

# Long non-coding RNAs and chromatin regulation

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Microarray analysis and new sequencing technologies have revealed that the majority of the genome is transcribed in many eukaryotes. Much of the RNA appears to be non-coding and an ongoing debate is how much of a functional role it has. Different mechanisms by which ncRNA can be regulatory have been described: direct ncRNA effects on transcription; recruitment of chromatin modifiers; formation of silent nuclear compartments. These have been documented chiefly in yeasts and mammals but examples are now appearing in plants. To date RNA-mediated transcriptional silencing studies in plants have focused on siRNAs, but data now show longer ncRNAs are also involved in this silencing. Roles for long ncRNAs in the phenotypic plasticity of plants are also suggested by whole genome analysis showing widespread effects of different external cues on ncRNA expression.

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Current Opinion in Plant Biology 2011, 14:168–173

This review comes from a themed issue on  
Genome studies and molecular genetics  
Edited by Jeffrey L. Bennetzen and Jian-Kang Zhu

Available online 17th December 2010

1369-5266/\$ – see front matter  
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DOI [10.1016/j.pbi.2010.11.006](https://doi.org/10.1016/j.pbi.2010.11.006)

### Introduction

Analysis of transcriptome complexity in nuclear and cytoplasmic RNA in the 1970s suggested the existence of large amounts of nuclear non-coding RNA (ncRNA) [1]. However, the predominant components regulating transcription were viewed, until recently, to be transcription factors and their cognate *cis* elements. This view has changed rapidly, in part through early discoveries in plants of the role of double-stranded and small RNA in transcriptional gene silencing [2]. A genome-wide perspective was introduced by the surprise finding that 30% of annotated genes in the Arabidopsis genome showed significant antisense RNA expression [3]. Deep sequencing of transcriptomes in many organisms has now revealed extensive ncRNA, including intergenic, aberrantly processed and antisense transcripts. This adds to the already extensive list of small RNAs including small

nucleolar (sno) RNAs, micro (mi) RNAs, small interfering (si) RNAs and in plants *trans*-acting si (tasi) RNAs and natural *cis* acting siRNAs. Some of the longer ncRNA may code for very small proteins [4], but it is rapidly turned over by degradation pathways such as the exosome [5] or nonsense-mediated decay [6] and therefore is of very low abundance, perhaps explaining why it has gone unnoticed for so long. The important question now is how much of this RNA has regulatory roles. Here, we will not try to review all aspects of non-coding RNA and refer the reader to excellent recent reviews on how non-coding RNA could impart specificity in mammalian chromatin regulation [7], the role of ncRNA in plants in general [8] and the importance of small RNAs in plant development [9]. Instead, we focus on the types of mechanisms whereby long (>100 nucleotide) non-coding RNA can effect chromatin regulation in eukaryotic systems, as these mechanisms are less well understood as compared to small RNA silencing pathways and will be of importance in plant research. We end by selecting one case study – regulation of the floral repressor *FLC* – where antisense transcripts are linked to chromatin regulation via two different histone modifications in independent flowering pathways.

### Mechanism of action of ncRNAs

Long ncRNAs can influence chromatin regulation and therefore gene expression in a number of ways (schematically represented in [Figure 1](#)).

