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The Diversity and Functions of Plant RNA Modifications: What We Know and Where We Go from Here

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Abstract

Since the discovery of the first ribonucleic acid (RNA) modifications in transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), scientists have been on a quest to decipher the identities and functions of RNA modifications in biological systems. The last decade has seen monumental growth in the number of studies that have characterized and assessed the functionalities of RNA modifications in the field of plant biology. Owing to these studies, we now categorize RNA modifications based on their chemical nature and the RNA on which they are found, as well as the array of proteins that are involved in the processes that add, read, and remove them from an RNA molecule. Beyond their identity, another key piece of the puzzle is the functional significance of the various types of RNA modifications. Here, we shed light on recent studies that help establish our current understanding of the diversity of RNA modifications found in plant transcriptomes and the functions they play at both the molecular (e.g., RNA stability, translation, and transport) and organismal (e.g., stress response and development) levels. Finally, we consider the key research questions related to plant gene expression and biology in general and highlight developments in various technologies that are driving our insights forward in this research area.

Contents

1. INTRODUCTION

Ribonucleic acid (RNA) molecules are some of the most fundamental molecular components in all living organisms. They are transcribed from genomic deoxyribonucleic acid (DNA) by RNA polymerase enzymes and are translated into proteins, providing a shorter-lived and highly regulatable intermediate in organismal gene expression regulation. During and/or after transcription, the basic building blocks of RNA—the four nucleotides adenine (A), uracil (U), cytosine (C), and guanine (G)—can be chemically modified by various enzymes, expanding the possible chemical composition of RNA molecules beyond what is encoded in the genome. Modifications can include methylation of the bases [e.g., N^6-methyladenosine (m^6A), N^1-methyladenosine (m^1A), and 5-methylcytosine (m^5C)], methylation of the ribose sugar (e.g., N_m and m^6A_m), or more complex reactions such as the oxidation of methylated bases [e.g., 5-hydroxymethylcytosine (hm^5C)]. The collection of RNA modifications, which brings functional, regulatory, and structural complexity to gene expression regulation, is commonly known as the epitranscriptome.

Although it is hard to pinpoint when RNA modification was first discovered, noncanonical nucleosides such as pseudouridine (Ψ) were first recognized in 1957, even before the various classes of RNA molecules [e.g., transfer RNAs (tRNAs)] had all been uncovered (22). With emerging technologies, we have vastly expanded our knowledge of the diversity and abundance of RNA modifications in various organisms, including plants, in the last few decades, which has been termed by some as the golden period for this field of study (39). Thousands of modified nucleotides in various RNA types such as messenger RNAs (mRNAs), tRNAs, ribosomal RNAs (rRNAs), microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) have been found in various cellular compartments including the nucleus, cytoplasm, chloroplast, and mitochondria (79). In addition to revealing the identities of the modifications, we have also begun uncovering the diverse functional roles of RNA modifications in plants. Progressing alongside mammalian counterparts, advances in the field of plant mRNA modifications continue, particularly with regard to m^6A, the most well-studied mRNA modification to date. With rapidly expanding knowledge of the core protein components that comprise the machinery responsible for writing, reading, and erasing RNA modifications, many key functional roles that the modifications play at both the molecular and physiological levels have been revealed. As such, despite being a relatively new field, epitranscriptomic regulation of RNA is emerging as one of the most important systems affecting key biological processes, including early development and reproductive processes as well as stress responses via unique molecular pathways in plant biology.

This review attempts to provide a high-level overview of the vast diversity of RNA modifications, the modification machinery, and the implicated functions of said modifications in plant species. Given the amount of attention garnered by m^6A research, many comprehensive review articles have been published that discuss in depth its profiling, machinery, and function in plants (4, 20, 46, 65). Other review articles have focused on earlier developments in the field of eukaryotic and plant RNA modifications that have been found in rRNAs, tRNAs, and mRNAs (130). Therefore, we focus here on the overall diversity of RNA modifications and more recent developments in the plant research community, while highlighting some of the key earlier studies monumental to this field of inquiry. Looking to the future, we also discuss the strengths and limitations of current studies focused on RNA modifications that are involved in plant gene expression regulation and future technological developments that have the potential to greatly enhance this field of study in the future.

2. DIVERSITY OF RNA MODIFICATIONS

The various types of RNA modifications include (*a*) simple covalent modification of the nucleotide bases (e.g., m^5C, m^6A, m^1A), (*b*) noncanonical base rearrangements (e.g., pseudouridylation), (*c*) 2′-*O*-methylation of the ribose (N_m), and (*d*) other more complex modifications (e.g., hm^5C) (123). To keep track of this ever-growing list of RNA modifications, a few research groups have attempted to compile and record them into databases (7, 12, 123). For instance, the RNA Modification Database (RNAMDB) was the most comprehensive list that was consistently updated from 1994–2011 (12). According to the last update of RNAMDB, there were approximately 128 RNA modifications identified in eukaryotic transcriptomes, of which ~40% were found in tRNAs, ~31% in rRNAs, and ~10% in protein-coding mRNAs. As recently as 2021, 334 (323 if you discount the unknown modifications that are listed) different nucleotide modifications have been characterized and listed in MODOMICS, a database of RNA modification pathways (7). A vast majority of these RNA modifications have been identified in noncoding RNAs (ncRNAs; e.g., tRNAs), but many key modifications have also been found to occur in mRNA molecules, having various effects on gene expression, and these are the focus of our discussion below.

3. THE DIVERSITY OF RNA MODIFICATIONS IN VARIOUS TYPES OF RNA

3.1. Transfer RNAs

tRNAs are small ncRNAs that are usually between 70–100 nucleotides (nt) in length and one of the most abundant classes of RNA molecules in cells. They are an indispensable part of the protein translation machinery responsible for correctly pairing with the 3-nt codon of the mRNA and bringing the correct amino acids to the ribosome for polypeptide synthesis. tRNAs have also been found to be the most highly modified RNA (20, 30), with between 10–20% of their nucleotides modified (83). Given the universally conserved role of tRNAs in translation, most studies focusing on eukaryotic tRNA structure and function have been done in yeast, and these foundational discoveries are assumed to be conserved across kingdoms. Therefore, high conservation of important tRNA modification sites has also been assumed throughout eukaryotic species as well as within species across all biological conditions (14). Some examples of highly conserved key modification sites are dihydrouridine (D) found commonly at positions 16 and 20, the Ψ found in the TΨC-loop (at positions 54 and 55) within the T-arm of tRNA molecules, and various modifications found in a number of tRNA anticodon regions (at positions 32, 34, and 37) (20). In the model plant *Arabidopsis thaliana* (hereafter *Arabidopsis*), a number of highly conserved Ψs have been validated in positions 13 (D stem), 27 (anticodon loop), 38 (anticodon loop), 39 (anticodon stem), and 55 (TΨC-loop) as well as N^1-methylguanonine (m^1G) in position 37 (52, 99) (**Figure 1**).

The first key study to specifically investigate plant tRNA modifications focused on *Arabidopsis* and a woody plant, hybrid aspen (*Populus tremula* × *tremuloides*). This study identified 21 modifications known to occur in eukaryotic tRNA molecules such as Ψ, D, methyladenosines (m^1A and m^6A), and m^5C, as well as 4 novel uncharacterized modified nucleosides using high-performance liquid chromatography (HPLC) (14). This study revealed a high level of conservation between plant tRNA modifications when compared to those in yeast and near-perfect conservation between the two studied plant species. In 2017, another group used liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) to systematically identify tRNA modifications in rice and *Arabidopsis* that are associated with stress response and development (114). In this study, they were able to detect 25 nucleoside modifications in tRNAs, 15 of which were methylation events. Certain tRNA modification sites were also shown to demonstrate specificity during precise developmental periods as well as different stress responses. This stress-specific variation in tRNA modification sites was corroborated by a very recent (2022) study that used a data-independent acquisition (DIA) mass spectrometry (MS) method to detect various RNA modifications in the highly modified wobble position of *Arabidopsis* tRNAs (50). They revealed that a number of these modification events, specifically 5-aminomethyluridine (nm^5U), 5-carbamoylmethyluridine (ncm^5U), 5-carbamoylhydroxymethyluridine ($nchm^5U$), and 5-methoxycarbonylmethyluridine (mcm^5U), increased in abundance upon salt stress (50). Additionally, levels of the mRNA transcripts predicted to encode writers of these modifications, *TRNA METHYLTRANFERASE 11* (*TRM11*) and *TRM9*, as well as *EPIDERMAL GROWTH FACTOR RECEPTOR-LIKE PROTEIN 1* (*AtELP1*) were also found to increase in abundance upon salt stress treatment, suggesting a direct mechanism involving these enzymes in regulating the specific tRNA modification events in the context of plant salt stress response (50). As we discuss in subsequent sections, proteins that are involved in adding these chemical modifications to specific RNA bases, in tRNAs and other classes of RNAs, are also being identified and studied in-depth in plant systems, providing important insights into these regulators of gene expression.

Overall, it seems that the core tRNA modification events are often conserved not just among plant species but throughout all eukaryotes. We are also beginning to reveal that modifications in tRNAs and other classes of highly conserved RNAs are not static and could play important roles in various stress responses, specific developmental transitions, and other important physiological events that affect plants (50, 114). With the advancement of technology, plant biologists can ask interesting research questions that go beyond the detection and validation of conserved tRNA modification sites and attempt to discover and quantify modifications in multiple biological contexts.

