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Review Paper

The impact of epitranscriptomic marks on post-transcriptional regulation in plants

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Abstract

Ribonucleotides within the various RNA molecules in eukaryotes are marked with more than 160 distinct covalent chemical modifications. These modifications include those that occur internally in messenger RNA (mRNA) molecules such as N^6-methyladenosine (m^6A) and 5-methylcytosine (m^5C), as well as those that occur at the ends of the modified RNAs like the non-canonical 5′ end nicotinamide adenine dinucleotide (NAD^+) cap modification of specific mRNAs. Recent findings have revealed that covalent RNA modifications can impact the secondary structure, translatability, functionality, stability and degradation of the RNA molecules in which they are included. Many of these covalent RNA additions have also been found to be dynamically added and removed through writer and eraser complexes, respectively, providing a new layer of epitranscriptome-mediated post-transcriptional regulation that regulates RNA quality and quantity in eukaryotic transcriptomes. Thus, it is not surprising that the regulation of RNA fate mediated by these epitranscriptomic marks has been demonstrated to have widespread effects on plant development and the responses of these organisms to abiotic and biotic stresses. In this review, we highlight recent progress focused on the study of the dynamic nature of these epitranscriptome marks and their roles in post-transcriptional regulation during plant development and response to environmental cues, with an emphasis on the mRNA modifications of non-canonical 5′ end NAD^+ capping, m^6A and several other internal RNA modifications.

Key words: epitranscriptome; RNA modifications; Post-transcriptional regulation; RNA stability; RNA methylation; NAD^+ capping

Introduction

To date, over 160 modifications have been identified to occur on the collections of RNAs found in a diverse range of biological organisms from bacteria to all eukaryotic organisms, adding another layer of information to the RNA molecules themselves subjecting them to an additional layer of regulation in the form of epitranscriptome-mediated post-transcriptional regulation [1]. Once thought to mostly be added onto the ribonucleotides of functional RNA molecules [e.g. transfer RNAs (tRNAs)], recent evidence has suggested that covalent modifications are also significantly present on messenger RNA (mRNA) bases. For instance, N^6-methyladenosine (m^6A) has been found to likely be the most abundant internal RNA modification within mRNA molecules [2]. Additionally, several other internal epitranscriptome modifications such as 5-methylcytosine (m^5C) and N^1-methyladenosine (m^1A) have also been identified on eukaryotic mRNAs, including plant transcriptomes along with their writer, reader and eraser protein complexes for a few of these modifications [3, 4]. Thus, covalent RNA modifications are a ubiquitous layer of information that can be added to the

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various classes of RNAs in eukaryotic transcriptomes, which can provide an additional layer of regulatory information on these molecules.

In addition to internal mRNA modifications, recent studies revealed that these molecules can also be modified at their 5′ ends through the addition of several non-canonical metabolite caps, including NAD^+, flavin adenine dinucleotide, uridine diphosphate glucose (UDP-Glc), uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and 3′-desphospho-coenzyme A (dpCoA) [5–8], resulting in the discovery of a 5′ end cap epitranscriptome. This discovery was driven by technological innovations that combined click chemistry with high-throughput sequencing to allow the transcriptome-wide detection of these non-canonically capped RNAs in bacteria, humans and plants [9–12]. Furthermore, the decapping enzymes that remove at least some of these non-canonical 5′ additions (erasers) have been identified in these organisms and include NudC, DXO1 and CD38 [9, 10, 13–16]. In bacteria, these non-canonical initiating nucleotides have been found to be incorporated into RNA during the process of transcription initiation, thus suggesting that RNA polymerase acts as the writer for these types of epitranscriptome marks [5, 7]. However, whether this form of incorporation is also functioning in eukaryotes where canonical 7-methylguanosine (m^7G) cap addition requires non-polymerase complexes of proteins is completely unknown, but likely to be distinct from this process in bacteria. Furthermore, which proteins can recognize and bind to these non-canonical cap additions as reader proteins also remains completely unknown in all systems. Thus, the 5′ end epitranscriptome adds an emerging and intriguing layer of potential post-transcriptional regulation where many areas of inquiry are still left to be explored.

In this review, we summarize the most current findings and areas of research interest for internal and 5′ end cap epitranscriptome modifications in plants, focusing on the internal modifications m^6A, m^5C and m^1A, as well as on non-canonical 5′ end NAD^+ capping (Figure 1). We also highlight recent discoveries on the impact of these various covalent RNA additions on RNA structure, stability and translation during plant development and response to environmental stresses.

Overview of the key complexes involved in depositing, removing and interacting with RNA modifications in plants

Growing evidence suggests that post-transcriptional modifications are dynamic and are regulated by distinct protein complexes. The three key molecular complexes for a dynamic epitranscriptomic mark are the writer proteins that deposit the chemical modification onto specific RNA bases, the reader proteins that recognize the specific RNA modification once it has been deposited, and the eraser proteins that are able to remove the modification. Some of these key proteins have been identified and characterized in plant cells (Figure 1). For instance, for m^6A methylation, which is the most prevalent internal RNA modification in mRNAs, the writers, readers and erasers have been identified for plant transcriptomes [17–20] (Figure 1A). In Arabidopsis, the writer complex has been found to consist of METHYLTRANSFERASE A (MTA, orthologue of human METTL3), METHYLTRANSFERASE B (MTB, orthologue of human METTL14), FKBP INTERACTING PROTEIN 37 (FIP37, orthologue of human WTAP), VIRILIZER (VIR, orthologue of human VIRMA) and the E3 ubiquitin ligase AtHAKAI (orthologue of HAKAI) [21–23]. Additionally, two proteins from the ALKBH family, ALKBH9B and ALKBH10B, have been identified as m^6A demethylases that remove m^6A from mRNA molecules [24, 25]. The existence of these m^6A eraser proteins suggests that the m^6A modification is reversible in nature, but the exact mechanism and importance of this potential reversibility needs to be further explored. Finally, at least four of the 13 total plants, YT521-B homology (YTH)-domain containing proteins ECT2, ECT3, ECT4 and CPSF30 were found to bind directly to m^6A through their YTH domains, and act as reader proteins for this epitranscriptomic mark [26–29] (Figure 1A). Furthermore, a current study demonstrates interaction between MTA and both RNA polymerase II and TOUGH (TGH), a component in pri-miRNA processing, suggesting that components of the m^6A writer complex might also facilitate binding of protein partners to methylated transcripts [30]. The orthologs of these complexes are found in various other plant species, including rice, corn and wheat [18]. Thus, for the m^6A covalent modification, the identity of writers, erasers and readers is well characterized across the plant kingdom. However, much is still to be uncovered about the functionality of this system in the selection and recognition of specific m^6A modification sites throughout the plant transcriptome.

