Occurrence and Functions of m^{6}A and Other Covalent Modifications in Plant mRNA[1][OPEN]

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Posttranscriptional control of gene expression is indispensable for the execution of developmental programs and environmental adaptation. Among the many cellular mechanisms that regulate mRNA fate, covalent nucleotide modification has emerged as a major way of controlling the processing, localization, stability, and translatability of mRNAs. This powerful mechanism is conserved across eukaryotes and controls the cellular events that lead to development and growth. As in other eukaryotes, N^6-methylation of adenosine is the most abundant and best studied mRNA modification in flowering plants. It is essential for embryonic and postembryonic plant development and it affects growth rate and stress responses, including susceptibility to plant RNA viruses. Although the mRNA modification field is young, the intense interest triggered by its involvement in stem cell differentiation and cancer has led to rapid advances in understanding how mRNA modifications control gene expression in mammalian systems. An equivalent effort from plant molecular biologists has been lagging behind, but recent work in Arabidopsis (*Arabidopsis thaliana*) and other plant species is starting to give insights into how this essential layer of posttranscriptional regulation works in plants, and both similarities and differences with other eukaryotes are emerging. In this Update, we summarize, connect, and evaluate the experimental work that supports our current knowledge of the biochemistry, molecular mechanisms, and biological functions of mRNA modifications in plants. We devote particular attention to N^6-methylation of adenosine and attempt to place the knowledge gained from plant studies within the context of a more general framework derived from studies in other eukaryotes.

Control of gene expression is of paramount importance in biology, and a variety of molecular mechanisms working at the DNA, pre-mRNA, mRNA, and protein levels have evolved to ensure that appropriate levels of activity of gene products operate at any given time. It has long been recognized that chemical modification of the transcription template, either of the DNA itself or of associated histone proteins, is key to the regulation of gene expression. Thanks to rapid progress in the last decade, it has now become clear that chemical modification of mRNA also plays crucial roles in the control of endogenous gene activity and in shaping the outcome of host-virus interactions. This Update provides a brief overview of mRNA modifications identified in plants and their viruses, and an account of what is known of their functional relevance and the molecular mechanisms underlying their functions. We also include a discussion of emerging controversies and outstanding questions in this field.

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SEEING MRNA MODIFICATIONS: HIGH-THROUGHPUT SEQUENCING DELIVERS THE BREAKTHROUGH

Although chemically modified nucleotides have been known to exist in RNA, even in mRNA, since more than 40 years (Holley et al., 1965; Desrosiers et al., 1974; Perry and Kelley, 1974; Boccaletto et al., 2018), the mRNA modification field has only taken off with the recent introduction of high-throughput sequencing-based methods to map modified nucleotides in specific mRNAs transcriptome wide (see Box 1 for a historical context of the development of the field). Three main categories of such methods enjoy widespread use (see Linder and Jaffrey [2019] for a detailed review focused on methods). (1) Antibodies specific to a modification are used to immunoprecipitate fragmented mRNA, and RNA sequencing (RNA-seq) is applied to input and immunoprecipitated RNA fractions. Significant enrichment then identifies mRNA intervals likely to contain a modified nucleotide (Dominissini et al., 2012; Meyer et al., 2012; Edelheit et al., 2013; Schwartz et al., 2013; Luo et al., 2014; Delatte et al., 2016; Dominissini et al., 2016; Li et al., 2016; Cui et al., 2017). (2) Modified nucleotides are specifically derivatized using unique reactivity with an appropriate compound, and the presence of the derived nucleotide is read either as a mutation signature or as a stop upon reverse transcription (Squires et al., 2012; Carlile et al., 2014; Lovejoy et al., 2014; Schwartz et al., 2014a; Li et al., 2015; David et al., 2017; Enroth et al., 2019; Sun et al., 2019; Zhang

ADVANCES

- Plant mRNAs contain post-transcriptionally modified nucleotides. Each modification occurs in a specific set of mRNAs, and may be found in specific sequence contexts.
- *N6*-methyladenosine (m^6A) is the major modified nucleotide in plant mRNAs, and is crucial for embryonic and post-embryonic phases of plant growth and development.
- m^6A is found in the 3′UTRs of thousands of plant mRNAs, and it is deposited by a deeply conserved, nuclear methyltransferase complex whose activity is coupled to transcription, at least in animals.
- Gene regulatory functions of m^6A in plants involve specialized RNA binding proteins with an m^6A-recognizing YTH domain. Three of these proteins (ECT2/ECT3/ECT4) exhibit apparent genetic redundancy in m^6A - dependent control of leaf growth and development.
- 5-methylcytidine (m^5C) facilitates phloem transport of mRNA in plants.