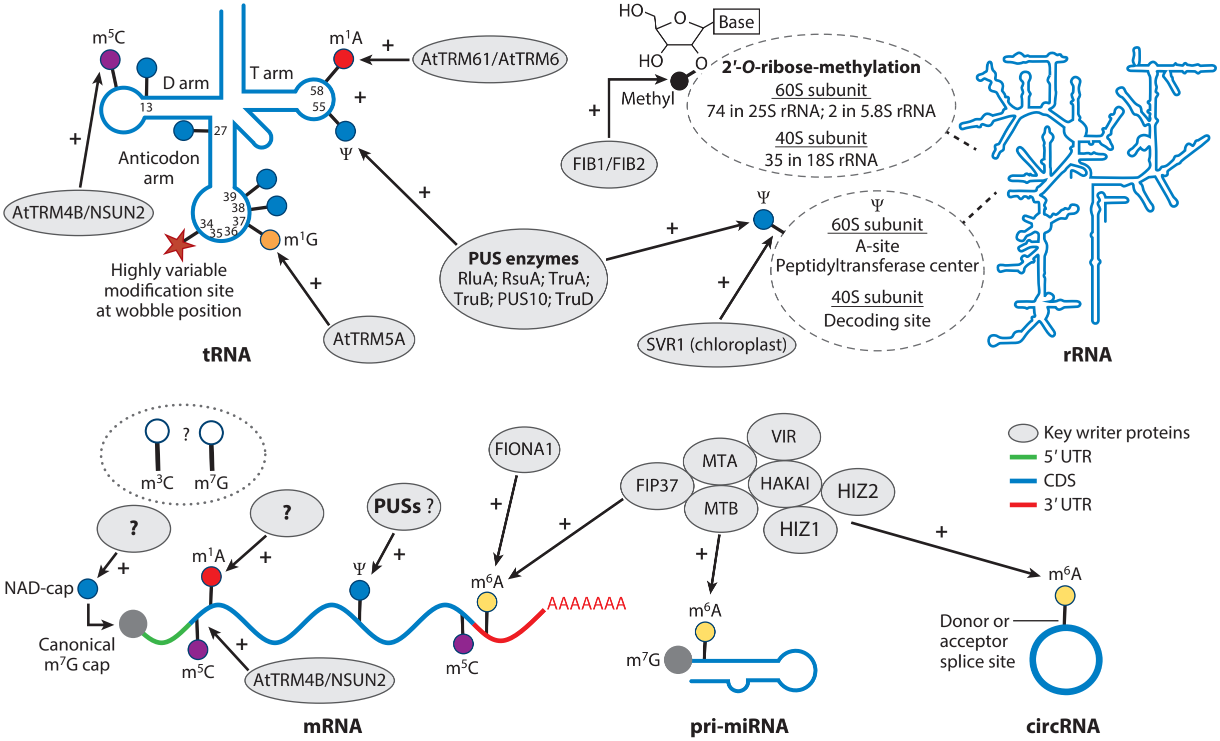


Figure 1

RNA modifications in various RNA types uncovered in plant studies. Various classes of RNA are depicted with known RNA modifications found on them. The gray ellipses represent the key writer proteins that have been identified in plants for the specific modifications as noted. For mRNA modifications, the transcript region [5′ UTR (*green*), CDS (*blue*), and 3′ UTR (*red*)] where each specific modification tends to be localized is noted. For tRNAs, there are hundreds of known and predicted modifications throughout their sequences in various eukaryotes. Thus, the modifications displayed are some key modification sites that have been validated in plant tRNAs. For rRNA, the two major categories of modifications and the regions within rRNA subunits where they are concentrated are shown inside the dotted circles. The m^3C and m^7G modifications found in the dashed oval above the mRNA molecule are two modifications that have preliminary support to be found internally on these molecules, while the m^7G cap moiety (*gray circle* on the 5′ end of mRNA) is known to be present on most eukaryotic mRNAs as noted in the figure. Abbreviations: CDS, coding sequence; circRNA, circular RNA; mRNA, messenger RNA; m^1A, N^1-methyladenosine; m^1G, N^1-methylguanonine; m^5C, 5-methylcytosine; m^6A, N^6-methyladenosine; m^7G, N^7-methylguanosine; NAD-cap, nicotinamide adenine dinucleotide cap; pri-miRNA, primary microRNA; PUS, pseudouridine synthase; rRNA, ribosomal RNA; tRNA, transfer RNA; UTR, untranslated region.

3.2. Ribosomal RNA

The most conserved RNAs across eukaryotic species, rRNAs are also highly modified, harboring various chemical moieties at specific base pair positions, important for the proper functionality of these RNA molecules (28). In fact, the precise addition of various RNA modifications at specific nucleotide positions in rRNA molecules is critical for the proper processing of these RNAs and subsequently their functionality in protein translation. In rRNAs, there are two major types of RNA modifications that have been identified, N_m and pseudouridylation, whose primary function is to maintain their secondary structure, and thereby stabilize their form to allow proper functionality of the rRNA molecules (119) (**Figure 1**). These modifications cluster near important sites, including those where the ribosome interacts with the mRNA that is being translated and the pocket that accepts the amino acid–charged tRNA molecules. For instance, a genome-wide Ψ profiling was performed on a plant model organism in 2019 for the first time using Ψ sequencing (Ψ-seq) in *Arabidopsis*. This study identified Ψ sites in the highly conserved and predicted regions of Ψ1000, Ψ1104, and Ψ1118 located in the decoding site of the 40S ribosome subunit as well as Ψ2844, Ψ2855, Ψ2870, Ψ2884, Ψ2945, and Ψ2965 in the peptidyl transferase center and A-site of the 60S ribosome subunit. Similarly, a study in 2021 profiled ribose methylation (N_m) in *Arabidopsis* for the first time and detected 35 methylation sites in 18S, 74 in 25S, 2 in 5.8S, and none in 5S rRNA (119). Of these N_m events, 12 were novel sites of this modification in eukaryotes, while a vast majority had previously been predicted or found in various other organisms. In the chloroplast and mitochondria, 5 identical modification sites were found. Specifically, Cm1351 and Cm1358 were identified in the small subunit (SSU) rRNA, while Cm1935, Gm2269, and Gm2571 were found in the large subunit (119). Given the high conservation of these modification sites, they are most likely to be catalyzed by small nucleolar ribonucleoproteins (snoRNPs), as has been found in other organisms.

In a recent 2022 study, a newly developed rRNA isolation method allowed researchers to obtain pure rRNA molecules, aiding in the identification of additional rRNA modification sites using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) (101). With this improved method, the group was able to detect for the first time in *Arabidopsis* 25S rRNA molecules 4 new methylation events—N^2,N^2-dimethylguanosine ($m^{2,2}G$), N^6,N^6-dimethyladenosine ($m^{6,6}A$), 3-methyluridine (m^3U), and N^7-methylguanosine (m^7G)—as well as two of these new methylation-based modification types ($m^{2,2}G$ and $m^{6,6}A$) in 18S rRNAs. Furthermore, these new modifications were also found in two monocot species, rice and perennial ryegrass, suggesting that they are prevalent across plant species. An additional study using m^7G mutational profiling sequencing allowed the detection of the previously uncharacterized but evolutionarily conserved eukaryotic m^7G modification site at position 1581 of the *Arabidopsis* SSU rRNA for the first time (29). While the roles of key conserved rRNA modification sites are likely to remain the same across most species, the roles of the novel plant-specific modification sites in relation to regulation of protein expression are likely of high interest and should be further researched.

It has long been assumed that because of the high conservation and low turnover of rRNA in a cell, modifications in rRNA are mostly added in the initial rRNA processing and maturation steps and are likely not a dynamic moiety within the host rRNA molecule. However, this dogma is being challenged by recent studies that incorporate more varied experimental contexts (e.g., abiotic stress), similar to those described above in regard to tRNA molecules. For instance, one recent cancer study demonstrated that rRNA in diffuse large B cell lymphoma cells was hypomethylated compared to rRNA in normal cells (56). Similarly, in plants, a chloroplast rRNA methyltransferase, RsmD, was found to play a role in cold stress tolerance potentially by altering translation levels (78). Overall, the identification of dynamic rRNA modification sites, including those that respond to specific stress responses, opens up a new area of inquiry into the functionality of these dynamic modification events and their ultimate effects on plant biology through effecting proper gene expression regulation. Future studies will need to be done to provide answers to these important research questions.

3.3. Messenger RNA

Historically, profiling of mRNA modifications using MS–based approaches has been difficult due to the low stoichiometry of mRNA in the total RNA population, often resulting in the contamination of potential mRNA samples with more highly abundant ncRNAs (e.g., rRNA). With the continued improvement of mRNA selection techniques and new, more sensitive and specific modification-profiling techniques, the field has focused much attention on studying RNA modifications that occur on mRNA molecules and their important biological effects. Below, we briefly discuss a number of RNA modifications that have been found to occur on eukaryotic mRNAs, focusing on the studies of these covalent mRNA additions in plant systems.

3.3.1. N^6-Methyladenosine.

m^6A was the first identified modification to occur on mRNA molecules and is also the most abundant internal modification on these transcripts (23). This modification has been predominantly found around stop codons and in the 3′ untranslated region (UTR) of mRNA transcripts (2, 24) (**Figure 1**). Due to its abundance and the availability of methods for profiling m^6A (more about this below), this chemical mark has taken center stage in the field of epitranscriptomics, with hundreds of studies in the last few years in plant systems including *Arabidopsis*, rice, tomato, wheat, and poplar, to name a few (59, 66, 136, 139, 141) (**Table 1**). In mammals, the deposition of m^6A by a complex of writer proteins seems to primarily happen in a loosely defined RRACH sequence context, but plant studies have also identified alternative m^6A motifs in these systems, including UGUA (117). Across *Arabidopsis* accessions, m^6A sites have been demonstrated to be highly conserved (132). m^6A, as briefly highlighted in later sections, has been found to affect mRNA stability and translation of transcripts among other molecular effects, providing mechanistic insights into its potency as an RNA regulator. Given the amount of attention garnered by m^6A, there have been excellent review articles that discuss in depth its profiling, machinery, and function in plants and that may be of additional interest to readers (see, for example, 4, 20, 46, 65).

3.3.2. N^1-Methyladenosine.

Similar to m^6A, m^1A modifications host an additional methyl group on a different nitrogen within the nucleotide base. A transcriptome-wide study in the flowering plant petunia demonstrated that m^1A seems be another highly abundant mRNA modification in plants (126). More specifically, transcripts originating from a total of 3,231 genes were found to display m^1A. This methylation-based modification was highly localized in the coding region, with a single highly enriched peak close to the start codon (**Figure 1**). Sequence motifs that contained an A surrounded by sequences with high GC content seemed to be key in the selection of potential m^1A methylation sites, like what was shown in a previous mammalian study (25, 126). Additionally, numerous m^1A sites showed dynamic gain and loss of the methylation event in response to treatment of petunia plants with the hormone ethylene, suggesting that this mark could be involved in regulating response to this hormone. However, more work is necessary to test that hypothesis. Finally, this study suggested that methyltransferase PhTRMT61A is likely responsible for depositing this epitranscriptome mark onto petunia mRNAs. The one major caveat to this and other current m^1A studies is that the transcriptome-wide mapping was done using a monoclonal antibody thought to be specific for this modification. However, subsequent experiments have suggested that it might also recognize m^7G cap modifications, which would explain the localization close to start codons (40). This caveat needs to be cleared up in the near future so the field can determine if current m^1A data are trustworthy or need to be replaced with data with more specificity.