For two other methylation-based RNA covalent modifications, m^5C and m^1A, some of the machinery involved in their deposition, removal and recognition has also been identified and studied. In the case of m^5C, the Arabidopsis tRNA-specific methyltransferase 4B and rice NOP2/SUN (NSUN)-domain family member, OsNSUN2, have been identified as the mRNA m^5C methyltransferases [3, 31, 32] (Figure 1B). Interestingly, while ALYREF and YBX1 are known m^5C reader proteins in mammalian systems [33, 34], there are currently neither known readers nor eraser proteins of m^5C in plant systems. However, the existence of reader proteins for this mark in other eukaryotic systems strongly suggests their presence in plants, and their discovery and characterization will require future research inquiry. In regards to the m^1A modification, which is found in both mRNAs and tRNAs, two recent studies have found that the tRNA-specific methyltransferase AtTRM61/AtTRM6 in Arabidopsis and PhTRMT61A in petunia act as m^1A writers in these plant transcriptomes [4, 35] (Figure 1C). Thus, all emerging evidence points to multiple internal mRNA modifications that act as epitranscriptome regulatory systems and have a full complement of writer, eraser and reader proteins. However, our current understanding is extremely limited concerning the other methylation-based internal epitranscriptome systems and much is still left to be uncovered.

Until recently, it was thought that the 5′ end of eukaryotic mature RNAs is only marked by canonical 7-methylguanosine (m^7G) caps, which act to protect the capped mRNAs from degradation, while prokaryotic RNAs lack this cap structure. In 2009, chemical screens using liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses revealed that NAD^+ can be attached to *Escherichia coli* and *Streptomyces venezuelae* RNA at the 5′ terminus [5]. More recently, a method called NAD captureSeq [9] was used to identify 5′ end NAD^+ capped RNAs in bacteria transcriptome-wide, and additional studies revealed that the enzyme nudix phosphohydrolase protein NudC is able to remove this non-canonical 5′ cap moiety from these RNA molecules. In 2017, the NAD captureSeq approach was applied to the transcriptome of human cells and revealed that eukaryotic RNAs also contain this non-canonical 5′ end NAD^+ cap. Additionally, just like in bacterial systems, these NAD^+ capped RNAs were also ‘deNADded’, a process that is carried out by the DXO/Rai1 decapping enzymes in eukaryotic cells [10]. These findings make it clear that RNAs can also be capped by other non-canonical

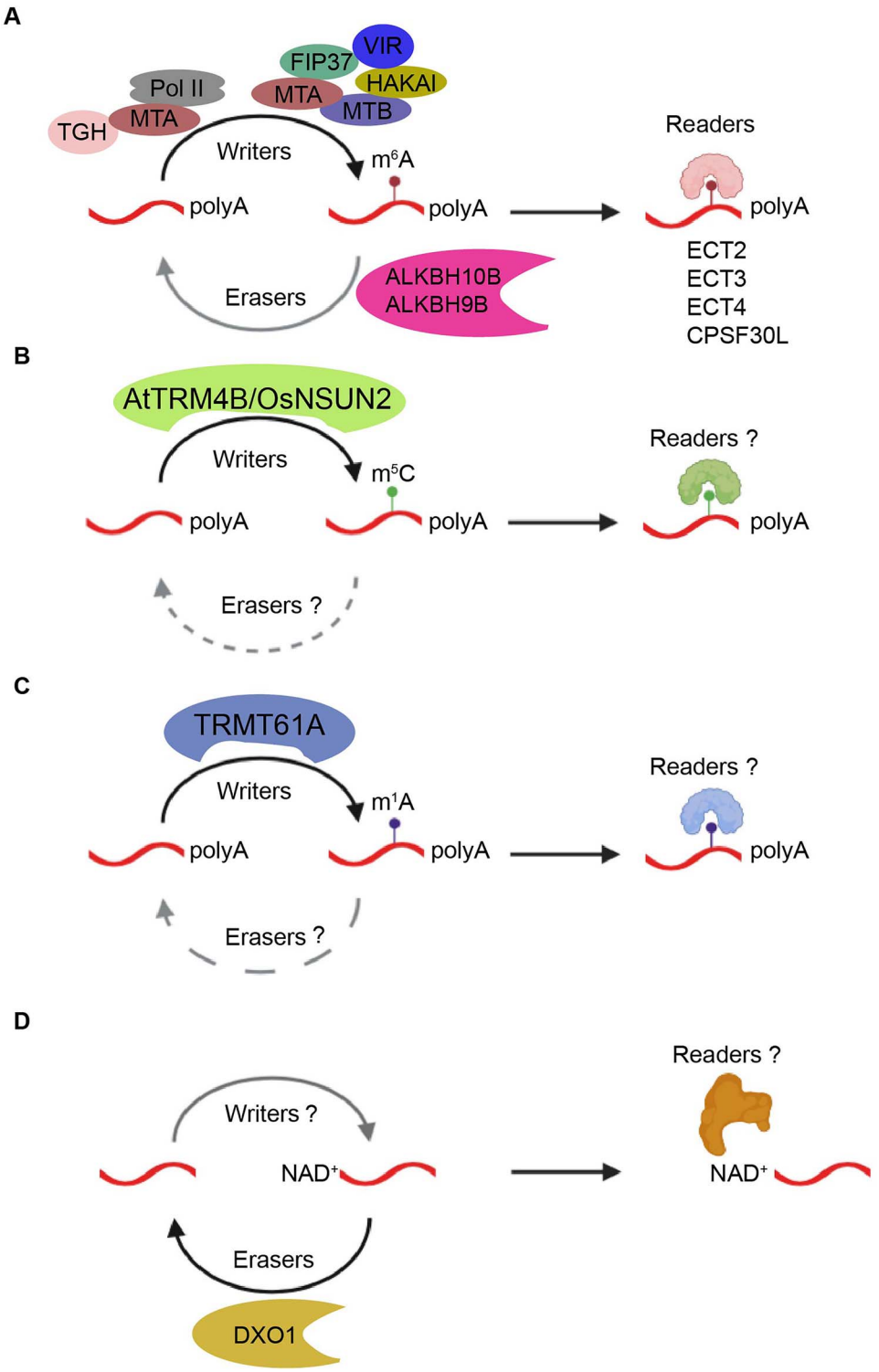

Figure 1. The known writers, erasers and readers of m^6A, m^5C, m^1A and 5′ end NAD^+ cap in plants. The nomenclature used for these protein names in the figure are those specific to plants.

metabolite molecules in diverse kingdoms of life from bacteria to humans [7, 8]. Additional studies in plant systems utilizing NAD captureSeq, NAD-seq and NAD tagSeq have identified the landscape of NAD^+ capping in the plant transcriptome [11, 12, 14] and also revealed that the plant ortholog of DXO1 also functions in NAD^+ decapping (deNADding) in Arabidopsis [13–15] (Figure 1D). Thus, the prevalence and importance of internal as well as 5′ end modifications on eukaryotic RNAs has been uncovered, revealing that multiple modification-based regulatory systems are functioning in eukaryotic transcriptomes.

