histone modifications. PRC2, which methylates histone H3K27, is required for *FLC* repression and is recruited to the *FLC* locus before silencing. Epigenetic changes in chromatin structure, particularly the alteration of histone modifications from H3K4me3, H3K36me3, and H2Bub1 to H3K27me3, alter the epigenetic state at *FLC* to repress *FLC* expression (reviewed in [10]).

Figure 3



Regulatory lncRNAs produced from the *FLC* locus. Diagram of the *FLC* locus [57]. The arrow indicates the *FLC* transcription start site and vertical bars denote the exons of the *FLC* sense transcript. COLDAIR lncRNA (purple) is transcribed in vernalization from the first intron of *FLC* in the sense direction, relative to *FLC* mRNA. COOLAIR (blue) and ASL (red) are transcribed in the antisense direction; the arrow indicates their transcription start site. COOLAIR AS I and II are alternatively polyadenylated, with a proximal poly(A) site in intron 6 and a distal poly(A) site in the sense promoter region. Blue boxes show AS I and II exons, and blue dotted lines correspond to the spliced regions. ASL is also alternatively spliced: red boxes depict ASL exons and red dotted lines indicate spliced regions. ASL spans the first intron of *FLC*. Brown dotted lines depict the R loops, which form over the promoter region of COOLAIR. An R-loop that extends from the COOLAIR promoter to the proximal polyadenylation site represses COOLAIR transcription.

Two different classes of lncRNAs transcribed from *FLC*, COLDAIR and COOLAIR, participate in epigenetic silencing of *FLC* (Figure 3) [28,52]. COLDAIR is a 5′ capped, non-polyadenylated lncRNA transiently induced by vernalization from intron 1 of *FLC* and transcribed in the same direction as *FLC* (Figure 3). COLDAIR physically associates with CLF (CURLY LEAF), the plant homolog of the PRC2 enzymatic component EZH2. Knockdown of COLDAIR compromised the cold-mediated enrichment of CLF and H3K27me3 at *FLC* and impaired *FLC* repression in response to vernalization, suggesting that vernalization requires COLDAIR [28]. COLDAIR was proposed to be required for PRC2 recruitment to *FLC* chromatin to initiate epigenetic silencing, similarly to the models proposed for the mammalian HOTAIR and Xist lncRNAs [39]. However, the fact that mammalian PRC2 binds to unrelated RNAs with high affinity suggests that lncRNAs alone are not sufficient to target PRC2 to initiate silencing [53•].

COOLAIR, a set of several alternatively spliced and polyadenylated lncRNAs (AS I and AS II, proximally and distally polyadenylated, respectively) arises from the 3′ end of *FLC* in an antisense direction relative to *FLC* [54]. Cold induces COOLAIR first, before COLDAIR and before the major accumulation of H3K27me3, and COOLAIR was originally proposed to act during the early phase of vernalization [54]. However, disruption of COOLAIR transcription does not disrupt vernalization [55]. Recent work showed that COOLAIR participates in acceleration of transcriptional shutdown of *FLC* during vernalization independently of PRC2 and H3K27me3 [10,56]. The removal of COOLAIR desynchronized the replacement of H3K36 methylation with H3K27me3 in the intragenic *FLC* nucleation region, suggesting that COOLAIR or the process of antisense transcription could be required to coordinate the switching of chromatin states [56].

COOLAIR participates in the vernalization and autonomous pathways to repress *FLC*. In the autonomous pathway the chromatin state of *FLC* is coupled to processing of COOLAIR [57]. The constituents of the autonomous pathway, FCA, FY, FPA, the cleavage polyadenylation machinery components CstF64 and CstF77, and the spliceosome factor PRP8, promote the choice of proximal polyadenylation site in processing of COOLAIR, favoring the production of AS I [57–59]. This affects the recruitment of the histone demethylase FLD (FLOWERING LOCUS D) to *FLC* resulting in H3K4me2 demethylation of *FLC* [60].

Recent work also discovered ASL (Antisense Long) transcript in early-flowering Arabidopsis ecotypes that do not require vernalization for flowering [29]. Distinct from other lncRNAs at *FLC*, ASL is a non-polyadenylated, antisense lncRNA >2000 nucleotides long, with two alternatively spliced isoforms. ASL is transcribed from

the same promoter as COOLAIR and their 5′ regions partially overlap. However, ASL spans intron 1, an important region for maintenance of *FLC* silencing, and it also overlaps with the region that gives rise to COLDAIR in the sense direction. The ASL transcript physically associates with the *FLC* locus and H3K27me3 [29], suggesting that ASL and COOLAIR play different roles in *FLC* silencing and perhaps in the maintenance of H3K27me3.

The exosome functions in RNA processing and two exosome components have important functions in lncRNA-mediated regulation of flowering. RRP6, the nuclear-specific catalytic subunit of the exosome complex, has exosome complex-dependent and complex-independent functions [61,62]. Arabidopsis RRP6L1 and RRP6L*2* (RRP6-Like) regulate expression or processing of both COOLAIR and ASL; this regulation is independent of the exosome core complex [29]. Although single RRP6L mutants had minor effects on COOLAIR, RRP6L double mutants caused *FLC* de-repression and delayed flowering. The pattern of down-regulation of AS I and II in RRP6Ls double mutant was somewhat similar to the pattern observed in mutants of 3′-end processing factors CstF64 and CstF77 [29,57], suggesting that RRP6Ls may participate in the 3'-end processing of COOLAIR.