3.3.3. 5-Methylcytosine.

m^5C is a modification of cytosine residues in various RNA molecules. In 2017, m^5C was profiled by two groups using bisulfite sequencing or immunoprecipitation in plants (18, 21). These studies found that transcripts from up to 4,396 protein-coding genes contained m^5C peaks and that this modification was primarily found in the coding region of these modified transcripts (**Figure 1**). Furthermore, this methylation event was found to localize most often in modified targets two nucleotides after the start codon and/or right before the stop codon (18), a pattern unique to plant mRNAs. These studies also demonstrated that m^5C tended to be

Table 1 A glimpse of recent studies (2021–2022) across diverse plant species, focusing on the transcriptome-wide analysis of the m^{6}A profile (or the m^{6}A methylome)

Year	Plant species	Sequencing approach	Conditions			Highlights/key findings	Reference(s)
			Tissues/development stage	Stress response			
				Biotic	Abiotic		
2021	Maize (*Zea mays*)	m^{6}A-seq	Aerial tissues from 14-day-old seedlings of a B73×Mo17 F_1 hybrid and its parental inbred lines, B73 and Mo17	NA	NA	Global m^{6}A profile between the hybrid and parents remained unchanged in terms of abundance and distribution. m^{6}A-modified genes in the hybrid were observed with nonadditive expression trends (potential role in heterosis).	67
2021	Watermelon (*Citrullus mucosospermus*)	m^{6}A-seq	Leaves of resistant 'R1288' genotype (48 hours postinoculation)	Cucumber green mottle mosaic virus	NA	m^{6}A hypomethylation was observed in response to the viral infection. The potential involvement of a demethylase, *ClALKBH4B*, was suggested.	45
2021	Diploid woodland strawberry (*Fragaria vesca*)	m^{6}A-seq	Fruits at three ripening stages	NA	NA	m^{6}A methylome is involved in the regulation of nonclimacteric fruit ripening. m^{6}A enrichment in the CDS of transcripts is involved in ABA biosynthesis and signaling pathway.	140
2021	Wheat (*Triticum aestivum*)	m^{6}A-seq	Leaves of resistant yannong999 and susceptible yannong24 varieties	Wheat yellow mosaic virus infection	NA	Global m^{6}A profiling revealed the main enrichment in 3′-UTR and stop codons across varieties. Transcripts that are differentially m^{6}A-methylated across varieties are potentially involved in plant–pathogen interaction.	136

(*Continued*)

Table 1 (*Continued*)

Year	Plant species	Sequencing approach	Conditions			Highlights/key findings	Reference(s)
			Tissues/development stage	Stress response			
				Biotic	Abiotic		
2021	Tomato (*Solanum lycopersicum*) cv. 'Micro-Tom'	m^6A-seq	Anthers at the tetrad stage	NA	Moderately low-temperature 10°C/10°C (day/night)	m^6A levels declined in mRNAs in anthers upon moderate low-temperature stress. There is a potential role of m^6A in tomato anther development under low-temperature conditions.	124
2021	Poplar (*Populus trichocarpa*)	Nanopore direct RNA sequencing; Validation by m^6A-seq and m^6A-REF-seq	Stem-differentiating xylem	NA	NA	m^6A profile observed at single-base resolution for all detectable transcripts, revealing m^6A enrichment in transcripts encoding proteins involved in wood formation.	34
2021	Sweet sorghum [*Sorghum bicolor* (L.) Moench]	m^6A-seq	Roots of salinity-tolerant M-81E and sensitive Roma genotypes	NA	150 mM NaCl for 24 h (salt stress)	Compared to the controls, the m^6A profile changed drastically for the sensitive genotype.	138
2022	Rice (*Oryza sativa*)	m^6A-seq	Shoots and roots of seedlings of salinity-tolerant genotype (FL478)	NA	120 mM NaCl for 48 h (salt stress)	m^6A enrichment was observed in the shoots in a tissue-specific manner under salt stress.	111
2022	Pear (*Pyrus bretschneideri*)	m^6A-seq	Leaves (12 h postinoculation)	Fire blight (*Erwinia amylovora*)	NA	Pathogen inoculation induced changes in the transcriptome-wide m^6A profile and increased the abundance of transcripts with m^6A.	44

(*Continued*)

Table 1 ***(Continued)***

Year	Plant species	Sequencing approach	Conditions			Highlights/key findings	Reference(s)
			Tissues/development stage	Stress response			
				Biotic	Abiotic		
2022	Apple [*Malus prunifolia* (Willd.) Borkh.]	m^6A-seq	Mature leaves of 1-year-old tree seedlings (1.5-m tall)	NA	Drought treatment at relative soil water content ~40%	Global m^6A profiling of drought-tolerant apple species revealed m^6A enrichment in both CDS and 3′ UTR under both control and drought conditions.	70
2022	Apple (*Malus domestica* cv. Roya Gala)	m^6A-seq	Leaves (15 days after inoculation)	Powdery mildew (*Podosphaera leucotricha*)	NA	m^6A methylomes of WT- and *MhYTP2*- (an m^6A reader cloned from PM-resistant apple genotype) overexpressing plants were analyzed. Corresponding m^6A marks promote *MdMLO19* and *MdMLO19-X1* mRNA degradation, potentially enhancing resistance against PM.	43
2022	Barley (*Hordeum vulgare* L. cv. Golden Promise)	m^6A-seq	Roots	NA	5 μM $CdCl_2$ for 7 days (cadmium stress)	Global m^6A profiling revealed hypermethylation upon Cd stress and m^6A enrichment primarily observed near stop codon and 3′-UTR.	98
2022	Tomato (*Solanum lycopersicum*)	m^6A-seq	Fruits at 12, 20, and 28 DAF	NA	NA	Global m^6A deposition increased (mainly in the 3′ UTR) during fruit expansion of tomato.	48

(*Continued*)

Table 1 (*Continued*)

Year	Plant species	Sequencing approach	Conditions			Highlights/key findings	Reference(s)
			Tissues/development stage	Stress response			
				Biotic	Abiotic		
2022	*Arabidopsis thaliana*, *Gossypium arboreum*, *Gossypium hirsutum*, *Glycine max*, *Phaseolus vulgaris*, *S. lycopersicum* *S. bicolor*, *Z. mays*, *Aegilops tauschii*, *Triticum dicoccoides*, *T. aestivum*, *O. sativa*, and *Physcomitrella patens*	m^6A-seq	Aerial parts of seedlings	NA	NA	Evolutionary conservation and divergence of m^6A profiles were studied across 6 monocots, 6 dicots, and a bryophyte. The m^6A methylomes were compared across 13 representative plant species spanning over half a billion years of evolution. New patterns and preferences of m^6A methylomes were observed with respect to ancient orthologous genes, polyploidization, etc.	75
2022	*A. thaliana*	Nanopore direct RNA sequencing	6-day-old WT and mutant (*fio1–2*) seedlings	NA	NA	Global m^6A profile of *fio1-2* (mutant for FIONA1, ortholog of human METTL16) revealed hypomethylated sites in CDS. FIONA1-mediated m^6A methylation is associated with *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) involved in regulation of flowering.	122

Abbreviations: ABA, abscisic acid; Cd, cadmium; CDS, coding sequence; DAF, days after flowering; MhYTP2, *Malus* YTH domain-containing RNA binding protein 2; m^6A, N^6-methyladenosine; m^6A-seq, m^6A-immunoprecipitation-sequencing; m^6A-REF-seq, m^6A-sensitive RNA-endoribonuclease–facilitated sequencing; NA, not applicable; PM, powdery mildew; UTR, untranslated region; WT, wild type.

associated with low translation output from mRNAs that contain this mark. A number of physiological functions, including affecting mRNA movement across graft junctions, have recently been uncovered and are discussed in greater detail below (see Section 5).

3.3.4. 3-Methylcytosine. Another cytosine modification found in plant mRNAs, 3-methylcytosine (m^3C), was initially identified in transcripts through the use of the computational prediction tool high-throughput annotation of modified ribonucleotides (HAMR). These findings were later validated on a handful of candidate transcripts using RNA immunoprecipitation (RIP)–quantitative polymerase chain reaction (qPCR) using a m^3C-specific antibody (105). However, a systematic profiling of this mark has not yet been done, and the functionality of this modification in mRNA has not been studied to date. More thorough studies of mRNA m^3C should be undertaken.

3.3.5. Pseudouridine. A common modification in rRNA and tRNA, Ψ was profiled in plants for the first time in 2019 (99). This study identified 322 protein-coding transcripts that contain Ψ (~1.21% of detectable mRNA). The distribution of this modification revealed that they are primarily localized within the coding sequence (CDS) region of mRNAs, with a few also scattered in the 5′ UTR (**Figure 1**). A study in mammals and yeast failed to identify a motif enriched around Ψ sites but concluded that the secondary structure context of the modified nucleotide likely plays a critical role in determining the locations of Ψ sites (13).

3.3.6. 7-Methylguanosine. m^7G is the canonical modified nucleotide that serves as the cap structure on the 5′ end of eukaryotic mRNAs (31) (**Figure 1**). Shortly after the discovery of this modified nucleotide serving as the cap structure in viral mRNAs, researchers also discovered that it was present in eukaryotic mRNAs (32, 33). Its role as the mRNA 5′ cap is crucial in the stability and translation of these molecules. In addition to the cap position, m^7G is also found internally in mRNA. Only recently, scientists have profiled m^7G in mammalian mRNAs using antibody-based pull-down approaches and found thousands of transcripts that contain internal m^7G sites (69, 134). Interestingly, these two studies provide conflicting results about the localization of internal m^7G sites, with one showing enrichment of this mark in the 3′ UTR in contrast to the 5′ UTR in the other study. In plants, mutational profiling followed by m^7G–mutational profiling sequencing (m^7G-MaP-seq) has been performed for tRNAs and rRNAs but not for mRNAs (29). This same study also looked for m^7G in *Escherichia coli* and yeast mRNAs but did not find any sites in this class of RNAs. These conflicting results challenge the validity of internal m^7G site discovery in eukaryotic mRNAs. Studies focused on plant mRNAs have not yet been conducted and could help resolve some of these open questions.