Technological advances have revealed the extent and features of RNA modification landscapes in plant transcriptomes

The study of RNA modifications in eukaryotic transcriptomes has made huge strides in the past two decades largely due to technological advancements in the fields of molecular biology and biochemistry. For instance, the approaches of LC–MS/MS, two-dimensional thin layer chromatography and dot blots with antibodies specific to a modification of interest have been used to quantify the total amount of m^6A, m^5C/hm^5C and m^1A in plant transcriptomes [3, 4, 21, 22, 36, 37]. These measurements have led to the estimate that 0.45–1.5% of all adenosines present in mRNAs are methylated as m^6A in different tissues of Arabidopsis plants [37]. Surprisingly, similar or even slightly higher levels of 0.28–1.82% of all adenosines within mRNAs were detected as methylated to m^1A in the transcriptomes of different tissues from petunia [4]. While these two adenosine methylation modifications are highly prevalent in plant transcriptomes, only 0.010–0.036% of all cytosine bases in mRNAs are modified to the m^5C form [3]. Thus, these biochemical methods were helpful in detecting the overall levels of these epitranscriptome modifications within the transcriptomes of plants and opened the door to questions about the function and regulation of these RNA modifications.

More recently, methods marrying immunoprecipitation and high-throughput sequencing such as m^6A-seq/meRIP-seq [38, 39], m^5C RIP-seq [40] and m^1A-seq/m^1A-ID-seq [41, 42] have been developed to profile sites of m^6A, m^5C and m^1A transcriptome-wide, respectively, in yeast, plant and mammalian systems [3, 4, 37, 43] (Figure 2A). Additional improvements involving a UV crosslinking step to these RIP approaches were used to detect m^6A sites at single-base resolution in some eukaryotic transcriptomes. The resulting methods were the photocrosslinking-assisted m^6A sequencing strategy (PA-m^6A-seq) [44], m^6A individual-nucleotide-resolution crosslinking and immunoprecipitation (miCLIP) [45] and m^6A crosslinking immunoprecipitation (m^6A-CLIP) [46]. Unfortunately, these improved methods have not been applied yet to the studies of m^6A in plant transcriptomes and may prove tricky to develop given the need for a UV crosslinking step since this treatment is often not efficient in plant cells. Nonetheless, these studies have revealed the transcriptome-wide distribution of m^6A modifications. Specifically, the m^6A distribution exhibited an enrichment in mRNA 3′ untranslated regions and near stop codons in Arabidopsis across multiple tissues [22, 43], consistent with the reported distribution in yeast, mammals and other plants such as rice and maize [38, 47–52]. Furthermore, an additional peak around the translation start codon was also observed in specific tissues [37, 53], which is similar to the overall m^6A distribution uncovered in Drosophila [54]. Most of the plant studies detected a conserved motif sequence 'DRACH' or 'RRACH' (where A is the modified m^6A site, D = A/G/U, R = A/G and H = A/C/U), identical to the finding in other species [22, 37, 38, 45]. Similar approaches have been used to map m^1A globally and have found that this mark is specifically enriched around mRNA start codons in plant transcriptomes [4]. Overall, these global m^6A and m^1A mapping techniques have uncovered the position and sequence context in which these marks occur in eukaryotic transcriptomes.

To study the m^5C modification, bisulfite sequencing has been used to map the sites of this modification transcriptome-wide at single nucleotide resolution in both animal and plant transcriptomes [31, 47]. Interestingly, these studies have revealed a m^5C distribution pattern of two peaks of this mark near the start and around the stop codon in Arabidopsis [3], but only a dominant m^5C peak around the start codon in the rice transcriptome [32, 55]. Although the overall distribution of m^5C has been mapped, there is still much to learn about its preferred sequence contexts and site selection in various plant transcriptomes.

While all the methods described above rely on antibody selection of the modified RNA, an antibody-independent method that detects RNA modifications through the use of RNA restriction enzymes has recently been developed. Specifically, recent studies have uncovered a novel m^6A sequencing technique that utilizes an m^6A sensitive RNA endonuclease enzyme MazF that recognizes the ACA motif and provides nucleotide resolution information on m^6A location. This method cleaves ACA motifs that do not contain m^6A but spares the ones that do. By comparing libraries of wild-type and mutants for the m^6A eraser protein FTO, it was validated that one can identify m^6A position at nucleotide resolution. Although this method has only been published in mammals [56], with the identification of the major m^6A writer and eraser proteins in various plant species, this method can be used to get a nucleotide resolution map of m^6A in an antibody-independent manner in these various plant transcriptomes (Figure 2B) and is likely to be utilized in future m^6A studies in plants.

Very recent advances in available high-throughput sequencing technology are now driving the field of RNA modification detection away from antibody-based detection such as meRIP-seq, which gives poor resolution on the positional information of m^6A within transcripts because these RIP-seq techniques utilize high-throughput sequencing techniques that produce short reads of amplified cDNA derived from template RNAs. In contrast, nanopore sequencing is an emerging sequencing technique that passes intact nascent RNA molecules through a protein pore that sits in a membrane across an electrical gradient that can identify the bases of the RNA as they pass through the pore in real time. The sensitivity of the electrical signal produced by the bases is key in helping identify the canonical A, U, G and C bases as well as the presence of modified bases such as m^6A [57, 58] (Figure 2C). Using nanopore direct RNA sequencing (DRS) to sequence *Arabidopsis thaliana* wild-type plants and comparing those results to sequencing data for the loss of function mutant for the m^6A writer protein VIR (*vir* mutants), it was found that the signature difference in signals associated with the presence of m^6A correlated very highly with m^6A sites recognized by antibody-based meRIP-seq approaches [59]. Overall, this study significantly improves m^6A site identification and resolution over traditional antibody-based approaches and demonstrates the future power of studies focused on using these sequencing technologies to detect and study RNA modifications on a global scale. This new generation of nanopore sequencing has been successful in reducing the historically high error rate of this technology by using better flowcells and capture technologies as well as improved base calling algorithms, which has made it useful in RNA modification detection. However, while Nanopore DRS can precisely detect internal RNA modifications (Figure 2C), it cannot be used to directly detect the signal of 5′ cap modifications since it is unable to sequence the last dozen nucleotides as the RNA end makes its way through the pore. Overall, it is likely that using DRS technologies for internal modification detection and characterization will be the preferred method for future studies of this class of epitranscriptome marks.

