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Covalent RNA modifications and their budding crosstalk with plant epigenetic processes

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Abstract

Our recent cognizance of diverse RNA classes undergoing dynamic covalent chemical modifications (or epitranscriptomic marks) in plants has provided fresh insight into the underlying molecular mechanisms of gene expression regulation. Comparatively, epigenetic marks comprising heritable modifications of DNA and histones have been extensively studied in plants and their impact on plant gene expression is quite established. Based on our growing knowledge of the plant epitranscriptome and epigenome, it is logical to explore how the two regulatory layers intermingle to intricately determine gene expression levels underlying key biological processes such as development and response to stress. Herein, we focus on the emerging evidence of crosstalk between the plant epitranscriptome with epigenetic regulation involving DNA modification, histone modification, and non-coding RNAs.

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Keywords

RNA modification, DNA methylation, Histone modifications, Non-coding RNAs, Plant epigenetics, N^6-methyladenosine (m^6A).

Introduction

It is well established that various covalent chemical modifications are added to RNA molecules in organisms throughout all kingdoms of life. This collection of covalent RNA modifications, referred to as the epitranscriptome of an organism, can significantly influence transcript fate [1]. With the advancements in high-throughput sequencing technologies, we have witnessed an exponential increase in the identification and characterization of the specific covalent modifications of RNA nucleotides that make up the epitranscriptome in plant transcriptomes. Notably, owing to its high abundance in messenger RNAs (mRNAs), N^6-methyladenosine (m^6A) has surfaced as the most well-studied epitranscriptomic mark across diverse plant species [2–4]*. In fact, the occurrence of m^6A in mRNAs of multicellular eukaryotes like mammals and plants is now quite clear [5–9]. Alongside many breakthrough studies from mammalian research, the plant research community has also made significant contributions in m^6A research. For instance, it was first established that m^6A is essential for embryo development in multicellular eukaryotic organisms from pioneering studies in the model plant *Arabidopsis thaliana* [10]. Similarly, several recent studies in animals and plants have investigated the role of m^6A marks on RNA metabolic fate via the involvement of the YTH-domain containing family of RNA-binding proteins as readers of m^6A. In fact, it was demonstrated for the first time that the YTH domain-containing protein ECT2 relocates into stress granules and not processing bodies in the context of plant response to heat stress [11]. Additionally, the plant YTH-family protein CPSF30 is the first known m^6A reader whose role in regulating mRNA polyadenylation site selection was established, along with its role in the nitrate signaling pathway [12]. While it is not possible here to highlight all the pioneering research within the landscape of plant epitranscriptomics, many recent reviews have extensively covered the scope and details of plant RNA modifications characterized to-date [3,4,13–16]; the role of associated writers, erasers, and readers [17,18]; the impact of specific RNA modifications during distinct RNA life cycle stages [2*]; and the regulation of key biological processes by important riboregulators [19–21].

Mirroring the epitranscriptome, the relatively extensively studied epigenome comprises the collection and distribution of covalent chemical modifications of DNA and histone proteins [22,23]. The epigenome has been found to influence genome structure and stability without any alterations at the genome sequence level, and consequently regulates plant response to both internal and external signals [24]. Global profiles of plant epigenomic marks such as DNA methylation (5-methylcytosine (5 mC)), histone modifications such as methylation, acetylation, and ubiquitination, and their impact on