3.3.7. 5′ Nicotinamide adenine diphosphate caps. The cellular metabolite 5′ nicotinamide adenine diphosphate (NAD^+) has also been found to be incorporated as a noncanonical 5′ end cap modification on specific collections of eukaryotic mRNAs, including plant protein-coding transcripts (**Figure 1**). First discovered to be added to the 5′ end of *E. coli* mRNAs, 5′ NAD^+ caps have also been found on mRNAs from all profiled eukaryotes to date (15, 51, 106). In *Arabidopsis*, high-throughput 5′ NAD^+ cap profiling was performed in 2019 using the NAD-capture sequencing methodology revealing 5,687 and 6,582 NAD^+-capped RNAs in the transcriptomes of seedlings and inflorescences, respectively (113). Another study that employed a less sensitive nanopore sequencing–based method identified only 188 mRNAs that contained a 5′ NAD^+ cap structure (133). To date, no protein involved in adding NAD^+ caps to mRNA 5′ ends (i.e., writer protein) has been identified. Thus, whether NAD^+ caps are incorporated at mRNA 5′ ends by RNA polymerase during transcription initiation or added posttranscriptionally needs further research and is one of the most important and pressing questions to answer in this field of inquiry. Interestingly, a more recent study in *Arabidopsis* has revealed that NAD$^+$ caps induce degradation of the transcripts onto which they are added. Thus, the addition or removal of this mark to specific transcripts in response to abscisic acid (ABA) treatment provided a mechanism for regulating the transcripts encoding important ABA response proteins (131). Overall, 5′ NAD$^+$ caps are a relatively abundant modification and negatively affect the stability of the transcripts onto which they are added in plant transcriptomes, specifically in response to stress stimuli. In total, additional follow-up studies are needed to provide more insight into the addition, removal, and recognition as well as the overall importance of this modification in plant transcriptomes.

3.4. Small RNA (smRNAs) and Other Noncoding RNAs

As technological advances have made it easier to purify the various types of RNAs and profile their chemical modifications in eukaryotes, the modification status of other classes of ncRNAs, not just rRNAs and tRNAs, including small interfering RNAs (siRNAs), miRNAs, circular RNAs (circRNAs), and small nuclear RNAs (snRNAs), are of interest. Previous studies have found that miRNAs and siRNAs are methylated on their terminal 3′ ends with a N_m moiety that is added by methyltransferase protein HEN1 (128). Additionally, a 2021 study revealed that loss of the major m^{6}A writer protein MTA results in an overall decrease in the levels of a specific subset of mature miRNAs, with a resultant accumulation of their primary miRNA (pri-miRNA) transcript, suggesting a direct positive role for m^{6}A in miRNA processing (6) (**Figure 2*c***). This study also probed the secondary structure of 11 pri-miRNA transcripts whose methylation levels were reduced in *mta* mutant plants compared to wild-type plants and identified a concomitant change in miRNA stem-loop structure in the absence of m^{6}A. Furthermore, the binding of pri-miRNAs to the miRNA processing protein HYL1 was significantly reduced in *mta* mutant plants. Overall, this study revealed a molecular mechanism for the role of m^{6}A in proper miRNA biogenesis, which is an important class of small regulatory RNAs (6) (**Figure 2*c***).

The unusual model system moso bamboo (*Phyllostachys edulis*) is the first plant species in which m^{6}A presence in circRNAs has been probed (115). To do this, the purification of circRNAs was optimized, which allowed m^{6}A presence on this class of RNA molecule to be interrogated using nanopore sequencing. This analysis revealed that 46 of the 428 detected circRNAs contained m^{6}A sites, and they tended to accumulate near the donor or acceptor splice sites of the exons they originated from (**Figure 1**). Additionally, the previously described (see Section 3.3.2) m^{1}A sequencing analysis uncovered 175 putative m^{1}A peaks in 134 circRNAs in the transcriptome of petunia corollas. However, these putative m^{1}A-modified petunia circRNAs have not yet been experimentally validated (126).

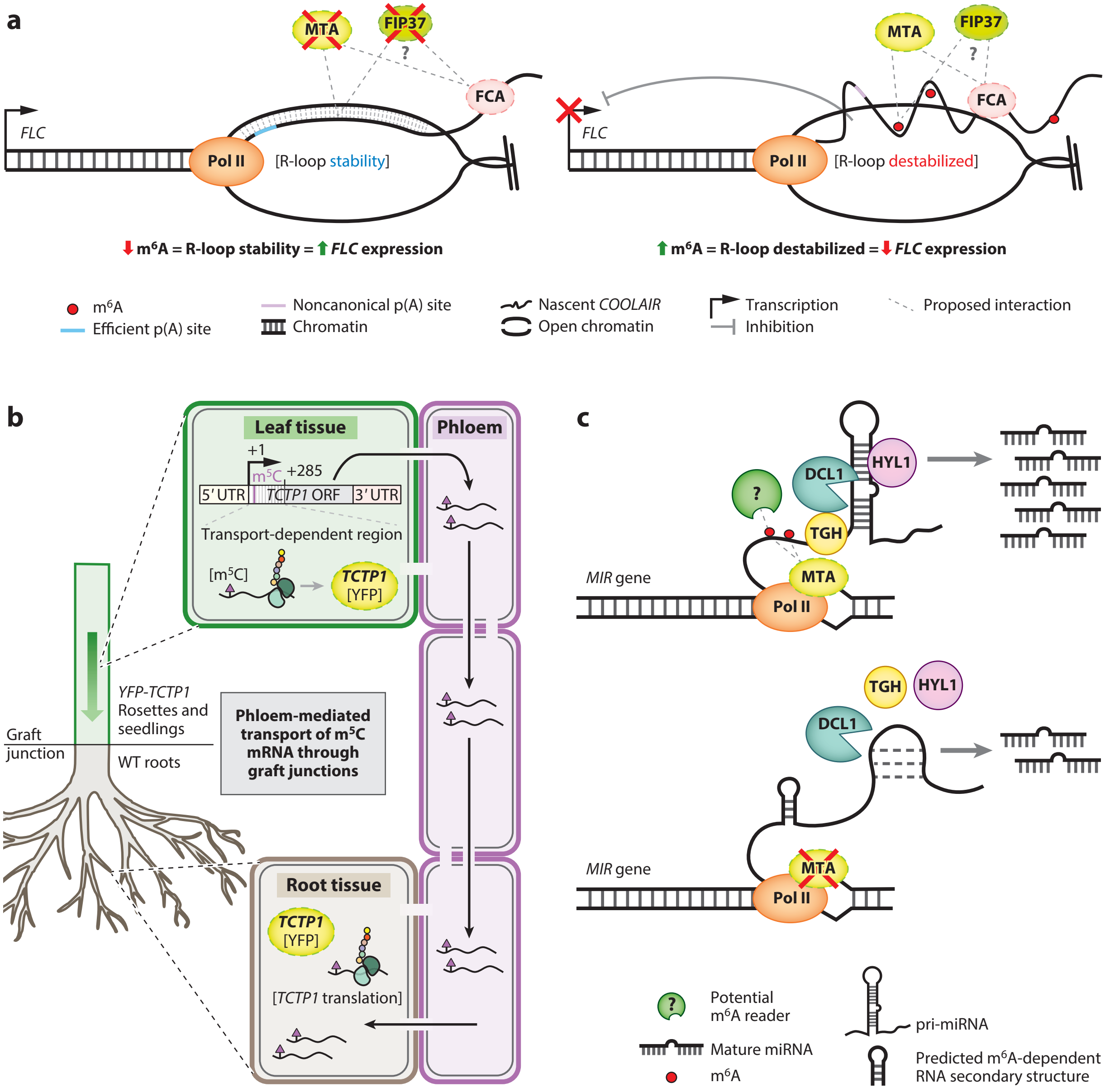
The epitranscriptomic regulation of lncRNAs has not been well investigated to date despite the presence of various modifications, including m^{6}A, m^{5}C, and m^{1}A, that have been shown in transcriptome-wide profiling studies (21, 55, 126). One key study from 2016 revealed that m^{6}A within the lncRNA X-inactive-specific transcript (XIST) is critical to its function in the transcriptional repression of X chromosome genes (82). Recently, the antisense lncRNA produced from the 3′ end of *FLOWERING LOCUS C* (*FLC*) locus, *COOLAIR*, has been demonstrated to also be m^{6}A methylated, and these modification sites are necessary for the proper functionality of this lncRNA in silencing the *FLC* locus to allow proper flowering in plants (121) (**Figure 2*a***).

Finally, a systematic profiling of Ψ in a plant transcriptome revealed that there were many snRNAs and small nucleolar RNAs (snoRNAs) as well as other RNAs that contain this mark (99). Methylation has also been found to be a prominent epitranscriptomic mark in eukaryotic snRNAs, where a total of nine methylation sites were discovered, including seven in U2, one in U4, three in U5, and eight in U6 (119). It is evident that we have only explored the tip of the iceberg when it comes to the diversity of RNA modifications in the various classes of eukaryotic

RNAs. However, along with increased focus on and dedication to profiling these epitranscriptome marks, it is essential to also perform the necessary functional studies to understand the importance of these modifications in plant systems.