In addition to the above described methods, there are other techniques that utilize chemical reactions to further modify RNA modifications, so that they can be detected by stalling of reverse

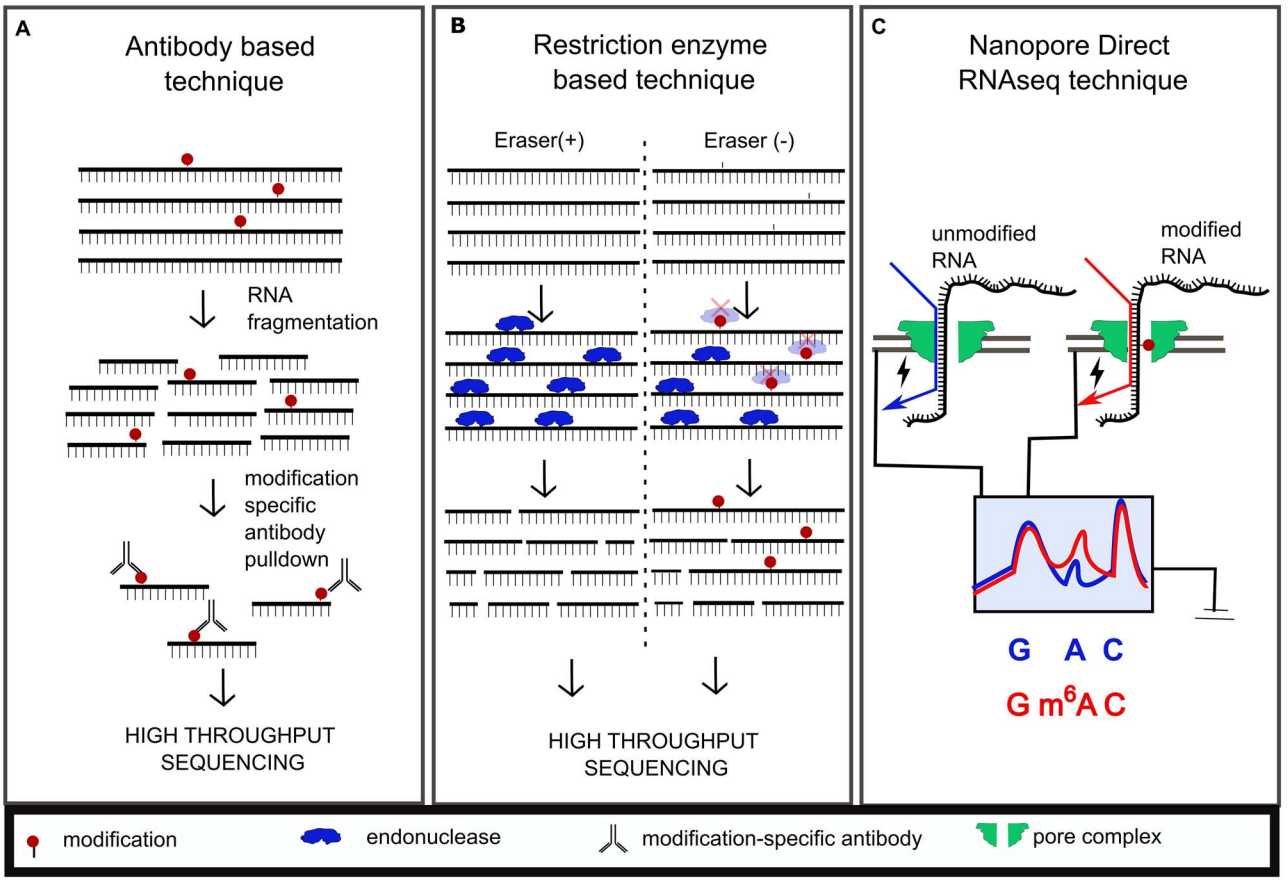

Figure 2. Overview of transcriptome-wide profiling of RNA internal modifications. (A) Antibody-based methods to profile RNA modification sites. (B) Antibody-independent profiling of RNA modifications using RNA endonuclease enzymes. (C) Nanopore DRS for detecting modified nucleotides.

transcriptase (RT) or be affinity purified from total RNA populations using click chemistry, the latter of which has been recently used in detecting and characterizing other less studied modifications. Here, we summarize three approaches for detecting NAD^+ capped RNAs based on marrying click chemistry to high-throughput sequencing [9, 60, 61] (Figure 3). To accomplish this goal, the NAD^+ cap is first modified with 4-pentyn-1-ol during ADP-ribosylcyclase (ADPRC) treatment and then labeled with biotin-azide for the NAD captureSeq and NAD-seq approaches or treated with a specific tagRNA-azide (25 nucleotides in length) during CuAAC treatment in the process of making NAD tagSeq libraries. For NAD captureSeq, the biotin-RNAs (NAD^+ capped transcripts) are subsequently captured using streptavidin beads and then directly ligated with adaptors, followed by reverse transcription all done on beads. The resulting cDNAs are released from the streptavidin sepharose beads by alkaline digestion, and these molecules are tailed with cytosine using TdT and CTP. Finally, a double-stranded DNA adaptor with a reverse complementary 2-nt G-overhang is ligated, and these ligated cDNA samples are ultimately PCR amplified for the final preparation of the sequencing libraries [9, 60]. For NAD-seq, the biotin-RNAs (NAD^+ capped transcripts) captured on streptavidin beads are eluted from the beads followed by RNA fragmentation, T4 Polynucleotide Kinase treatment and adaptor ligation. These adapter-ligated RNAs are then turned into cDNA using RT and amplified by PCR for final sequencing library preparation [14]. Finally, for the NAD tagSeq approach, total RNA samples are tailed with poly(A) using poly(A) polymerase and purified with oligo dT beads. The tagRNA-azide molecules (NAD^+ capped transcripts) are affinity purified. These affinity-purified tagRNA-azide molecules are prepared for Nanopore sequencing [12, 61]. The combination of these three methods has been used to identify NAD^+ capped RNAs transcriptome-wide in plants, which includes mostly NAD^+ capped mRNAs but also some miRNA precursors, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and long non-coding RNAs (lncRNAs) [11, 12, 14].