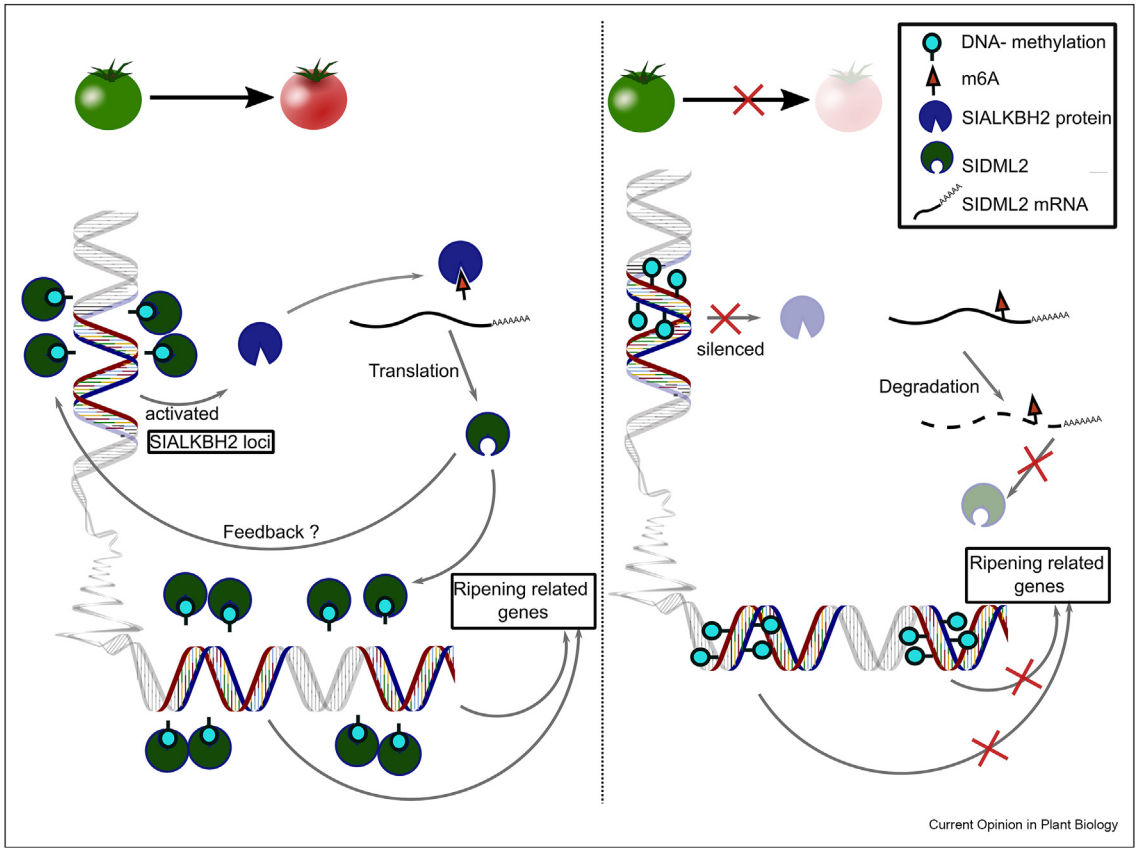
chromatin remodeling have been widely investigated [25–27]*. In addition to the canonical regulators (including proteins such as writers, erasers, and readers [28]) of these epigenomic marks, non-coding RNAs such as long non-coding RNAs (lncRNAs) [29,30], microRNAs (miRNAs) [31–33], and more recently chromatin-associated RNAs (caRNAs) [34–36] have been found to interact with the epigenetic machinery to specifically modulate gene expression at both transcriptional and post-transcriptional levels in plants.

With both the epigenome and epitranscriptome serving as layers in regulation of gene expression in plants, crosstalk between the two and the subsequent effects of these interactions on regulatory processes, are only beginning to be studied. To provide a glimpse of this new research dimension in plants, we highlight some recently surfaced promising links of the plant epitranscriptome with DNA methylation, histone modifications, and ncRNAs as modulators of gene expression at the epigenetic level. We also discuss their importance and direction for future inquiry in plants.

The interplay between RNA and DNA modifications in plants: m^{6}A, 5 mC, and the fruit ripening nexus

DNA methylation is a conserved epigenetic mark in plants that has been associated with maintenance and regulation of genome stability, thereby, affecting gene expression during development and stress response [26,37]. Across eukaryotes, 5 mC is the most widely studied DNA methylation mark [38]. In plants, genome-wide mapping of 5 mC has revealed the distribution of this mark can vary across different cell types and in response to varying environmental conditions, which can lead to important biological consequences [39–41]. As a dynamic process in plants, DNA methylation can be passively lost or diluted during successive DNA replication events [37]. Alternatively, active demethylation can occur by the involvement of 5 mC DNA glycosylases/lyases such as REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2), and DML3 to inhibit hypermethylation at genomic locations and allow proper levels of gene expression [26,37].

Figure 1



RNA and DNA demethylation machinery in tomato ripening: During tomato ripening, the promoter of the *SIALKBH2* gene gets demethylated and activated. The SIALKBH2 protein demethylates m^{6}A on the *SIDML2* transcript, which prevents it from being degraded. SIDML2, a DNA demethylase activates ripening related genes and also possibly feeds back to activate the *SIALKBH2* locus.