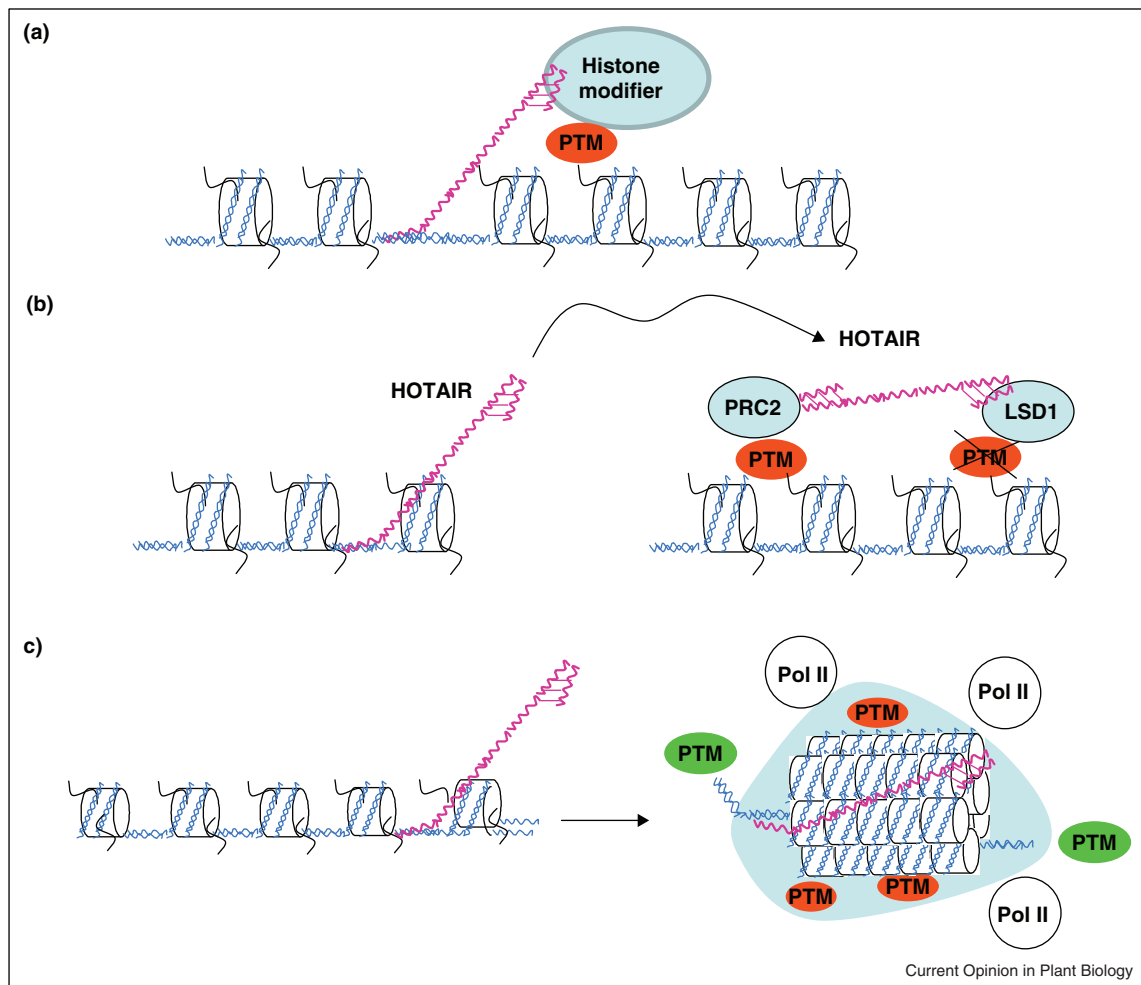
#### Direct ncRNA effects on transcription

There are many examples, especially in *Saccharomyces cerevisiae*, where transcription of convergent or overlapping genes results in transcriptional or promoter interference via physical interactions between transcribing polymerases [10]. However, the ncRNA molecule itself, can influence transcription via a number of mechanisms: antisense transcripts generated in the vicinity of promoters have been found to facilitate sense transcription in *S. cerevisiae* [11]; transcripts formed over regulatory elements have been shown to influence adjacent gene expression [12]; ncRNAs have been found to globally and directly inhibit RNA polymerase by binding in the active site and blocking the early steps of transcription initiation, [13,14]; a new class of ncRNAs, known as enhancer-ncRNAs (eRNAs), function in *cis* and activate neighbouring genes [15]. Whether these represent distinct mechanisms or modifications on a theme is currently unknown.