In addition to these experimental approaches, significant developments in computational processes and algorithms have given rise to various computational approaches to detect RNA modifications. In fact, a number of techniques that can identify and categorize multiple modification types in a single analysis have been developed and used for studying these important epitranscriptome systems in plants. For example, a computational method termed high-throughput annotation of modified ribonucleotides (HAMR) has been developed that can be used to identify and categorize the ~45 distinct types of RNA modifications that affect the Watson:Crick base pairing edge of the modified ribonucleotides [62]. To do this, the algorithm analyzes any existing or new RNA-seq dataset for bases where RT misincorporations occur in 3 or 4 nucleotide substitution patterns but with high-quality sequencing scores. Thus, these multi-nucleotide substitution patterns are not single nucleotide polymorphisms or RNA editing but are the byproduct of RT misincorporation events caused by modification-induced errors at these nucleotide positions. The HAMR algorithm has been used to predict the existence of 3-methylcytocine (m^3C) and

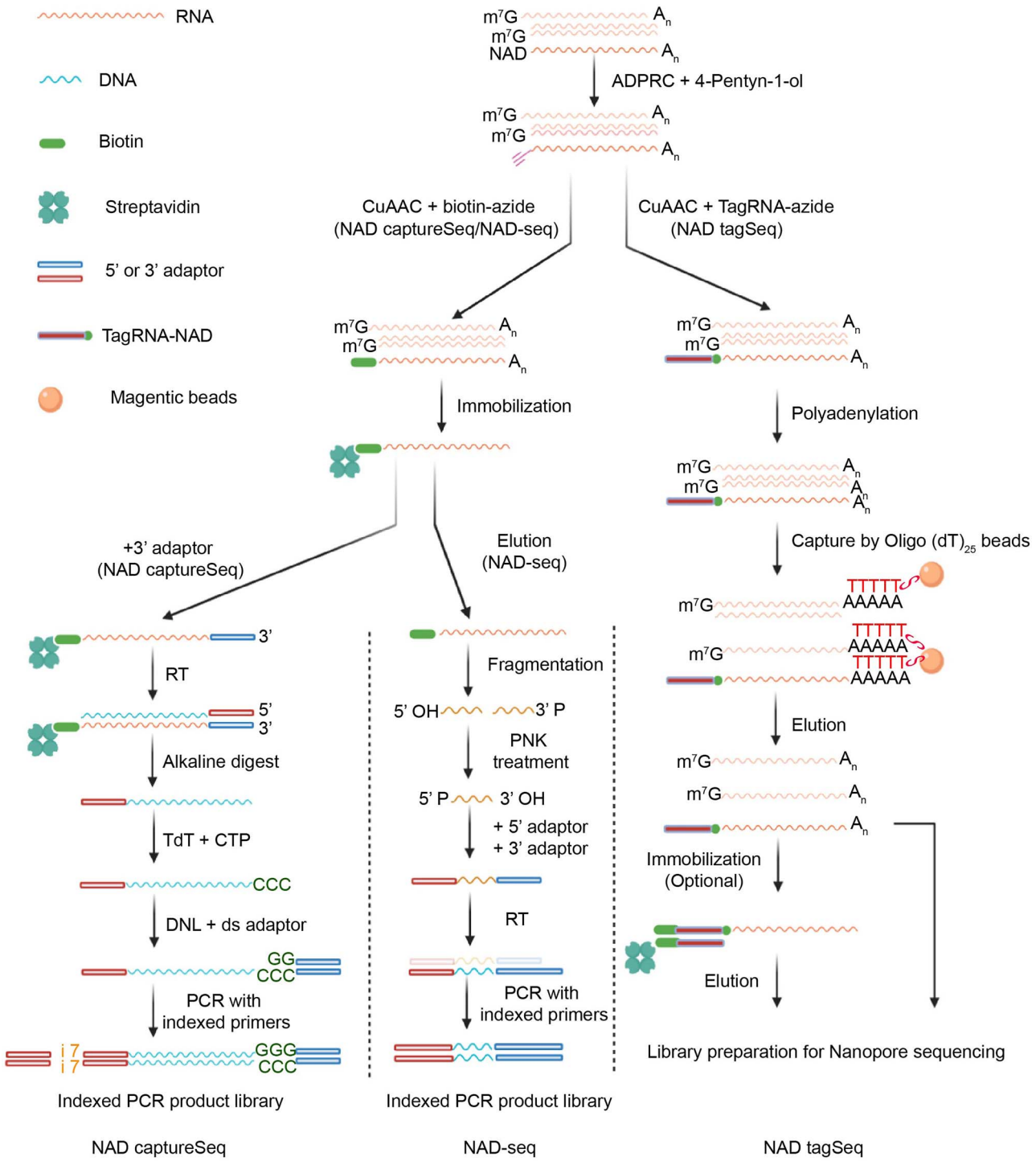

Figure 3. Descriptions of click chemistry-based methods for identifying NAD^+ capped RNAs including NAD captureSeq, NAD tagSeq and NAD-seq.

1-methylguanosine (m^1G) modification sites in plant mRNAs [62]. Additionally, new machine learning-based techniques have been published that can predict specific covalent RNA modification sites in plant transcriptomes. These R toolkits called plant epitranscriptome analysis (PEA) and PEA-m5C are shown to accurately predict m^6A and m^5C, respectively, in *A. thaliana* with around 70–80% accuracy, which is significantly higher than previously published prediction software [63, 64]. These algorithms use known modification data and the features around the site of modification in existing datasets as training data, which allows them to then predict the presence of these modifications on new RNA transcriptome data. PEA-m5C was able to predict that m^5C accumulates to its highest levels 4 nucleotides downstream of mRNA start codons, something that was not generally known. The development of the variety of technical and computational methods is expanding both the breadth and depth of epitranscriptomic information in RNA and is helping uncover some of the developmental and physiological functions

associated with these RNA modifications. Thus, these types of high-throughput computational approaches will be useful in future studies aimed at reanalyzing the wealth of RNA-seq data that we have for multiple eukaryotic organisms, especially for covering numerous plant transcriptomes.

Plant development and stress responses regulated by RNA modification-mediated post-transcriptional regulation

To determine the effects of epitranscriptome-mediated regulatory systems on plant biology, genetic studies using plant mutants containing lesions in the genes encoding RNA modification machinery have provided important new insights into the potential roles of covalent RNA modifications in regulating fundamental plant developmental processes and biotic and abiotic stress responses (Figure 4). For example, the disruption of MTA function, the key m^{6}A methyltransferase in Arabidopsis, leads to embryo lethality [21], which is also true when any of the other core components of the m^{6}A writer complex MTB, FIP37 and VIR are also missing through genetic ablation [65, 66]. These findings indict the critical role of the m^{6}A modification in key processes of embryonic development. Even when bypassing the embryonic lethality of *mta* loss of function mutants by expressing MTA under the control of the embryo-specific *ABI3* promoter (*mta ABI3:MTA* plants), the resulting transgenic plants demonstrate numerous additional developmental defects, including dwarfing, late flowering and numerous abnormalities in floral organ number, size and identity [67]. Additionally, the study of plants with a viable hylomorphic allele of *FIP37* indicated that the m^{6}A modification regulatory system functions in the control of shoot stem cell fate, since these mutants displayed massive overproliferation of the shoot apical meristem [22]. It was also found that plants containing the weak allele *vir-1*, which contains a hypomorphic mutation in the *VIR1* gene, exhibited defective root cap formation, lateral root development and aberrant cotyledon development [23]. Furthermore, *MTB* RNAi lines exhibit defective vascular development in the root [23]. In total, these results reveal that the deposition of m^{6}A on target RNAs is required for proper post-embryonic development in both the plant shoot and root.