Surprisingly, RRP6Ls appear to function as the main regulators of ASL synthesis or biogenesis, as their mutants show little or no ASL transcript. This observation is intriguing since RRP6 is a 3′–5′ exoribonuclease and RRP6 defects usually result in over-accumulation of various RNAs due to failures of RNA degradation or processing. However, recent work reported that a surprisingly high number of yeast mRNAs also showed decreased abundance in *rrp6Δ* mutants [63••]. Similarly, in humans, inactivation of the RRP6 homolog dramatically reduces the levels of Xist, which functions in X-chromosome inactivation [64].

RRP6Ls also affect epigenetic modification of *FLC*; for example, RRP6L mutants exhibit decreased levels of H3K27me3 and lowered nucleosome density at the *FLC* locus, correlated with *FLC* de-repression and flowering delay in these mutants. RRP6L1 physically associated with the ASL transcript and directly interacted with the *FLC* locus, suggesting that RRP6L proteins may participate in the maintenance of H3K27me3 via regulation of ASL. Thus, RRP6Ls participate in the regulation of synthesis or biogenesis of *FLC* lncRNAs and might also act in different *FLC* silencing pathways by regulating diverse antisense transcripts [29].

COOLAIR transcription is affected by R-loops, which form over the COOLAIR promoter region, and a mutant of TF AtNDX showed de-stabilized R-loops and increased COOLAIR transcription [65]. However, the increase in COOLAIR transcription in this mutant was also accompanied by increased *FLC* expression and delayed flowering; thus, the role of R-loop formation over the COOLAIR promoter in the regulation of *FLC* remains unclear. The formation of R-looped structures can arise from failure of transcriptional termination [66], which itself serves as a mechanism for co-transcriptional exosome recruitment through the noncanonical 3′ end-processing pathway [63••]. RRP6 also participates in resolving deleterious R-loops in mammalian cells [36••], suggesting that plant RRP6Ls may act similarly in the processing of *FLC* antisense transcripts and participate in resolving R-loops. These observations suggest that the lncRNA-mediated regulation of *FLC* is even more complex than previously thought.

Many mammalian lncRNAs play crucial roles in bringing together proteins, RNA, and DNA to actively shape three-dimensional nuclear organization (Figure 1f) [67••,68,69]. Although information about the role of lncRNAs in nuclear architecture in plants is only beginning to emerge, several studies hint that this mechanism might also act in plants. First, the RdDM pathway may contribute to higher-order chromatin structure through collaborating with the MORC proteins. Arabidopsis MORC6 has been proposed to provide ATPase activity for DMS3, a component of DDR complex, to form a functional analogue of a cohesin-like protein required for X-chromosome inactivation in mice. In accord, mutant plants deficient in MORC1 and MORC6 show decondensation of pericentromeric heterochromatin [70]. Second, the *FLC* promoter and 3′ terminator regions form a short-distance interactions known as gene loops [71,72]. Similar chromatin loops formed between the locus that gives rise to the APOLO lincRNA and the distant genomic regions of the *PID* gene, and APOLO lincRNA affects the spatial association of these loci. The dynamics of the APOLO region loop formation is controlled via RdDM, active DNA demethylation, and Polycomb complexes [9•]. *FLC* alleles also physically cluster during epigenetic silencing in vernalization, forming long-distance interactions, and this process is dependent on the PRC2 *trans*-acting factors VRN5 and VERNALIZATION 2 [73]. However, the role of lncRNAs in this process remains unclear.

The example of *FLC* illustrates the diverse, complex, and essential roles that lncRNAs play in plants. Moreover, although many studies have improved our understanding of the functions of lncRNAs, emerging work has only begun to reveal the mechanisms that regulate lncRNAs, illustrating the key importance of transcription and RNA-processing activities in this regulation.

Conclusions and outlook

In the short time since the discovery of pervasive transcription, studies in plants, animals, and fungi have significantly

expanded our knowledge of lncRNA biology, particularly in identification of different categories of lncRNAs. By contrast, much remains to be understood about lncRNA functions and mechanisms of action, particularly in plants. Remarkable progress has been made in elucidating the roles of plant lncRNAs in regulation of flowering time and in RdDM. However, the roles of very few other plant lncRNAs have been explored to date. Details on the regulation of synthesis and biogenesis of lncRNAs in plants also remain scant. Ongoing and future work to balance our understanding by identifying factors controlling the expression and biogenesis of lncRNAs and integrating this knowledge with the information learned about the components functioning with these lncRNAs will provide crucial insights into the mechanisms of lncRNA function. Moreover, addressing all angles of the problem will also enable synergistic advances that will allow plant lncRNAs to be better understood. Many discoveries are waiting to be unearthed for myriad plant lncRNAs.

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- • of special interest
- •• of outstanding interest

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