#### Recruitment of chromatin modifiers

Long ncRNAs also guide the site-specific recruitment of chromatin-modifying enzymes [7]. How long ncRNAs

Figure 1



Possible mechanisms of action for ncRNAs. (a) Recruitment of histone modifiers to specific target genes by ncRNAs (purple) acting in *cis* and inducing post-translational modifications (PTMs) of histones (in red, those associated with silent chromatin and in green, those associated with active chromatin). (b) ncRNAs functioning in *trans*. The mammalian ncRNA HOTAIR silences a Hox locus on a different chromosome through interaction with two different histone modifiers: Polycomb Repressive Complex 2 (PRC2), which methylates H3K27 and a histone demethylating complex (known originally as LSD1 but now KDM1), specific for H3K4me2. (c) Formation of a silent nuclear compartment. Long non-coding RNAs can mediate the formation of nuclear domains that are enriched in histone modifications associated with silent chromatin (red) and deficient in RNA polymerase II (Pol II) and histone modifications associated with active chromatin (green).

impart specificity to chromatin modifications is a matter of intense analysis and current views include sequence-specific (RNA:DNA) recruitment, allosteric activation of the modifying enzymes or a scaffold function [7]. Classic examples of ncRNAs that recruit histone modification machinery are the mammalian Xist, Kcnq1ot1 and Air ncRNAs, all required for allele-specific epigenetic silencing of multiple genes in *cis* within large chromosomal domains [16–18]. Examples of more local activity include the cold-induced epigenetic silencing of PHO84 in yeast [19]. Specific secondary structures of ncRNAs are a common theme in many of these mechanisms. A stem and loop structure was identified for the repeated motif known as “Repeat A” of the Xist RNA, responsible for the recruitment of PRC2 complex to the inactive X [20]

and it seems to be conserved in other ncRNAs involved in recruitment of the PRC2 complex [21].

ncRNAs can guide site-specific recruitment of chromatin-modifying enzymes in *trans*, i.e. on different chromosomes. This function is illustrated beautifully by the 231 ncRNAs transcribed from human HOX loci. These are sequentially expressed along a developmental axis with their expression demarcating broad chromosomal domains of differential histone methylation [22]. One of these is the 2.2 kb HOTAIR ncRNA, which originates from the HOX C locus and represses transcription across 40 kb of the HOX D locus, on a different chromosome, via recruitment of the Polycomb Repressive Complex 2 (PRC2). This type of silencing is likely to have

widespread consequences as 20% of the large intergenic ncRNAs in the human genome are physically associated with PRC2 [23]. HOTAIR also associates with a second histone modification complex involving a histone demethylase activity significantly increasing the possible complexity of the outcomes associated with ncRNA/chromatin modifier interactions [24\*\*].

#### Formation of silent nuclear compartments

ncRNAs also play architectural roles through participation and/or formation of specific nuclear compartments [25]. The association of ncRNAs with specific chromosomal domains can lead to their compartmentalization into a nuclear region inaccessible to RNA Pol II. The chromatin in these domains tends to be enriched in the histone modifications associated with repressed chromatin (H3K27 and H3K9 methylation) and devoid of modifications associated with active chromatin (acetylation/H3K4 methylation). The first examples were Xist [26\*] and Kcnqot1 [17,27,28] and in both cases, formation of the silent compartment mediated by the ncRNA was found to be an early event in the silencing process. A modular architecture seems to be a common theme for ncRNAs with different parts of the ncRNAs able to have different roles. This is exemplified by the stem-loop structure of Rep A of the Xist RNA being required for the recruitment of the PRC2 complex [16] but not in the Xist-dependent formation of the silent compartment [26\*].

Although no similar nuclear compartments have yet to be described in plants RNA-mediated transcriptional silencing in plants does appear to involve nuclear compartmentalization. Several components of the RNAi machinery appear to be enriched in nuclear bodies that associate with the nucleolus [29,30].

#### Non-coding RNA and siRNA silencing

To date RNA-mediated transcriptional silencing studies in plants have focused on siRNAs [9]. These are reviewed elsewhere in this issue so here we discuss recent data that suggests longer ncRNAs are also involved in this silencing. The paradigm for how ncRNA links with chromatin regulators came from dissection of the mechanism for RNAi-mediated heterochromatin formation established in *Schizosaccharomyces pombe*. Centromere silencing was disrupted in RNAi mutants revealing a role for siRNA in heterochromatin formation. However, Pol II-generated heterochromatic transcripts were also found to be required in combination with the siRNAs. Non-coding RNAs thus serve a dual function in *S. pombe* centromere silencing, serving as both precursors to siRNA and scaffolds that interact with siRNAs to recruit chromatin-modifying factors [31,32].