It is not only the m^{6}A writer complex that is required for proper plant development. It has also recently been found that the disruption of ALKBH10B, the demethylase that oxidatively reverses m^{6}A methylation (m^{6}A eraser), results in mutant *alkbh10b* plants with delays in flowering and repressed vegetative growth [24], indicating that the removal of m^{6}A is also vital for plant development. Relatedly, two other studies demonstrated that the YTH domain-containing m^{6}A reader protein ECT2 is required for normal trichome branching [27, 28]. An additional project focused on m^{6}A reader proteins found that three closely related ECT proteins, ECT2, ECT3 and ECT4, are required for correct timing of leaf formation and normal leaf morphology [26]. Interestingly, the trichome branching phenotype was also displayed by *mta* conditional knockout plants as well as plants overexpressing *FIP37*, revealing that m^{6}A-mediated regulatory module, including deposition and recognition of this mark, is involved in proper trichome development in plants [66, 67].

In addition to the impact of m^{6}A-mediated regulation on plant development, current studies have also revealed that this epitranscriptome mark has critical functions in plant response to abiotic and biotic stress (Figure 4). Specifically, it has been demonstrated that the lack of the m^{6}A eraser protein ALKBH9B (*alkbh9b* mutant plants) reduces plant infectivity by Alfalfa Mosaic Virus (AMV). In fact, this study found that *alkbh9b* mutant plants displayed significantly reduced levels of both virus RNAs and viral coat proteins as compared with wild-type plants. This is likely due to the lack of m^{6}A removal from the AMV genome by AtALKBH9B, and thus, the AMV genome likely maintains this mark which might protect it from being recognized as foreign RNA molecules [25]. Additionally, it was recently demonstrated that m^{6}A-mediated post-transcriptional regulation is also necessary for proper salt stress response in plants. Specifically, it was found that this epitranscriptome mark is added to transcripts encoding salt stress response regulators to stabilize and increase protein output from these RNAs to allow proper response to this important abiotic stress [43, 68]. Thus, m^{6}A is not only a regulator of numerous developmental processes but also of numerous abiotic and biotic stress responses in plants.

There is also emerging evidence that other epitranscriptome marks have important functions in development and stress response (Figure 4). For instance, Arabidopsis *trm4b* mutant plants that lack the m^{5}C writer protein TRM4B display shorter primary roots due to reduced cell division in the root apical meristem [3, 31]. Additionally, these *trm4b* mutant plants also demonstrate increased sensitivity to oxidative stress [31]. Relatedly, when the rice RNA m^{5}C methyltransferase OsNSUN2 is removed, the resulting *osnsun2* mutant plants are significantly more vulnerable to heat stress [32]. Additionally, recent findings determined that the removal of the Arabidopsis m^{1}A writer proteins AtTRM61 or AtTRM6 resulted in mutant plants displaying embryo arrest and seed abortion [35]. Relatedly, the depletion of the petunia m^{1}A writer PhTRMT61A resulted in mutant plants that displayed abnormal leaf development [4]. Overall, it is becoming increasingly clear that internal mRNA modifications direct important post-transcriptional regulatory programs to affect numerous plant developmental processes throughout their life-cycles as well as both biotic and abiotic stress responses.

It is not just internal mRNA modifications that have been found to have profound effects on plant development and stress response. In fact, 5' NAD$^+$ capping also has functions in plant development and stress response (Figure 4). For instance, Arabidopsis *dxo1* mutant plants that lack the NAD$^+$ decapping enzyme DXO1 have been found to exhibit multiple growth defects in both vegetable and reproductive developmental stages, including dwarfing, light green coloration signaling lower levels of total chlorophyll, and decrease fertility giving smaller seed sets [13–15]. It was originally thought this might be a consequence of increased small RNA processing from protein-coding mRNAs that accumulate in the *dxo1* mutant plants, but inhibiting processing of these small regulatory RNAs in this mutant background does not relieve these developmental defects [13, 14]. Thus, the increased smRNA levels from protein-coding mRNAs do not contribute to the developmental defects of *dxo1* mutant plants. Interestingly, the dwarfism phenotype of these mutant plants can partially restored by growing them at moderately high temperatures (e.g. 28°C), as well as crossing them with mutant plants lacking regulators of immunity in Arabidopsis such as NPR1 and EDS1. These results indicate that DXO1 also contributes to repressing autoimmunity, as well as developmental processes [15]. Finally, it was recently found that *dxo1* mutants exhibit strong insensitivity to seed germination inhibition mediated by the plant stress hormone abscisic acid (ABA) as compared with wide-type plants, revealing that DXO1 is also required for the proper response to the