In tomato, an initial investigation of potential epitranscriptome and epigenome interplay during the process of fruit ripening revealed some interesting findings (Figure 1). Previously, wide-spread 5 mC demethylation of the genome was associated with the transcription of well-established fruit ripening genes in tomato [42,43]. Subsequently, Zhou et al. [44]** explored the epitranscriptome-wide m^6A profile of tomato mRNAs and observed an overall decline in this covalent modification during fruit ripening. Previously, 5 mC was found to potentially regulate the transcription of the *SlALKBH2* locus, which encodes an m^6A eraser (RNA demethylase) in this plant. This m^6A eraser protein can in turn bind with the *SlDML2* mRNA that encodes a 5 mC eraser protein (DNA demethylase) in tomato, thereby, facilitating its m^6A demethylation and consequently, promoting the stability of this transcript. In their study, the authors used a CRISPR/Cas9 gene editing system to target exons of *SlALKBH2*. While their subsequent analysis of three resulting independent mutants clearly validated the indispensability of SlALKBH2 protein function for normal fruit ripening in tomato, it also revealed decreased *SlDML2* mRNA levels and increased m^6A modification on this specific mRNA in the mutant plants. However, mechanistically, the reciprocity of *SlALKBH2-SlDML2* demethylation mediated by the proteins encoded by the opposite transcript currently relies heavily on the findings of transient expression assays done in *Nicotiana benthamiana* leaves. Thus, further investigation in tomato is required to consolidate the proposed DNA-RNA demethylation regulatory model. Nevertheless, overall, these findings draw our attention to the importance of demethylation at both the epigenomic and epitranscriptomic levels, and the potential of epigenomic and epitranscriptomic feedback loops regulating fundamental processes, such as fruit ripening in climacteric fruits.

Likewise, efforts are being made to understand the regulation of ripening in non-climacteric fruits, such as strawberry, at both the epigenomic and epitranscriptomic levels. Similar to tomato, strawberry exhibits a striking overall trend of DNA hypomethylation during fruit ripening [45]. Mechanistically, the global reduction in DNA methylation in tomato has been attributed to increased expression levels of transcripts encoding DNA demethylases, such as *SlDML2* [43]. However, in strawberry, Cheng et al. [45] observed no such expression trend for *SlDML2* homologs, *FveDME1*, *FveROS1.1*, *FveROS1.2*, and *FveROS1.3*. Instead, transcripts of 5 mC writers (DNA methyltransferases) such as DOMAINS REARRANGED METHYLASEs (DRMs) (e.g., *FvDRM1.3* and *FvDRM3.1*) were downregulated. DRMs are *de novo* DNA methyltransferases that are involved in the RNA-directed DNA methylation (RdDM) pathway in plants [46] (reviewed in [37]). Cheng et al. [45] further examined the expression profile of transcripts encoding the other components of this pathway such as those associated with siRNA biogenesis (*Pol IV*, *RDR2*, and *DCL3*) and siRNA-guided DNA methylation (*Pol V*, *AGO4/6*, and *DRM2*) and found that they were downregulated along with RdDM-dependent 24 nucleotide (nt) siRNA products. From these observations, it was concluded that the RdDM pathway rather than a DNA demethylation pathway is involved in reprogramming of 5 mC during strawberry ripening. At the epitranscriptomic level, Zhou et al. [47] have recently investigated m^6A profiles in strawberry during ripening. Unlike the climacteric fruit model plant (tomato) that likely involves m^6A demethylase *SlALKBH2* in positive regulation of ripening, the non-climacteric fruit model plant (strawberry) exhibits an indispensable involvement of m^6A methyltransferases, *MTA* and *MTB*, in positive regulation of strawberry fruit ripening, which was demonstrated by agroinfiltration-mediated transient transformation of strawberry fruit resulting in delayed ripening in *MTA or MTB* RNAi fruits and early ripening in *MTA-* or *MTB*-overexpressing fruits compared to the respective control fruits (agroinfiltrated with empty vectors). Finally, although the results of Zhou et al. [44]** could support the proposal of a nexus between DNA and RNA (de)methylation in tomato ripening, this research team did not observe any differential m^6A profile for transcripts of DNA methyltransferases involved in the RdDM pathway in strawberry ripening [47]. Overall, more work is needed to understand the interconnections between epigenomic and epitranscriptomic machinery that is involved in regulation of ripening in non-climacteric fruits, while conclusive evidence of this nexus directly in planta is needed for climactic fruit ripening.