4. DIVERSITY OF REGULATORY PROTEINS: WRITERS, READERS, AND ERASERS

Various profiling studies have attempted to demonstrate a direct relationship between specific RNA modifications and their associated molecular and physiological functions. However, as we



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Graphical representations of three examples in which RNA modifications impact distinct classes of RNA. (*a*) Model depicting the influence of the RNA modification m^6A on *COOLAIR* R-loop stabilization at the *FLC* locus (suggested model of Reference 102 results). *COOLAIR*-associating protein FCA facilitates the deposition of m^6A within nascently transcribed *COOLAIR* transcripts. m^6A deposition promotes R-loop destabilization and the formation of dynamic nuclear condensates, which favors RBP-mediated p(A) at noncanonical p(A) sites, and eventually results in *FLC* suppression. Alternatively, when either MTA, FIP37, or FCA are disrupted, the *COOLAIR* R-loop is stabilized, the efficient p(A) site is favored, dynamic nuclear condensation is not promoted, and *FLC* transcript abundance increases. (*b*) Depiction of the influence of RNA modification m^5C on phloem-mediated mRNA transport. The *TCTP1* ORF harbors a region close to the start +1 site that is essential for m^5C modification and subsequent mRNA transportation (suggested model of Reference 125 results). Phloem-mediated mRNA transport through graft junctions is m^5C-dependent for the *TCTP1* transcript and others like it. (*c*) Model depicting the influence of RNA modification m^6A on miRNA maturation. The m^6A writer protein MTA associates with Pol II and the pri-miRNA processing protein TGH. m^6A deposition promotes the recruitment of further pri-miRNA biogenesis proteins DCL1 and HYL1 to promote mature miRNA processing. Lack of m^6A causes a decrease in pri-miRNA structure and cofactor binding and subsequent decrease in mature miRNA biogenesis (suggested model of Reference 6 results). Abbreviations: DCL1, DICER-LIKE 1; HYL1, HYPONASTIC LEAVES 1; miRNA, microRNA; mRNA, messenger RNA; m^5C, 5-methylcytosine; m^6A, N^6-methyladenosine; ORF, open reading frame; p(A), polyadenylation; Pol II, polymerase II; pri-miRNA, primary microRNA; *TCTP1*, *TRANSLATIONALLY CONTROLLED TUMOR PROTEIN 1*; TGH, TOUGH; UTR, untranslated region; WT, wild-type.

discuss in the subsequent sections, trying to infer function from correlative associations between an mRNA modification and a particular function results in an overgeneralization at best. Depending on the context and the specific modified RNA molecule, modifications can sometimes perform contradictory functions even in the same cell. The key to understanding the biological significance and context specificity is to identify and characterize the RNA modification machinery—specifically, the RNA-binding proteins (RBPs) that add, recognize, or remove the chemical modifications, commonly referred to as writers, readers, and erasers, respectively (54). We describe what is known about these proteins in the context of specific RNA modifications below.

4.1. Writers

Writers are proteins or protein complexes that carry out the catalytic function of adding chemical modifications to specific bases in various RNA molecules. Some of the first writer proteins were identified for their roles in adding the N_m modification to the sugar ring of nucleotides in tRNAs and rRNAs. In rRNAs, methylation is primarily directed by box C/D snoRNA-guided enzymes (76, 95). In *Arabidopsis*, the fibrillarin proteins FIB1 and FIB2 have been shown to associate with C/D snoRNAs and are involved in the process of N_m writing (119). By contrast, tRNA N_m modifications are mostly carried out by standalone proteins. In rice, OsTRM13 is a newly identified tRNA 2′-*O*-methyladenosine methyltransferase whose expression is increased upon salt stress (112). It is primarily found in the nucleus, suggesting that the process of adding this modification is localized to the nucleus (112). As noted above, another common tRNA and rRNA modification is Ψ, which is primarily added by pseudouridine synthase (PUS) enzymes through both box-H/ACA-dependent and RNA-independent mechanisms. In 2022, a comprehensive study to identify these enzymes was performed for *Arabidopsis* and maize. This study uncovered six subfamilies of PUSs: RluA, RsuA, TruA, TruB, PUS10, and TruD (120). Before this search, SUPPRESSOR OF VARIEGATION 1 (SVR1) was the only well-documented PUS. SVR1 is localized to the chloroplast and likely functions in chloroplast protein biosynthesis, since *svr1* loss-of-function mutants display reduced pseudouridylation of chloroplast rRNAs (129).

In addition to N_m and Ψ, writers for important base modifications of tRNA and rRNA are also being identified. For instance, m^1G modification of position 37 of the tRNA is highly conserved from *E. coli* to plants, and its deficiency causes increased frameshift mutations. In 2019, the first plant homolog of the methyltransferase TRM5 that mediates this modification in animals was identified in *Arabidopsis* as AtTRM5a (42). This study also highlighted the importance of this protein in proper plant vegetative and reproductive development (52). Similarly, the putative m^{1}A writer AtTRM61/AtTRM6 complex was identified in 2020, and its role in N^1-methylation at the A58 position on initiator methionyl-tRNA (tRNAiMet) has been confirmed (100). Without this methylation event, there is no protein synthesis, and thus the plants lacking AtTRM61 or AtTRM6 display embryonic lethality. Various other tRNA methyltransferases both known and putative have been compiled in the PlantRNA database, and it would be worthwhile to confirm their role in tRNA modifications (17). Given the significant number of modifications that occur in rRNAs and tRNAs, there are still many more modification writer proteins that need to be identified and characterized, a challenge for RNA modification research for many years to come.

Simple base modification, such as methylation events, are the most commonly identified modifications to date in mRNA molecules. Significant advances have been made in regard to the identification of mRNA modification writer proteins in both mammalian and plant systems, particularly for m^{6}A. Specifically, in 2008, MTA was identified as the main m^{6}A methyltransferase in plant systems (139). Since then, the m^{6}A writer complex of proteins has expanded and includes the core components MTA, MTB, FIP37, and VIR (86, 88). Additionally, plant scientists were the first to discover a role for the HAKAI protein in the m^{6}A methylation pathway as a writer protein and demonstrated that loss of HAKAI led to a reduction of up to 35% of total m^{6}A levels (88). It is becoming increasingly clear that the addition of this specific modification is a tightly regulated process. For instance, HIZ1 and HIZ2 are newly discovered components of the *Arabidopsis* m^{6}A writer complex that associate with the complex through a direct interaction with HAKAI (135). Despite low primary amino acid sequence homology, HIZ2 has been proposed to the be the long-sought plant homolog of ZC3H13, a known writer complex protein in mammals, due to the significant reduction in m^{6}A levels displayed by plants that lack this protein. The function of HIZ1 in this complex is more elusive, as mutant plants lacking this protein display no visible phenotypes, while overexpression of the protein results in decreased m^{6}A levels. Therefore, HIZ2 has been proposed to regulate m^{6}A deposition by the core m^{6}A writers through its interaction with the complex through HAKAI. In addition to this core complex, another m^{6}A methyltransferase, METTL16, has been shown to add m^{6}A to ncRNAs, lncRNAs, and mRNAs in eukaryotes (116). Recently, the ortholog of this protein was identified in *Arabidopsis* as the FIONA1 protein (107). FIONA1 has been found to add m^{6}A onto the U6 snRNA and a small collection of mRNAs, as evidenced by the reduction of m^{6}A in these specific RNAs in loss-of-function mutant plants. However, whether FIONA is truly independent of the larger m^{6}A writer core complex is yet to be established and needs to be further investigated.

Despite significant progress in characterizing m^{6}A writer proteins, the writers of other mRNA methylation events, such as m^{1}A and m^{5}C, are to date unknown. Some potential candidates are those proteins known to add these marks to tRNAs. For instance, there are two types of m^{5}C writers: tRNA aspartic acid methyltransferase 1 (TRDMT1) and tRNA-specific methyltransferase 4 (TRM4/NSUN2) (10). In support of some of these proteins functioning in mRNA m^{5}C deposition, a recent study demonstrated that *Arabidopsis* tRNA-specific methyltransferase, AtTRM4B, was likely to function as an mRNA m^{5}C writer in addition to its known function of adding m^{5}C methylations on tRNA at positions C48, C49, and C50. In support of this hypothesis, m^{5}C levels in root mRNAs were reduced in the absence of this protein (18). To date, there are no known mRNA m^{1}A writers, and identifying these proteins and the importance of this mark in plant biology needs to be further investigated. Overall, one of the greatest gaps of knowledge in the field of RNA modifications is our current understanding of the parameters required for the precise deposition at very specific mRNA nucleotides for the various RNA modifications by these writer proteins. Identification and careful characterization of the writer proteins/complexes are needed to understand this specificity in modification deposition, which is important for our basic understanding of these systems as well as for allowing them to potentially be used as tools for future crop improvement.

4.2. Readers

Similar to the progress made in understanding the machinery writing RNA modifications, the study of mRNA modification readers, proteins with RNA modification–binding domains, is limited to one or a few RNA marks. Advances in plant biology have focused on m^6A readers, where across plant species, m^6A reader proteins containing the YTH domain have been identified. To understand in detail the m^6A reader machinery, we direct the reader to previous reviews focusing on this topic (e.g., 60). In brief, there are 13 members in the YTH family in *Arabidopsis*. Similar to their mammalian counterparts, these reader proteins are divided into two clades: YTHDC and YTHDF (90), with 2 and 11 members in *Arabidopsis*, respectively. While the core residues that form the m^6A-binding YTH domain are conserved between mammalian and plant m^6A readers, plants contain an expanded set of m^6A readers with higher amounts of variation compared to the set of mammalian orthologs. Intriguingly, 1 of the 2 YTHDC proteins in plants is a larger isoform of the highly conserved member of the eukaryotic polyadenylation complex CLEAVAGE AND POLYADENYLATION SPECIFICITY FACTOR 30 (CPSF30) (46). CPSF30 is found mostly in the nucleus of the cell, whereas members of the YTHDF clade ECT2, ECT3, and ECT4 are found in the cytoplasm and in stress granule–like structures during heat stress response (3, 90, 97). These reader proteins have important roles in both the molecular and physiological processes, as discussed in the subsequent sections. Beyond *Arabidopsis*, 12 YTH domain–containing protein-coding genes are found in *Oryza sativa*, 9 in tomatoes, and 39 in the hexaploid species common wheat (corresponding to 13 homologous triads) (127).

Very few reader proteins of other mRNA modifications have been identified specifically in plants. As a first step, a focus on alternative functions of readers in plant models may be useful. For example, in mammals, it was shown that the YTH domain is not specific to m^6A and may function in binding to m^1A (19), revealing a dual functionality that should be explored in plants. Continued mining of bona fide mammalian readers in plant genomes coupled with biochemical verification will push our understanding even further. Through leveraging existing discoveries in mammalian systems with homologous counterparts in plants and pushing for identification and characterization of novel RNA modification–binding proteins through RNA immunoprecipitation experiments, we can shape a more holistic picture of these critical players in the field.