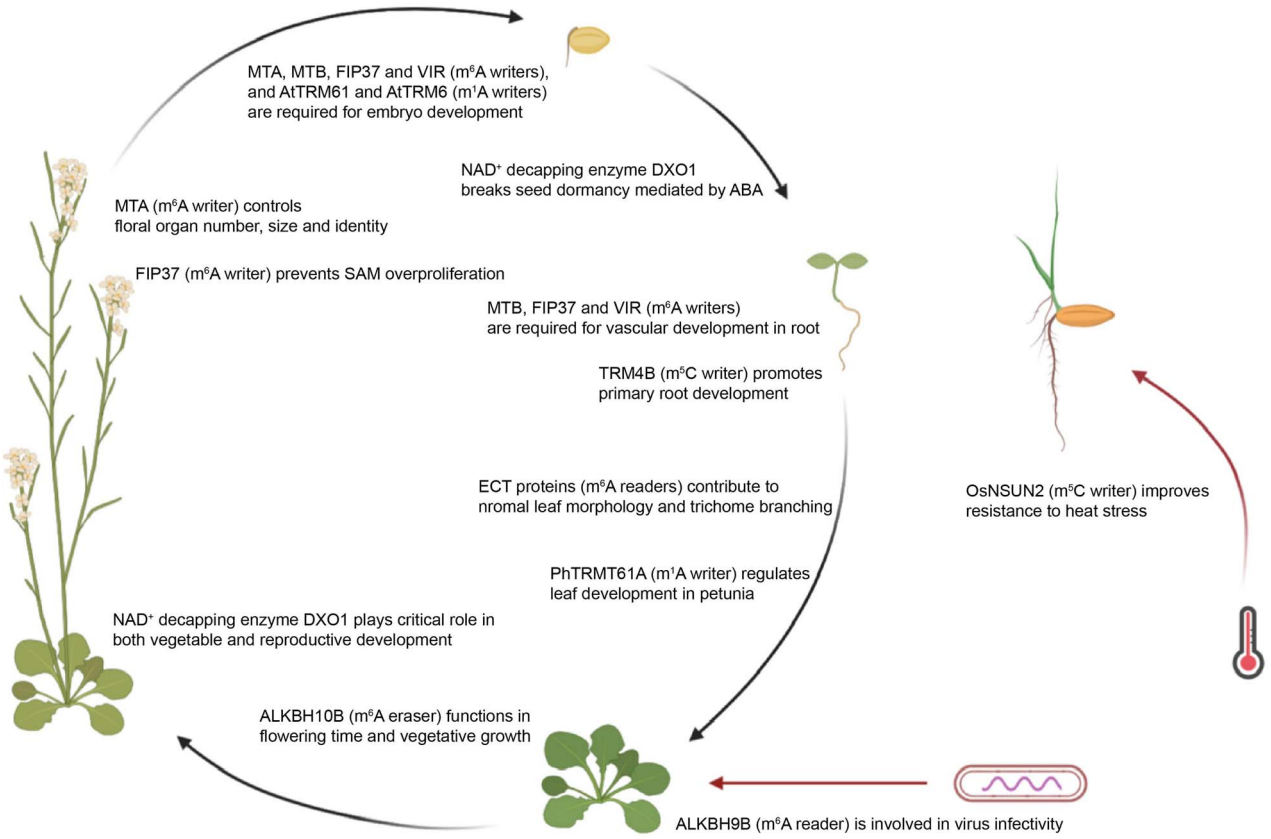

Figure 4. Plant development and stress responses regulated by various RNA modifications.

stress response hormone ABA [14]. Thus, it is likely that 5′ end modifications also have important roles in both plant development and stress responses like has been observed for internal medication-mediated regulatory systems.

It is notable that, in general, the phenotypes of plants containing copies of the writer and eraser proteins for the various RNA modifications with catalytic mutation have not been investigated in most cases. In fact, as noted above, most studies have been done on mutant plants that have completely lost the function of these important proteins for RNA modification addition and removal. Currently, several genome-editing approaches based on CRISPR-Cas systems, such as base editors and prime editing, have been developed for precise genetic modification and have been applied to plants, which will provide more effective tools for studying the molecular and physiological phenotypes caused by point mutations in the catalytic domains of these proteins [69]. The production and future study of these catalytic mutant plant lines will be extremely important for determining the direct effect of RNA modifications on plant development and stress responses in the future.

The molecular impacts of RNA modifications on post-transcriptional regulation

The combination of transcriptome-wide profiling approaches for these epitranscriptomic modifications with other global approaches such as protein interaction profile sequencing (PIP-seq), RNA-seq, ribosome profiling and approaches to measure RNA degradation intermediates [e.g. genome-wide mapping of uncapped and cleaved transcripts (GMUCT)] has uncovered the effects of RNA modifications on RNA secondary structure, pri-miRNA processing, RNA translation, transcription termination, RNA transport and RNA degradation in plants [5, 30, 32, 43, 55, 68] (Figure 5). For instance, a number of recent studies have demonstrated that the m^{6}A modification can both stabilize and destabilize specific modified transcripts in sequence, position and developmental context-specific manner [22, 27, 43, 68]. In fact, two related studies using the combination of m^{6}A-seq, RNA-seq and GMUCT revealed that m^{6}A sites protect the modified transcripts from ribonucleolytic cleavage thereby stabilizing these RNAs. The first study then went on to demonstrate that this mechanism of inhibit ribonucleolytic cleavage was important for stabilizing transcripts encoding salt response regulators when plants were subjected to long-term salt exposure. The follow-up study took these initial findings further and added additional PIP-seq data to reveal that salt-specific m^{6}A deposition resulted in an associated loss of RNA secondary structure which ultimately resulted in increased in mRNA stability and protein output from mRNAs encoding important salt and water stress regulators [43, 68]. Another recent study revealed that m^{6}A deposition on primary miRNA transcripts was necessary to promote miRNA processing through proper formation of RNA secondary structure as well as a direct protein–protein interaction between MTA, the major m^{6}A methyltransferase and TGH, a miRNA biogenesis factor [30]. Interestingly, the m^{6}A reader protein CPSF30L has also recently been shown to function in proper transcriptional termination [29], and this function requires the presence of m^{6}A on the transcripts that are improperly terminated in the absence of this m^{6}A binding protein. Finally, this m^{6}A modification is not