Based on the tomato ripening case study, the interplay between the epigenome and epitranscriptome needs to be further explored in additional biological processes, as the potential of these combined regulatory mechanisms is immense. Even in the context of fruit ripening, Zhou et al. [44]** noted that in addition to their current findings, SlALKBH2 could potentially modify transcripts of other ripening-associated genes in tomato, which needs further investigation. Moreover, at the epigenomic level, SlDML2 could activate several ripening-related genes as evidenced by the drastic negative impact on fruit ripening in a loss-of-function *sidml2* mutants [43]. Therefore, much work is still to be done in this context and other biological processes to get at the true importance of the interplay between the epigenomic and epitranscriptomic pathways in plants.

Correlating histone and RNA modifications in plants: H3K36me2 and m^6A

Prominent chemical modifications like methylation, acetylation, and ubiquitination of histone protein residues occur post-translationally. These modifications are well known to regulate gene expression by influencing chromatin accessibility and subsequently activation or repression of transcription [48]. Based on an extensive

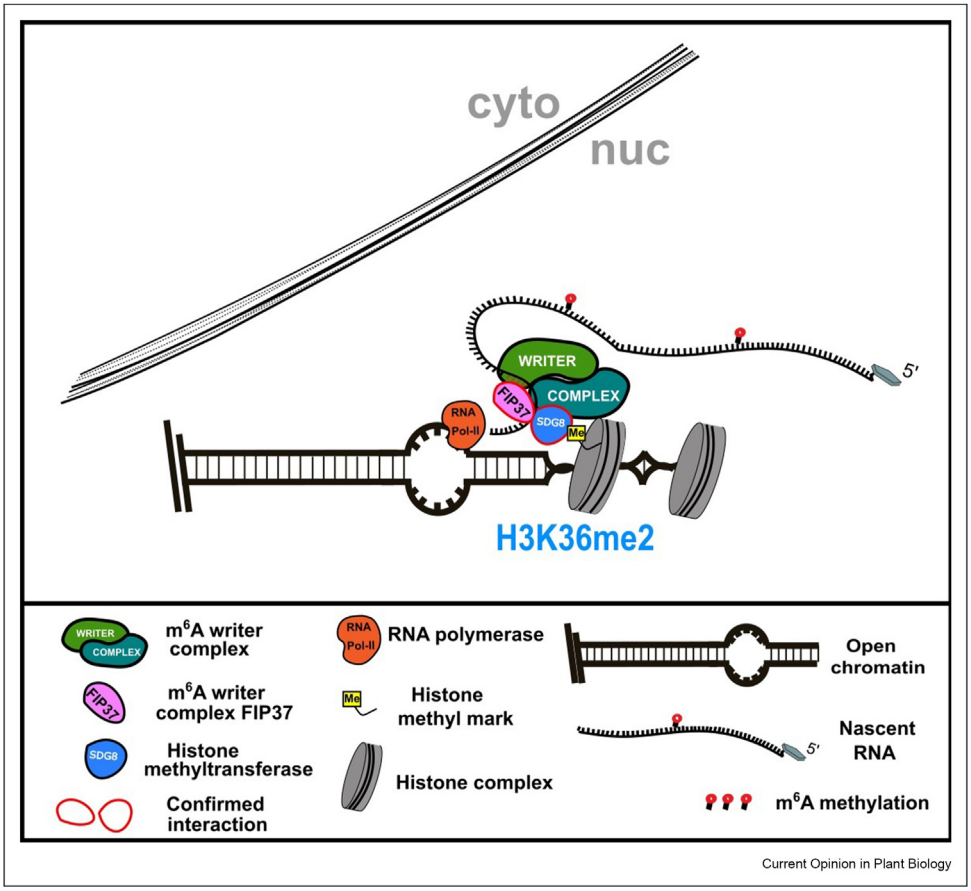
review of *Arabidopsis* histone post-translational modification ChIP-seq data, Leng et al. [27] have highlighted a genomic positioning system (or genomic distribution) that potentially guides different stages of RNA Pol II transcription such as initiation; early and productive elongation; and termination. Interestingly, histone modification marks can not only recruit and/or guide plant RNA Pol II transcription, but can also potentially recruit RNA modification complexes to specifically deposit chemical modifications on RNAs in eukaryotes. The link between histone modifications and m^6A was first explored in mammals, and m^6A deposition on mRNAs was found to be associated with tri-methylation on histone H3 lysine 36 (H3K36me3) [49]. However, the same mechanism of potential direct recruit of methyltransferase components by this specific histone modification has not been demonstrated in any plant system to date. Inspired by this initial study, Shim et al. [50]** conducted a similar, but first such analysis in plants, based on comparisons of global distribution of seven *Arabidopsis* histone marks (H3K4me2, H3K4me3, H3K9Ac, H3K18Ac, H3K27me3, H3K36me2, and H3K36me3) as