siRNA-induced transcriptional silencing in plants is characterized by the involvement of distinct plant-specific RNA polymerases, Pol IV and Pol V [33]. Pol

IV is required for siRNA production [34,35] and Pol V transcribes short non-coding transcripts adjacent to the silenced regions. These can be at least ~200 nt in size, initiate from multiple sites, have triphosphates or 7meG caps at their 5' ends, and lack poly A tails [36\*\*] and appear to serve as a scaffold to trigger the chromatin silencing perhaps by base-pairing with associated siRNAs and guiding AGO4 to target loci [37]. Pol IV and Pol V function are sufficient for silencing high copy number repeats but transcription of ncRNA by RNA Pol II is important for silencing the low-copy-number intergenic loci [38\*\*]. These act as non-coding scaffold transcripts that recruit the siRNA:AGO4 effector complex and Pol V to the target locus. The Pol II-dependent long ncRNA also act in the amplification of silencing by recruiting the siRNA-producing polymerase Pol IV [38\*\*]. Thus, intergenic transcription of long ncRNA by Pol II serves two functions in siRNA-transcriptional gene silencing in plants as it does in *S. pombe*.

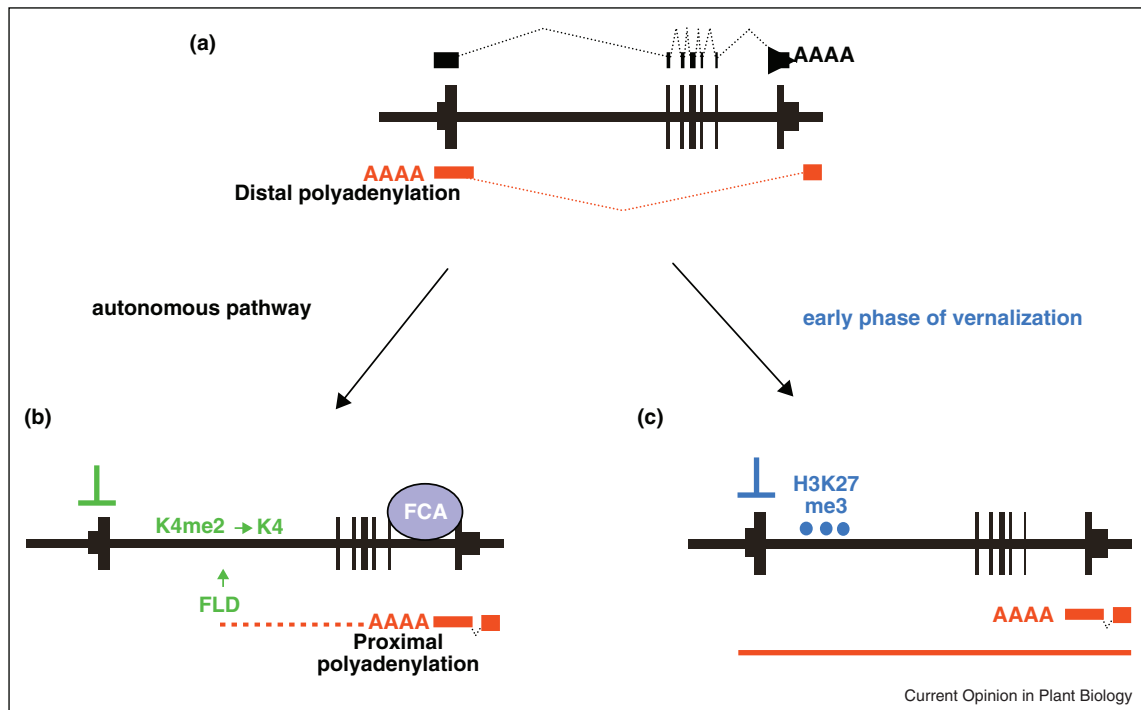
#### A specific case: the non-coding RNAs and chromatin regulation at *FLC*

The next few years are likely to see a huge increase in the number of plant genes being regulated by ncRNAs in the mechanisms described above. However, to date, our most detailed understanding comes from dissection of the regulation of the floral repressor locus *FLC*. Two different floral pathways that serve very different functions in the regulation of the transition to flowering have been found to involve long ncRNA-mediated chromatin silencing (Figure 2). One of these pathways adds a new regulatory ncRNA mechanism not previously described in other organisms – the triggering of chromatin silencing via targeted 3' processing of antisense transcripts.