4.3. Erasers

Many RNA modifications are dynamic in nature and can be selectively removed by proteins deemed erasers (87). Compared with the advances made in understanding RNA modification writers, there is very limited knowledge of eraser proteins. The majority of research has focused on the removal of methyl marks, and the ALKB family of nonheme Fe (II)/alpha-ketoglutarate-dependent dioxygenases (ALKBH family) catalyzes this demethylation process. In mammals, ALKBH1 and ALKBH3 function as erasers of m^1A (91) from tRNAs, while ALKBH5 is involved in mRNA m^6A demethylation. ALKBH9B and AKBH10B in *Arabidopsis* and SlALKBH2 in tomato have been experimentally shown to remove methylation from mRNA (27, 71, 141). Additionally, when expressed ectopically, human demethylase enzyme FTO seems to function as a potent demethylase in plant species rice and potato, where its overexpression caused reduction in overall m^6A levels, resulting in significant physiological phenotypes. However, the plant ortholog of FTO has not yet been discovered, suggesting that there may not be complete conservation of mammalian RNA demethylase proteins across species. Similarly, the dynamic NAD^+ cap modification on mRNA can also be removed. It has been shown that DXO1 in *Arabidopsis* acts as a de-NADing protein and is critical in the NAD-regulated stability of particular mRNAs (51, 131). For most RNA modifications in plants, the identities and functional specificity of eraser proteins are widely unknown, representing a large gap in this field. Understanding how these marks are removed is equally as important as defining their addition. Genetic manipulation of known eraser proteins, coupled with mining and biochemical experimentation of novel erasers, is necessary to fully understand the importance and function of each modification.

5. FUNCTIONS OF RNA MODIFICATIONS

With growing information about the modifications and their writer, eraser, and reader proteins, the functional roles of RNA modifications have only recently been uncovered in plants, mostly focusing on the role of m^6A in various biological processes.

5.1. Molecular Functions

Here, we highlight our current understanding of the molecular functions of RNA modifications in plants and discuss areas where more exploration is needed.

5.1.1. Translation. The best-known example of an internal modification affecting downstream translation of an mRNA happens in mammals where a single m^6A mark in the 5′ UTR of mRNA is able to directly bind to eukaryotic Initiation Factor 3 (eIF3) and initiate translation (73). Similarly, in a mammalian system, m^6A reader protein YTHDF1 was shown to directly interact with eIF1 and promote translation (and not stalling) of methylated mRNA (110). In plants, transcripts that are more enriched for methylation have recently been shown to be more highly ribosome-bound upon cold stress, correlating the presence of m^6A with higher gene expression (38). However, mechanistic understanding of m^6A and translation in plants is entirely unknown. In maize, global m^6A methylation was shown to be negatively correlated with translation, except in transcripts where the m^6A mark was found on the 5′ end, which demonstrated a positive correlation with translation (68). These data suggest a position-specific role for m^6A in translation, which deserves more investigation in other plant models. Additional RNA modifications have been implicated in translation. For example, profiling m^5C in *Arabidopsis* revealed that the transcripts containing m^5C were high in monosomes and low in polysomes, suggesting that m^5C is found in mRNA with low translational activity (18). rRNA and tRNA modifications affect translation but do so via other molecular mechanisms such as tRNA recognition and translational fidelity. For example, m^1A modification of position A58 on the tRNAiMet is important in adding the initiating methionine amino acid on a protein chain. The lack of this modification stalls all protein translation and causes sterile seeds in *Arabidopsis* (62, 100). The vast majority of mechanistic insights linking translation and RNA modifications has been in mammalian systems. In order to understand the functional implications of RNA marks, genetic manipulation coupled with proteomics is needed to move past correlative studies in plant systems.

5.1.2. RNA stability. The connection between m^6A and RNA stability has been of significant interest for quite some time. Early studies in mammalian systems demonstrated a correlation between increases in mRNA half-life in response to inhibiting m^6A addition chemically (11) or m^6A writer complex downregulation (5, 37, 109). In plants, transcriptome-wide investigation of the effects of m^6A on RNA stability was done in the near-complete absence of the main m^6A writer protein, MTA, and its direct interacting cofactors, FIP37 and VIR (8, 86, 91), or by increasing the levels of the m^6A eraser ALKBH proteins (27). These studies have demonstrated that plants with depleted levels of m^6A display a trend of m^6A targets being less abundant in tissues with reduced levels of this mark (2, 80, 92), suggesting m^6A is a stabilizing modification in plant mRNAs. However, other studies have reported an increase in the stability of methylated transcripts in plants that are m^6A deficient (92) or have an overabundance of ALKBH eraser proteins (27). Overall, it appears that m^6A can both positively and negatively affect the stability of specific target mRNAs, and as is suggested by mammalian studies, this all likely depends on which YTH domain–containing protein binds the specific methylated transcript. Specifically, binding of the m^6A sites by a YTHDC family protein results in the degradation of that target mRNA, whereas recognition by the YTHDF proteins tends to result in stabilization of the methylated mRNAs (26, 80). In support of this model in plant systems, the YTHDF family protein ECT2 has been found to facilitate the stability of its bound target mRNAs in the cytoplasm of *Arabidopsis* cells (117). Overall, in order to truly understand the effects of m^6A on RNA stability, studies of the plant m^6A-binding ECT proteins need to be the focus, as their interaction with the methylation in specific target transcripts likely dictates the outcome for that specific RNA molecule. Thus, detailed studies of the plant ECT proteins should be a major future research focus for this field.

Beyond m^6A, our understanding of the role of other mRNA modifications in RNA stability is limited. For instance, the presence of m^1A on petunia transcripts and m^5C on *Arabidopsis* mRNAs was positively correlated with mRNA stability (21, 126). Additionally, recent findings have revealed a link between 5′ NAD^+ capping and tissue- and hormone response–specific mRNA stability, where NAD^+-capped RNAs have a significantly higher rate of turnover compared to the same transcripts that do not contain this noncanonical cap (131). Overall, the relationship between RNA modifications and transcript stability is complicated. Continued experimentation to evaluate the functional impacts that single RNA modifications have on mRNA stability in plants is necessary. These future studies should focus on transcriptome-wide pulse-chase experiments that allow accurate assessment of mRNA stability over significant time periods in appropriate wild-type and mutant backgrounds.

5.1.3. RNA secondary structure. Changes in RNA secondary structure can have important downstream effects on transcript stability and translation. As mentioned earlier (see Section 1), RNA modifications are known to play key roles in stabilizing the secondary and tertiary structures of tRNA and rRNA molecules. They do so by directly affecting the base pairing of the nucleotides as well as the way the RNAs interact with the surrounding environment by altering the thermodynamic properties of specific regions surrounding the modification in the RNA molecule (77).

Recent research using the global RNA secondary structure–probing protein interaction profile sequencing (PIP-seq) approach has shown that m^6A-modified regions of mRNAs display a negative correlation with base-pairing probability, suggesting a lower overall RNA secondary structure in these regions (55). These findings suggest that m^6A is likely hindering the formation of secondary structure in the areas of RNA where this modification is found (55). Similarly, in vitro studies have shown that m^1G and m^1A disrupt helical secondary structures by disrupting base pairing. Overall, it is likely that many RNA modifications can exert their effects on modified transcripts by disrupting the formation of their proper folding patterns. This is an area of inquiry that should be pursued with future research.

5.1.4. Transport. The roles of mRNA modifications in RNA transport are only recently coming to light. A recent pivotal study demonstrated that m^5C is necessary for the transport of specific methylated mRNAs across graft junctions via movement through the phloem (108, 125). When these transcripts are insufficiently m^5C methylated by treating plants with a chemical that inhibits the addition of this mark, two highly phloem-transported transcripts, *TRANSLATIONALLY CONTROLLED TUMOR PROTEIN 1* (*TCTP1*) and *HEAT SHOCK HOMOLOGOUS PROTEIN 70.1* (*HSC70.1*), displayed significantly reduced movement in the absence of this mark (125) (**Figure 2*b***). Thus, m^5C appears to be indispensable for targeting the movement of specific transcripts in the phloem, but it is unknown if other RNA modifications function in similar transport or global localization processes. Further studies of the effects of RNA modifications on RNA movement and transport should be actively explored.

5.1.5. Polyadenylation and splicing.

In plants, the unique YTHDC protein translated from the longer isoform of the *CPSF30* gene is involved in coupling m^6A methylation to the regulation of mRNA polyA (46, 97). Recent studies have shown that losing the function of the longer CPSF30 (CPSF30-L) protein that contains the m^6A-binding YTH domain leads to a shift in polyA site selection for many nitrate signaling–related transcripts. This polyadenylation defect could only be recovered with endogenous CPSF30-L but not with a m^6A-binding defective mutant of CPSF30 (46). Furthermore, it was found that CPSF30-L binds to an m^6A site in far-upstream elements (FUEs), which then favors selection of the proximal polyA site, whereas loss of this protein generally lengthens the 3′ UTR region because of enhanced distal polyA site selection (97).

FIONA1 is an *Arabidopsis* U6 m^6A methyltransferase that adds m^6A at specific nucleotides in U6 snRNA, a core component of the spliceosome. While in mammals and fission yeast the lack of this protein leads to changes in splicing, a study in *Arabidopsis* showed that this phenotype is not displayed by plants that lack the FIONA protein (107). Determining why this methylation event is not required for the splicing of plant transcripts as compared to those from other eukaryotes will be interesting. Additionally, determining the effects of RNA modifications on mRNA splicing is definitely of significant interest in the community, and at least one study has suggested that there is a linkage between RNA modifications and alternative splicing (105), which needs to be more carefully explored in the future.