compared to published m^6A mRNA profiles [51,52]. Surprisingly, their study revealed a strong correlation between H3K36me2 and m^6A mRNA localization patterns in this model plant. More specifically, Shim et al. observed that both H3K36me2 and m^6A are enriched at the 3′ end of genes/transcripts, respectively [50]**, and this specific gene 3′ end distribution for H3K36me2 has been confirmed by additional studies [27]*. Relatedly, the authors also investigated the plausible interplay between H3K36me2 and m^6A machineries and reported a direct interaction between the histone methylation writer, SET DOMAIN GROUP 8 (SDG8/AT1G77300) [53] and an important m^6A writer complex component, FKBP12 interacting protein 37 kDa (FIP37/AT3G54170) [54]. However, it must be added here that the authors did not observe physical interaction between any other H3K36me2 metabolic enzymes and m^6A writer complex components (such as MTA and HAKAI) that were tested in the study suggesting the more than likely involvement of other regulatory RNA- and chromatin-binding proteins and transcription factors in site-specific m^6A deposition. Additionally, the authors have not validated their *in silico* findings by performing comprehensive *in vivo* assays such as extensive bimolecular fluorescence complementation and/or co-immunoprecipitation. This and a significant amount more experimental investigation still needs to be conducted *in planta* to determine the interplay between epigenomic marks and m^6A deposition on plant transcripts. Furthermore, studies in multiple plant species should be undertaken to determine the interplay between specific histone modifications and m^6A, as such interactions might show some specificity between various plant species. Similar studies in the context of plant stress responses might yield important new insights in regards to these epigenomic and epitranscriptomic interactions. Nonetheless, taken together, this work describes the first likely direct interaction between known components of epigenomic and epitranscriptomic writing machinery in a plant system, and thereby highlights a potential role of the histone mark H3K36me2 in recruiting or providing a bridging mechanism for the m^6A writer complex to chemically modify mRNAs co-transcriptionally in plants (Figure 2). In general, significant future research focus should be dedicated to determining the exact mechanisms directing specific deposition of m^6A to the 3' end of plant transcripts.