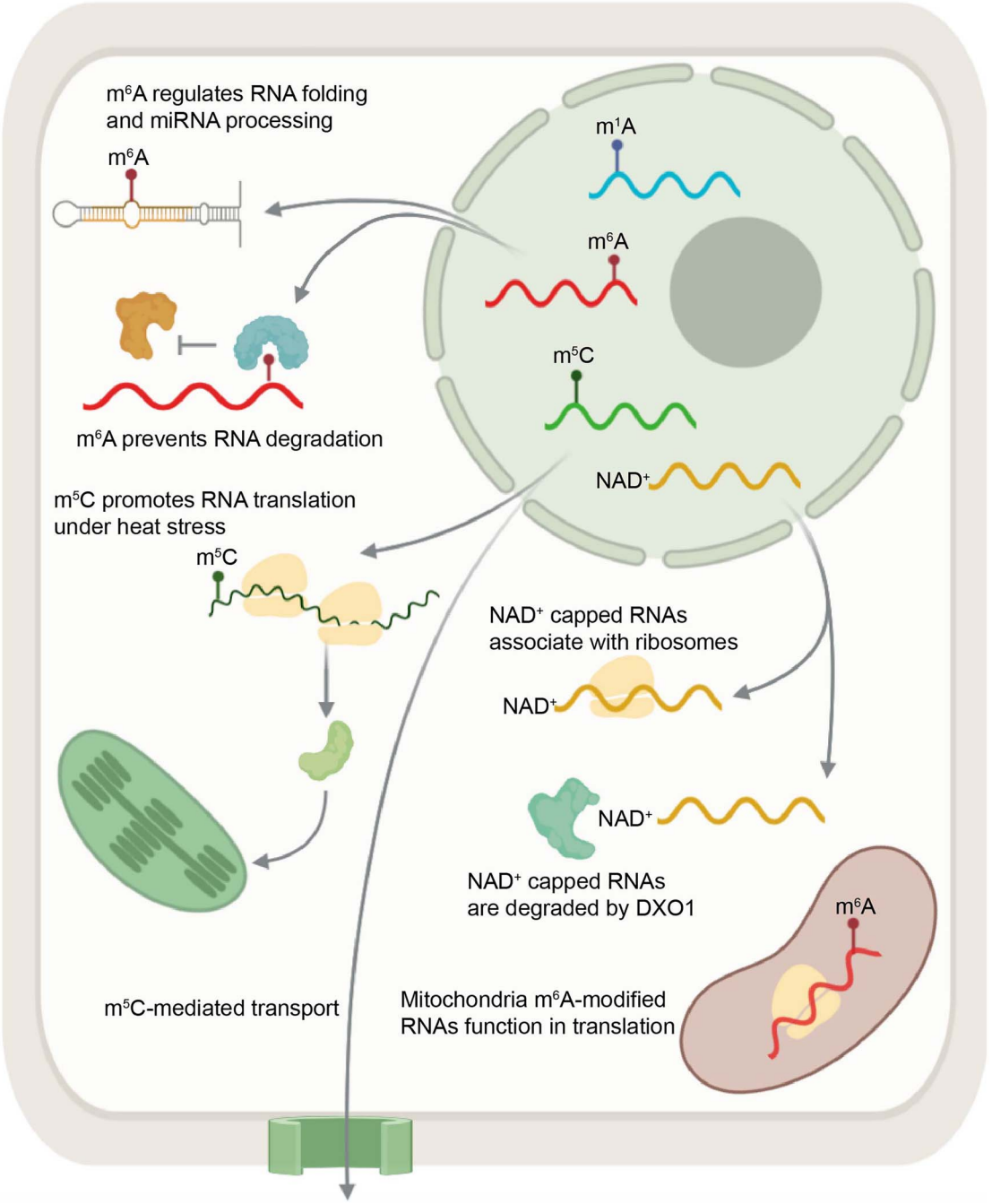

Figure 5. The impact of RNA modifications on post-transcriptional regulatory processes.

just present on nuclear mRNAs but has recently been shown to also be present in Arabidopsis and cauliflower mitochondrial RNAs. Intriguingly, the presence of the m^6A modification on mitochondria transcripts seems to positively modulate their translatability [70]. Thus, m^6A has multiple mechanisms by which it exerts various post-transcriptional regulatory effects on the transcripts containing this epitranscriptome mark, and it seems that there is still much to be uncovered by this powerful regulatory RNA feature.

The other internal modification whose mechanisms of action have been interrogated in plants is m^5C. For example, recent studies have found that m^5C-marked transcripts tend to be more stable and be more highly translated into proteins (Figure 5). In fact, a recent report found that m^5C methylation promoted the translation of heat-induced mRNAs when plants were subjected to high-temperature treatments, and was important for heat resistance in wide-type rice plants [32]. Another study revealed that mRNAs containing m^5C methylation were more stable as compared with non-methylated RNAs, and these modified RNAs tended to be enriched in the fraction of graft-mobile transcripts that move from shoot to root. These findings suggest that m^5C is a stabilizing mark functions in mRNA transport throughout the plant body [55]. Thus, internal RNA modifications have been found to affect every aspect of the RNA lifecycle.

Similar to internal RNA modifications, 5′ end NAD^+ capping has been found to affect the multiple aspects of RNA metabolism, with the most clear effect of this modification being on RNA stability (Figure 5). In fact, a recent study demonstrated that NAD^+ capped transcripts tend to be significantly more unstable as compared with those transcripts that do not contain this modification and this destabilizing effect is necessary to allow proper plant response to the stress hormone ABA [14]. These findings in plants are consistent with the reported destabilizing function of this non-canonical 5′ end modification in the human transcriptome [10]. Interestingly, two additional studies have found that some NAD^+ capped transcripts are associated with polysomes suggesting that this modification could also play some role in regulating protein translation [11, 12]. Overall, it is clear that NAD^+ capping affects numerous molecular aspects of RNA metabolism. However, this is still much to learn about this non-canonical 5′ end addition. For instance, we still do not know how NAD^+ caps are added to mRNAs in plant transcriptomes. Furthermore, we have no idea how and by which proteins this modification is bound in eukaryotic transcriptomes. These are just a couple of questions that require further investigation in plants and in eukaryotes more general.

Conclusions

All recent evidence supports the hypothesis that epitranscriptomic marks provide a novel layer of post-transcriptional regulation on the ultimate fate of RNA molecules. Genetic studies focused on characterizing the writers, erasers and readers of modifications such as m^6A, m^5C, m^1A and 5′ end NAD^+ capping have revealed insights into the roles of RNA modifications in plant embryonic, vegetative and reproductive development, as well as response to external stimuli such as viruses and aberrant temperatures. Furthermore, the use of multiomics approaches has demonstrated the impact of the epitranscriptomic code on RNA stability, translation, secondary structure and transport in plant cells. However, our understanding of the epitranscriptome and its unlimited potential for post-transcriptionally regulating plant transcriptomes is still in its infancy and requires concerted efforts to fully categorize the epitranscriptome and its multiple regulatory effects, especially in plants. This is because determining the marks and their molecular mechanisms behind the dynamic regulation of the transcriptome during different developmental stages and in response to various environmental stimuli and perturbations is likely to be powerful tools for use in improving plant productivity and resistance to both biotic and abiotic stresses during this era of agricultural response to global climate change.

Key points

- The phenotypes of plants containing mutations in the genes encoding known writers, erasers and readers of specific RNA modifications reveal the roles of these epitranscriptomic marks in plant development and stress responses.
- Technological advances have allowed the transcriptome-wide identification of m^6A, m^5C, m^1A and NAD^+ cap modifications.
- Multi-omics data reveal the molecular effects of epitranscriptomic marks on RNA secondary structure, transport, translation, functionality and stability.

Abbreviations

m^6A, N^6-methyladenosine, m^5C, 5-methylcytosine, m^1A, N^1-methyladenosie, NAD^+, nicotinamide adenine dinucleotide, mRNAs, messenger RNAs, m^7G, N^7-methylguanosine, LC–MS/MS, liquid chromatography–tandem mass spectrometry, DRS, direct RNA sequencing, RT, reverse transcriptase, HAMR, high-throughput annotation of modified ribonucleotides, PIP-seq, protein interaction profile sequencing, GMUCT, genome-wide mapping of uncapped and cleaved transcripts, ABA, abscisic acid.

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