5.1.6. Epigenetic regulation.

The interplay between epigenetic and epitranscriptomic regulation of gene expression is a relatively novel field of inquiry in biology. In plants, the first study to investigate this relationship looked at the association of histone modifications with m^6A and showed that the H3K36me2 modification of a gene body correlated with m^6A levels of its transcripts (93). Furthermore, the H3K36me2 writer SET DOMAIN GROUP 8 (SDG8) protein interacts with m^6A writer complex protein FIP37 and potentially guides this mRNA modification. Another important piece of evidence of such interplay came from tomato, where m^6A is implicated in fruit ripening via a possible epigenetic and epitranscriptomic crosstalk via the m^6A demethylase SlALKBH2 and a DNA demethylase, SlDML2 (141). Specifically, during ripening, DNA demethylases remove DNA methylation marks from the *SlALKBH2* locus, which ultimately leads to increased abundance of the SlALKBH2 protein. This m^6A eraser protein then removes m^6A from the *SlDML2* RNA transcript, thereby stabilizing those mRNAs and subsequently resulting in increased abundance of this DNA demethylase protein. Increased SlDML2 further activates the loci of many additional ripening-related genes (141).

Additionally, m^6A writer MTA plays a key role in the function of epigenetic regulators, including ncRNAs such as miRNAs and lncRNAs. Specifically, both m^6A and MTA have been implicated in the lncRNA-mediated epigenetic regulation of flowering suppressor FLC locus where m^6A in antisense RNA COOLAIR promotes the formation of nuclear condensates that eventually lead to the suppression of FLC (63) (**Figure 2*a***). It is also known that MTA directly interacts with polymerase II (Pol II), which has been confirmed by using both immunolocalization and proximity ligation assays (6, 94). It is therefore likely that MTA regulates miRNA and mRNA m^6A methylation co-transcriptionally. Using Förster resonance energy transfer (FRET)–fluorescence lifetime imaging microscopy (FLIM) interaction assays, it was shown that MTA also interacts with the early miRNA processing protein TOUGH (TGH) and promotes maturation of miRNA (6) (**Figure 2*c***). Questions remain as to where the eraser complexes are localized within the nucleus and if there is a potential for crosstalk between other types of epigenetic pathways such as histone modifications and m^6A erasers. These are questions that need to be addressed in future research endeavors.

5.2. Diversity of Functions that RNA Modifications Play in Plant Developmental and Physiological Processes

RNA modifications and the machinery required for their deposition are critical for proper plant development and various physiological responses. Below, we will discuss the findings relevant to the importance and functions that RNA modifications have been found to have in plant development and specific physiological processes.

5.2.1. Development. It has been found that alteration or complete loss of specific marks (e.g., m^6A) on a diverse set of RNA in numerous eukaryotic species results in dramatic phenotypes ranging from embryonic lethality to organ-specific defects. For instance, m^6A is essential for both embryo and later-stage development in many multicellular eukaryotes, with total loss of m^6A resulting in the failure of a developing embryo to progress past the globular stage in *Arabidopsis* (139). Even decreases in the levels of this mark in plants can result in significant alteration to flowering time and plant morphology (9, 27). Similarly, the m^1A mark at tRNA position 58 (A58) in *Arabidopsis* is essential for proper early plant development, with deficiency or depletion of the enzymes responsible for this m^1A deposition leading to embryo arrest and seed abortion (100). On rRNAs, N^4-methylcytidine (m^4C) in the 16S chloroplast rRNA in *Arabidopsis* is also critical for development, with disruption of this specific modification site causing defects in growth, seed yield, and photosynthetic activity (104). The recently described m^6A deposition on the U6 snRNA and a subset of polyA RNA by the m^6A methyltransferase FIONA1 in *Arabidopsis* offers another example in which disruption to this machinery results in significant alteration to flowering and photomorphogenesis (107).

Beyond *Arabidopsis*, m^6A deposition in rice has recently been directly linked to auxin biosynthesis during microsporogenesis and has been shown to be essential to local auxin production in anthers during male meiosis (16). Excitingly, in trees, the previously described relationship between ECT proteins and development was investigated (117), and this study found an important role for these m^6A-binding proteins in tree development. Furthermore, alterations to the m^6A methyltransferase MTA in *Populus trichocarpa* showed significant changes in trichome density and root development (66). Thus, in all plants, RNA modifications have evolved as essential regulators of plant development. Moving forward, the use of tissue- and organ-specific gene expression analysis tools to interrogate RNA modification and their associated machineries will allow for a more concentrated focus on the specific developmental processes they affect in various plant systems.

5.2.2. Abiotic stress response. Due to their sessile nature, plants are exposed to various environmental conditions and have therefore evolved complex mechanisms to respond to environmental stress. Epitranscriptomic regulation can potentially serve as a way to quickly and efficiently fine-tune gene expression in plant systems as they respond to various stressors. In plants, the loss-of-function mutants of various members of the m^6A writer complex, MTA, MTB, VIR, and HAKAI, demonstrate salt-hypersensitive phenotypes upon salt stress treatment (47). In another study, m^6A profiling in *Arabidopsis* showed changes in the m^6A methylation profile of thousands of stress-related transcripts upon salt stress treatment (2). Moreover, m^6A-methylated transcripts encoding salt and osmotic stress response proteins were found to be more stable in the context of plant salt stress response (2). Relatedly, a recent study looking at cold stress response in *Arabidopsis* also demonstrated a similar phenomenon, whereby cold stress–responsive transcripts showed increased m^6A enrichment upon stress treatment with a concomitant increase in transcript abundance and translation (38). In the tree genus *Poplar*, the overexpression of m^6A methyltransferase PtrMTA resulted in increased drought tolerance in these transgenic plants (66). Furthermore, a plethora of new studies have provided additional links between m^6A and stress responses in a wide range of plant species (**Table 1**). However, studies that reveal mechanisms related to the functional specificity of m^6A regulating specific stress-related transcripts are much needed in plants to understand how this mark might serve as a potential tool for future crop improvement.

Outside of m^6A, the m^5C modification in rice has been implicated in heat stress via regulation of the writer protein OsNSUN2 by this stressor (102). Specifically, it was found that a loss-of-function mutation of OsNSUN2 displayed heat stress hypersensitivity as compared to wild type. Relatedly, the OsNSUN2 protein was found to methylate transcripts encoding proteins involved in photosynthesis and heat stress responses.

For a long time, tRNA and rRNA modifications were considered to be static chemical decorations and not dynamic chemical additions (20). In recent years, evidence has emerged that these highly abundant ncRNAs might have important roles in plant stress responses, which calls for more focused studies investigating the roles of epitranscriptome pathways in these processes. Fittingly, N_m modifications of tRNAs have been implicated in salt stress tolerance in rice because increased abundance of the writer protein OsTRM13 makes plants more resistant to this stress (112). Specifically, this study demonstrated that overexpression of OsTRM13 led to rice plants with increased salt tolerance, while knocking down this protein led to the opposite hypersensitivity phenotype (112). Furthermore, important modifications near the wobble position of tRNA anticodon regions have been found to be induced upon alkylation or oxidative stress and are known to affect translation of stress response transcripts (41). In rice and *Arabidopsis*, levels of RNA modifications were measured under drought, cold, and salt stress using LC-MS/MS (114). This study found that four methylated nucleosides, Am, Cm, m^1A, and m^7G, were responsive to these stresses in tRNA molecules. Relatedly, hierarchical clustering of RNA abundance values of mRNAs encoding various RNA modification pathway proteins as compared to the modification for which they are responsible has demonstrated that the transcripts encoding many of the predicted methyltransferases are correlated with the level of the corresponding modification, suggesting that writer protein levels play a key role in the dynamic levels of specific marks. In plants, profiling of rRNA modifications in the context of stress response had not been performed until recently. In 2022, exposure of *Arabidopsis* plants to cadmium (Cd^{2+}) was found to lead to changes in the levels of the rRNA modifications $m^{6,6}A$ in 18S rRNA and $m^{2,2}G$, $m^{6,6}A$, and m^7G in 25S rRNA (101). Whether the change in levels is via altered activity or expression of the writer proteins/complexes needs to be further explored. There is no doubt that abiotic stresses cause changes in the RNA modification of various mRNAs, rRNAs, and tRNAs in plants. The biggest challenge in the field is to unravel the molecular mechanisms that explain these observations of stress-induced epitranscriptome dynamics and subsequent effects on gene expression regulation in plant systems.

5.2.3. Immune response. In *Arabidopsis*, a series of detailed analyses investigating the role of m^6A demethylase ALKBH9B in modulating alfalfa mosaic virus infection provided a direct link between RNA modifications and biotic stress response in plants. ALKBH9B has been shown to directly bind and demethylate viral RNA, thereby affecting the infectivity of alfalfa mosaic virus

(71). More recent work detailing the functional subdomains of ALKBH9B (1) and pathology of infection in the presence and absence of this eraser (72) has provided a detailed example of a host's RNA modification machinery directly impacting viral modification levels and subsequent abundance. In wheat, transcriptome-wide m^6A profiling revealed significant changes in mRNA and m^6A levels on transcripts involved in defense response (136), offering initial insight into the link between systemic plant response to pathogen challenge and RNA modification in an important crop species. Biotic stress response modulation through RNA modifications is vastly understudied, but early studies highlighting obvious links between RNA modification and host response have revealed that this is a ripe area for future research.

6. RECENT AND IMPROVED METHODOLOGIES FOR DETECTING AND QUANTIFYING RNA MODIFICATION SITES

The interest of the RNA modification research community to directly identify and characterize all of the various RNA modifications has driven the development and constant improvement in detection methods for these chemical moieties. However, there are still major limitations to MS and transcriptome-wide analytical approaches, including limitations in detection sensitivity, resolution, stoichiometry, and accuracy of RNA modification site identification, especially in plant systems. We highlight numerous methods below, many of which have not been previously covered in plant modification review articles (86, 130), and highlight areas of potential improvement in the future.

6.1. Mass Spectrometry– and Chromatography-Based Approaches

As a direct method to analyze nucleic acids at the nucleoside/nucleotide levels, MS can allow the identification of both known and novel/unknown RNA covalent modifications (58). However, the conventional MS-based strategy requires reduction of polynucleotide RNA molecules to mononucleosides/nucleotides, and thus, it cannot provide information about the location of the chemical modification with respect to the transcript. Therefore, strategies where oligoribonucleotides are analyzed by MS/MS are being explored. For instance, miRNAs have been successfully analyzed based on the application of LC-MS/MS (57). Recently, through the use of LC-ESI-MS/MS, 10 and 12 different chemical modifications in *Arabidopsis* 18S rRNA and 25S rRNA were detected, respectively (101). Interestingly, of these detected rRNA modifications, $m^{2,2}G$, $m^{6,6}A$, m^7G, and m^3U were identified and reported as being present in rRNAs for the first time in plants based on the findings from this new, more sensitive methodology. These findings highlight the potential of LC-ESI-MS/MS in exploring the unknown realm of the plant epitranscriptome, including those found in both protein-coding RNAs and ncRNAs.