There are additional intriguing connections between H3K36me2 and various RNA modifications. For instance, this histone mark has been associated with intron retention in rice [55], and previous studies in mammalian systems have demonstrated m^6A enrichment associated with alternatively spliced introns [56]. However, this link needs further exploration specifically in plant systems. In support of this potential cross talk in plants, a previous study focusing on mRNA modifications that alter Watson-Crick base pairing in the *Arabidopsis* transcriptome found this class of RNA covalent additions to be associated with alternatively spliced introns, suggesting their involvement in the regulation of splicing [57]. Overall, there is suggestive evidence for the co-transcriptional interplay between covalent base modification of cognate mRNAs in an H3K36me2-dependent manner that could potentially regulate various transcription and RNA-processing events (e.g., alternative splicing), thereby strongly effecting gene expression in plants. These connections need further inquiry in future research projects.

The effects of over-expressing mammalian mRNA demethylases in plants

The identification of genetic linkage between *FTO*, which encodes an RNA demethylase, and human obesity and diabetes sparked renewed excitement in research focused on covalent RNA modifications [58]. This demethylase has been demonstrated to remove the RNA modifications m^6A, m^6A_m, and m^1A from mRNAs in human cells [58–60], but this functionality likely does not have any connection to human metabolic phenotypes. Besides, FTO exhibits context-dependent activity that varies between tissues, subcellular localization, and based on RNA secondary structure and sequence (reviewed extensively in [61]). Recent studies on the role of FTO in RNA demethylation in mammalian systems are making it clear that this demethylase targets transcripts that are chromatin associated (caRNAs) and encoded by repetitive elements. These findings provide a mechanism for gene expression regulation mediated by RNA demethylation by FTO through increasing chromatin accessibility and

Figure 2



Histone modification H3K36me2 is correlated with RNA modification m^{6}A in *Arabidopsis*. Comparison of the distribution of histone modification within gene body regions with mRNA m^{6}A deposition revealed a correlation between histone mark H3K36me2 and m^{6}A in populations of transcripts. Biochemical analysis revealed an interaction between the m^{6}A writer complex protein FIP37 (AT3G54170) and the histone modifying enzyme SDG8/ASHH2 (AT1G77300) a well-known H3K36 modifying enzyme. Depicted here is a proposed model for the co-transcriptional deposition of m^{6}A through histone modification binding.

transcription [62]. In plants, there are currently no known FTO orthologs. Recently, Yu et al. [63]** artificially introduced an over-expression cassette encoding human FTO into rice and potato plants. Intriguingly, the ectopic expression of this RNA demethylase led to a nearly 50% increase in the grain (rice) and tuber (potato) yield and biomass of the two crop plants. Furthermore, improved physiological traits such as increased root growth, tiller formation, photosynthesis efficiency, and drought tolerance were observed in *FTO* rice plants. Additionally, demethylation of m^{6}A was observed in mRNA, repeat RNA, non-ribosomal RNA, and non-ribosomal nuclear RNA in the *FTO*-transgenic plants. The transgenic plants also exhibited a decline in the levels of transcription-repressing histone marks (such as H3K9me2 and H3K27me3), which facilitates an open chromatin state in these plant genomes. Based on these findings, the researchers speculated that m^{6}A demethylation of caRNAs (such as repeat RNAs) results in a more open chromatin state resulting in the observed increased transcript abundance for mRNAs that encode proteins involved with the processes linked to the improved plant traits [63,64]. This model is supported by the recent findings of similar effects of this demethylase in mammalian cells [61,64]. However, more work is needed to directly prove that the FTO demethylase can target similar collections of caRNAs in plant cells. Additionally, identifying whether there is a plant demethylase that functions similarly encoded by plant genomes is also an area for future research inquiry. Overall, this study highlights the potential of epigenomic and epitranscriptomic crosstalk, which might be a key feature to leverage in the pursuit in achieving a positive impact on crop improvement in the areas of production and yield and potentially improved stress response, as was exhibited by the FTO-expressing rice plants. Additionally, identifying a plant demethylase with similar function and effects could aid in such

studies by providing a plant protein to be used for potentially improving various crop plants as compared to using proteins from mammalian systems.

Towards understanding covalent modifications in regulatory non-coding RNAs involved in plant epigenetic processes

Regulatory non-coding RNAs such as miRNAs, lncRNAs, and caRNAs play important roles in several epigenetic regulatory mechanisms underlying gene expression modulation in plant transcriptomes [23,29,30,34]. Although widespread reciprocal associations have been observed among regulatory RNAs and epigenetic modifications at histone, DNA, and RNA levels for regulating complex gene expression mechanisms of several diseases in mammals [65,66], in plants, such correlations are still in need of direct inquiry. For instance, the human lncRNA *XIST* has been found to be strongly m^6A methylated and in the absence of the m^6A writer, *METTL3*, the functionality of the lncRNA, which is to silence specific target genes, was compromised [67]. A similar mechanism was demonstrated for the first time in plants for the lncRNA *COOLAIR* [68] and the m^6A modification was similarly found to be associated with its mechanism of action of triggering chromatin silencing [69]**. Specifically, *COOLAIR* is known to function in forming a R-loop (a DNA-RNA hybrid that includes the displaced single-stranded DNA) at the *Arabidopsis* floral repressor locus *FLC*. *COOLAIR* also is known to directly interact with the RNA binding protein FLOWERING CONTROL LOCUS A (FCA) and the 3′ RNA processing factor FY (WDR33) to promote its own proximal polyadenylation, which allows the subsequent recruitment of other proteins such as histone demethylases (FLD) that ultimately come together to repress *FLC* expression. This FCA-mediated *FLC* repression was found to be promoted specifically by m^6A modified *COOLAIR*. In the absence of m^6A methyltransferase (MTA) activity, FCA was functionally suppressed and *FLC* expression was increased. Additionally, the authors suggested that m^6A deposition on *COOLAIR* likely altered its secondary structure to promote the FCA-*COOLAIR* interaction. They also found that m^6A enhances FCA nuclear condensate formation *in vivo*, which in turn provides increased local concentrations of FY (WDR33) to cease transcription and allow resolution of the R-loop. Finally, FY physically interacts with the H3K4me1 demethylase FLD to induce chromatin silencing at *FLC* [69]**. Thus, in this context, the modification status of lncRNAs has important functional consequences for their recruitment of chromatin modifying enzymes and the ultimate regulation of their target loci.