At *FLC*, sense and antisense RNAs are co-expressed in a tightly co-ordinated manner with the end of the sense transcript aligning with the beginning of the antisense, and the end of the antisense aligning with the beginning of the sense transcript. The autonomous floral promotion pathway works through the action of the RNA binding proteins FCA and FPA. Both proteins independently function to promote 3' processing at the proximal polyadenylation site in the antisense *FLC* transcript. This in turn triggers histone K4 demethylase activity in the body of the gene that induces *FLC* transcriptional down-regulation of both sense and antisense strands [39,40\*\*,41\*\*]. Loss of any of the autonomous pathway components results in use of the distal poly (A) site and loss of the histone demethylation, and thus increased transcription. Judging from studies of RNA silencing in other organisms this linking of co-transcriptional RNA processing of ncRNAs with chromatin structure and transcription rate may be a common mechanism.

In the vernalization pathway, *FLC* is silenced by prolonged cold and this is epigenetically maintained for the

Figure 2



Linkage of ncRNA and chromatin modifications at *FLC*. (a) High expression of *FLC* is associated with polyadenylation of the *FLC* antisense transcript at the distal site and high levels of H3K4me2. (b) Low expression of *FLC*, through activity of the autonomous pathway, is associated with polyadenylation at the proximal site, which triggers FLD activity, demethylation of H3K4me2 and transcriptional down-regulation. (c) In the early phase of vernalization short cold induces up-regulation of *FLC* antisense, repression of *FLC* sense and H3K27me3, which epigenetically maintains the silencing through the rest of development.

rest of the life cycle. One of the earliest events in this silencing is a large increase in abundance of the antisense transcripts [42<sup>\*\*</sup>]. Antisense transcripts initiated from the *FLC* antisense promoter region were found to be sufficient to induce cold-dependent silencing of a GFP sensor construct, suggesting that these ncRNAs (which have been called COOLAIR after the now famous human predecessor HOTAIR) are important for the cold-induced transcriptional silencing of *FLC*. These antisense transcript changes occur before and independently of the Polycomb function that epigenetically maintains the silencing. Although the ncRNA action and Polycomb function (associate with increased H3K27me3 levels) are genetically separable there are important links. The transient accumulation of the antisense RNA becomes more long-term in Polycomb mutants and COOLAIR transcripts do physically associate with Polycomb components (De Lucia, Swiezewski and Dean unpublished). Elaboration of the complexity of this mechanism is likely to establish important concepts in the Polycomb field generally.

## Conclusion

It is relatively early days in the field of long non-coding RNA and chromatin regulation in plants but the explosion

of data in other eukaryotic systems showing the importance of ncRNAs in gene regulation, and examples such as *FLC*, suggest it will be a major area very soon. Whole plant transcriptome analysis has revealed interesting non-coding RNA expression [43,44]: their synthesis can be under circadian clock control [45], and be influenced by a number of abiotic factors [46,47]. How these environmental influences have such genome-wide effects is unknown at present. How the plant ncRNAs interact with small RNA pathways also requires much more analysis [48]. The clear potential of ncRNAs to induce chromatin changes that may be epigenetically inherited suggests they could play a significant role in the phenotypic plasticity of plants to environmental change. ncRNAs might play a bridging role in this plasticity through their ability to interact with different chromatin complexes. Very low abundance long ncRNAs may also be a common feature in many other transcriptional silencing mechanisms such as paramutation [49]. What is clear is that due to the paradox of requiring transcription in order to get transcriptional silencing, ncRNAs are likely to play a central role.

## Update

While preparing this manuscript an additional *FLC* non coding RNA (COLDAIR) has been reported to be

involved in *FLC* silencing during vernalization (Heo and Sung, *Science*, 2 December 2010, 10.1126/science.1197349).

## Acknowledgements

We thank Szymon Swiezewski and Clare Lister for critical reading of the manuscript and BBSRC (BB/C517633/1) and European Research Council (grant no. 233039) for financial support. The authors apologize for those publications that were not cited due to limitations of space.

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