Despite these advances, careful attention must be paid to the importance of highly pure RNA samples. One such purification methodology, developed by Tang et al. (101) for purifying rRNAs, is based on $polyA^+$ RNA depletion followed by agarose gel electrophoresis to eliminate potential contamination from bacterial species in order to analyze *Arabidopsis* (plant) rRNA. Thus, development of both precise purification techniques and highly sensitive MS-based modification detection methodologies is critical for future research projects.

In addition to detection, proper and precise quantification of RNA modification levels is key to enhancing our understanding of these marks. This is especially true concerning the specific classes of RNA modifications that are found in mRNAs, given the lower stoichiometric abundance of these RNAs when compared to rRNA and tRNA. Therefore, when analyzing mRNA modifications specifically, extreme efforts need to be invested in directed sample preparation before conducting MS analyses.

A number of thin layer chromatography (TLC) approaches have also been developed for quantifying RNA modification levels in RNA molecules. For instance, a TLC-based approach has been developed for quantifying m^6A levels that occur in a gibberellic acid (GA) sequence context within polyA$^+$-selected mRNA. This method displays high accuracy in its quantitation of these instances of m^6A but is limited by the sequence context specificity and need for very pure mRNA samples (8). As an example of a quantitative TLC-based method for determining m^6A levels in an RNA sequence of interest, SCARLET (site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography) was developed. This methodology employs a target-specific chimeric splint oligo in conjunction with single-stranded DNA (ssDNA) oligos to direct cleavage and radioactive labeling to a specific RNA sequence that can then be quantified for the levels of a specific modified nucleotide using TLC (65). This approach can be rather tedious and requires the use of radioactive labeling of the RNAs being targeted for modification-level quantitation, but it is highly accurate. To date, very few plant studies have used SCARLET or related methodologies to quantify modification levels in specific RNA molecules. For future plant RNA modification studies, obtaining the quantification of modification levels of individual transcripts is critical. With this in mind, a high-throughput label-free MS- or TLC-based quantification method would revolutionize future RNA modification studies in plant systems. Overall, more work is needed for precise quantification of RNA modification levels, especially those found in mRNAs, an area in need of research.

6.2. Sequencing-Based Methods

Antibody-dependent high-throughput sequencing–based methodologies, including m^6A and m^6A-MeRIP sequencing, have enabled researchers to produce the first transcriptome-wide maps of m^6A and a few other modifications in multiple eukaryotic organisms (24, 74). Although these approaches have provided a number of important discoveries in the field of RNA modification biology, issues regarding the cross-reactivity of various antibodies, a lack of providing modification stoichiometry, issues with sensitivity and compromised resolution (i.e., peaks as compared to single nucleotide resolution), and limitations of input RNA material have all resulted in slight detractions to these transcriptome-wide approaches.

To circumvent some of these methodology problems, a number of additions and improvements have been made to these antibody-based sequencing approaches. For instance, a cross-linking before immunoprecipitation (CLIP) step (i.e., cross-linking m^6A antibodies to these modification sites in RNA molecules) was incorporated into the m^6A antibody-based sequencing methodologies to enhance resolution down to the single nucleotide level while maintaining a global scale of identification (53, 61).

Additionally, recent antibody-independent, single-nucleotide resolution sequencing strategies that are applicable at a transcriptome-wide scale have been developed for m^6A mapping and are based on the sensitivity and specificity of this modification's RNA endoribonuclease, MazF (MAZTER-seq and m^6A-REF-seq) (36, 49, 137). MazF cleaves RNA immediately upstream of unmethylated ACA sequences, while, in the presence of m^6ACA, the RNase activity of this enzyme is inhibited. Presently, these two techniques only allow the detection of an m^6A profile for a very specific sequence context. Development and implementation of these methods in plant systems will be a necessary focus for future RNA modification studies.

Beyond the debate over antibody-dependent and -independent sequencing–based technologies for understanding the plant epitranscriptome, next-generation long-read direct RNA sequencing (DRS) using Oxford nanopore sequencers has emerged as a potent and comparatively unbiased approach that does not rely on reverse transcription or amplification steps in sequencing substrate preparation (35). All that is needed for epitranscriptome profiling with this technology is to develop the analysis tools for identifying the differences in current flow moving through the nanopores that result in errors in the called nucleotide at that position for specific modified ribonucleotides as compared to those that have not been modified or are marked with a different modification. In a recent *Arabidopsis*-focused study, Parker et al. (81) conducted both nanopore DRS and Illumina high-coverage short-read RNA sequencing to revisit transcriptome- and epitranscriptome-level complexity in this model plant. While they could verify the m^6A profile predicted by errors in the base calling for the nanopore DRS data, they also noted that the base-calling software (in this case, Guppy version 2.3.1) is extremely critical for determining errors in base calling and thus for RNA modification detection during downstream analysis for this type of sequencing-based approach.

Although there are now numerous m^6A-focused tools for using nanopore DRS sequencing experiments for studying this modification, including training data sets (generated based on sequencing in vitro transcription products with and without m^6A) and multiple computational tools (e.g., Nanom^{6}A and EpiNano) for using DRS data analysis to identify m^6A sites (34, 64), there are still caveats to this approach, even for m^6A. Specifically, the in vitro transcribed training data sets might not be the best model for developing analysis algorithms due to the lack of sufficient sequence variability in these molecules, and many of the currently available analysis tools have specific biases based on their underlying computational model and learning approaches. Nanopore DRS analysis has recently been extended to tRNA samples, and these studies have revealed that errors in DRS database calling corresponded to the expected modification sites of tRNAs, including conserved Ψ positions in the TΨC-loop (103). Overall, with the development of additional tools that may be used to analyze the complete diversity of RNA modifications found throughout the different RNA types in eukaryotes, the long-read DRS-based approaches provide a parallel high-throughput means for the discovery of RNA modification sites at single-nucleotide resolution in the transcriptomes of diverse plant species. Moving forward, this is another area of research that is worthy of our attention.

6.3. Computational Methods

With the continuing improvements in MS and sequencing technologies, scientists have generated an unprecedented collection of transcriptomic and epitranscriptomic data over the past decade. Therefore, it is imperative to highlight the progress made in computational technology development, without which it is impossible to fully and accurately explore the potential epitranscriptomic information stored in these data. For instance, one computational approach, HAMR, makes use of base-calling errors to predict RNA modifications globally using any high-throughput sequencing data where the library generation was done with a reverse transcriptase enzyme that lacks proofreading ability (89). More specifically, these mismatches are introduced into the complementary DNA library by the reverse transcriptase enzyme when it improperly incorporates an incorrect base at the site of an RNA modification due to the misrecognition of the base caused by the presence of the covalent modification. This package then measures the rate and pattern of this misincorporation and predicts RNA modification identity using empirical data based on tRNA modification sites that are fed to the machine-learning algorithm as a training data set. This method has been used in *Arabidopsis* and was successful in predicting novel m^3C sites in mRNA molecules, which were subsequently validated (105). More recently, another machine learning–based algorithm, PEA-m5C, was developed in *Arabidopsis* for transcriptome-wide prediction of m^5C sites using sequence features in and around experimentally validated m^5C sites (96). The success of these types of prediction-based approaches is likely to increase as we generate more accurate and reliable training data sets for plants.

To truly understand the functions of RNA modifications, we also need techniques to identify the targets of writers, readers, and erasers of these modifications. Recently, a mismatch detection–based technique called HyperTRIBE was used to detect the binding sites of the m^{6}A reader proteins ECT2 and ECT3 (4). In this technique, the protein of interest is translationally-fused to an RNA-editing enzyme, ADARcd, and is then ectopically expressed in the plant of interest. ADARcd introduces several mutations on the RNA nucleotides proximal to the RBP interaction sites that can then be detected as mismatches in downstream analyses of the resulting RNA-sequencing data, thereby allowing the identification of interaction sites of the RBP of interest (in the case of ECT proteins, these are m^{6}A sites) (85). Although generalizable to all RBPs, this method looks ideal for identifying targets of RNA modification writers, readers, and erasers as it eliminates the need for an antibody and can get nucleotide-level information where the reader proteins interact. Additionally, studies using this technology would greatly improve our understanding of the binding and targets of the writers, readers, and erasers of various RNA-modification systems and should be a focus for future research projects.

In the field of MS, the analysis of the resulting spectra obtained from MS/MS analyses of oligoribonucleotides can be highly complex and convoluted. Development of recent tools, such as NucleicAcidSearchEngine (NASE), has provided a platform to comprehensively analyze RNA MS/MS data for multiple chemical modifications, while also taking into consideration the false discovery rate estimation for this type of data (118). As described in previous sections, detection of RNA modifications using nanopore sequencing has its limitations. Recent advances in how nanopore reads are processed, specifically for m^{6}A detection, have resulted in more reliable prediction. Using *Arabidopsis* sequences, Deeplearning Explore Nanopore m^{6}A (DENA), an analysis package based on deeper novel neural networks, relying on training data sets generated by in vivo data from wild-type and m^{6}A-deficient plants, appears to be a powerful tool for the direct identification of this RNA modification (84). Since these developments can impact all fields of biology, plant biologists can lead the way in developing the needed technologies and approaches, while also focusing on adapting those that have already been developed in other systems.

7. CONCLUSION

The field of plant epitranscriptomics research is quickly gaining steam and uncovering new and exciting functionalities for these covalent RNA additions in plant systems (**Table 1**). The current methodologies are sufficient to create progress in the field. However, for truly transformative findings in the field of plant epitranscriptomics, more high-throughput and sensitive methods with increased specificity and resolution are needed for driving discoveries forward. We have outlined some ideas for future methods improvement, but even more importantly, the plant community cannot solely rely on adopting methods developed for original use in other eukaryotic organisms but must pioneer future methods development. Only in these ways can critical insights be made that will increase our understanding of the overall importance of RNA modifications in plant systems and provide the critical findings that will drive our ability to translate these basic findings to future crop improvement applications.

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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