With the ongoing progress in plant lncRNA research [30,70], it will be worthwhile to explore the m^6A-related lncRNA signature and examine its involvement in regulatory processes. Interestingly, in their study based on the high-throughput annotation of modified ribonucleotides (HAMR) pipeline in *Arabidopsis*, Vandivier et al. [57] found RNA modifications other than m^6A, which impact the Watson-Crick base pairing of the modified nucleotides, to be associated with distinct classes of non-coding RNAs such as lncRNAs, miRNAs, and snoRNAs in addition to mRNAs. Studying such modifications will likely further widen the scope of our understanding of lncRNA-mediated epigenetic regulation in plants and how they are involved in regulating plant growth and stress responses.

Finally, the involvement of the RNA modification machinery in regulation of miRNA biogenesis has also been recently reported. Specifically, Bhat et al. [71]** found that in the absence of m^6A deposition, the biogenesis of at least one-fourth of *Arabidopsis* miRNAs was affected. Mechanistically, the authors observed that an *Arabidopsis* m^6A writer complex component, MTA, deposits m^6A marks on small subset of primary miRNA transcripts (pri-miRNAs), which consequently induces the formation of the most favorable secondary structure for subsequent miRNA processing. This further facilitates direct interaction of MTA and TOUGH to recruit the Microprocessor complex to pri-miRNAs and in turn enable their processing [71]**. Importantly, this study utilizes m^6A-RNA immunoprecipitation followed by sequencing (m^6A-IP-seq) for the detection of m^6A marks on pri-miRNAs, a technique that has limitations associated with m^6A stoichiometry, specificity, and sensitivity. Therefore, future studies based on antibody-independent methods [72] should also be conducted for subsequent analysis in this direction, especially with the known low levels of pri-miRNA abundance within the plant transcriptome and the high levels of RNA needed for m^6A-IP-seq. Additionally, it will be interesting to further explore if the miRNA-m^6A interaction is reciprocal, that is, whether and how miRNAs regulate the expression levels of important components of the m^6A machinery such as MTA in plants. Overall, considering the new findings on methylation status of various classes of ncRNAs in plants, we have only begun accumulating evidence towards unraveling the interplay between the epitranscriptome and epigenome, and this crosstalk should be an area of intense research focus in the future.

Concluding remarks

The exploration of the crosstalk between the epitranscriptome and epigenome has resulted in increasingly complicated and crowded models involving the deposition, removal, and subsequent function of DNA/histone and RNA modifications in mammals, particularly in the context of complicated diseases such as cancer [66]. Here, we have highlighted current research in plant systems, which attempts to understand specific mechanisms where the epigenome and epitranscriptome

plausibly interact and influence one another. While insightful, these limited studies have hardly scratched the surface of this new and exciting field of inquiry. Research in this area needs to be more comprehensively and carefully explored in plant research systems, including in the context of their stress response systems. We have highlighted areas of future research focus that should be addressed in this area and that will improve our understanding in this important area of inquiry. Additionally, future *in planta* investigation of the epigenomic and epitranscriptomic regulators not merely in isolation but also in combination would be critical in enhancing our current understanding of plant gene expression regulation and the resulting plant phenotypes, which could potentially be leveraged as a potent strategy for crop improvement. In conclusion, increasing our understanding of the interplay between the epigenome and epitranscriptome will improve our understanding of how these interactions affect plant gene expression regulation and how it can be targeted for future crop improvement applications in the context of yield, biomass, and improved stress response.

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Declaration of competing interest

No conflicts of interest declared.

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