et al., 2019b). The cross-linking immunoprecipitation (CLIP) variant miCLIP constitutes an important special case in this category: a modification-specific antibody is UV cross-linked to purified RNA, allowing mutation-dependent mapping of cross-link sites (Ke et al., 2015; Linder et al., 2015) as in other CLIP techniques (Ule et al., 2018). (3) Differential RNA cleavage at modified versus unmodified nucleotides is used to identify modified sites based on the percentage of sequence read-throughs and stops at each nucleotide (Birkedal et al., 2015; Garcia-Campos et al., 2019; Zhang et al., 2019d). In addition to those three types of methods, direct RNA sequencing by Oxford Nanopore Technology (Garalde et al., 2018) has recently been used successfully to map N^6-methyladenosine (m^6A) sites based on systematic base-calling errors at modified sites (Liu et al., 2019; Parker et al., 2019; Yang et al., 2019). In general, methods in categories 2 and 3 produce modification maps with nucleotide resolution, while methods in category 1 produce intervals containing a modification. It remains an active field of research to develop methods to detect modifications for two reasons. First, new methods for the detection of known RNA modifications are in high demand, because no single method developed thus far perfectly combines the desired sensitivity and specificity: high-confidence modification sites should ideally rely on results obtained by two orthogonal methods (Zhang et al., 2019b). Indeed, antibody-based methods in categories 1 and 2 have been used since 2012 to detect m^6A sites (Dominissini et al., 2012; Meyer et al., 2012; Schwartz et al., 2013; Luo et al., 2014; Ke et al., 2015; Linder et al., 2015), but the recent development of RNase cleavage-dependent m^6A mapping (Garcia-Campos et al., 2019; Zhang et al., 2019d) shows that the limited sensitivity of antibody-based methods led to substantial underestimation of the number of m^6A sites in the transcriptome. Second, it is possible that some mRNA modifications await discovery, thus necessitating the development of methods for their detection.

MRNA MODIFICATIONS IDENTIFIED IN PLANTS AND OTHER EUKARYOTES

In plants, there are now reports on mapping of three distinct covalent nucleotide modifications in the bodies of mRNA: m^6A (Li et al., 2014c; Luo et al., 2014; Wan et al., 2015; Shen et al., 2016; Duan et al., 2017; Anderson et al., 2018; Miao et al., 2019; Parker et al., 2019), 5-methylcytidine (m^5C; Cui et al., 2017; David et al., 2017; Yang et al., 2019), and pseudouridine (Ψ; Sun et al., 2019). Other types of mRNA modifications, including C-U editing of mitochondrial and plastidial mRNAs (Shikanai, 2006), alternative 5′-caps (Kiledjian, 2018; Wang et al., 2019), and untemplated addition of nucleotides to mRNA 3′-ends (De Almeida et al., 2018), will not be covered here. The discovery of mRNA modifications in plants is lagging behind that in animal cells, in which at least six additional modifications have been mapped and, in some cases, also functionally analyzed (Boccaletto et al., 2018). These modified nucleotides include inosine (I; Bass and Weintraub, 1988; Shevchenko and Morris, 2018), internal (as opposed to cap) 7-methylguanosine (m^7G; Zhang et al., 2019b), cap-proximal 2′-*O*,N^6-dimethyladenosine (m^6A_m; Wei et al., 1975a; Linder et al., 2015; Mauer et al., 2017; Boulias et al., 2019), N^1-methyladenosine (m^1A; Dominissini et al., 2016; Li et al., 2016, 2017b; Safra et al., 2017), 4-acetylcytidine (ac4C; Arango et al., 2018), 5-hydroxymethylcytidine (hm^5C; Delatte et al., 2016), and 2′-*O*-methylation (any nucleotide [Nm]; Furuichi et al., 1975; Wei et al., 1975b; Dai et al., 2017; Bartoli et al., 2018). For at least one of these modifications, m^7G, plant homologs exist of the enzyme responsible for its introduction into tRNA (Alexandrov et al., 2002) and some mRNA sites (Zhang et al., 2019b). For others, such as ac4C and hm^5C, the identified modifying enzymes do not have direct counterparts in plants.

Regardless of the existence of close homologs in plant genomes of RNA-modifying enzymes identified in mammalian cells, it is possible that chemical modifications in plant mRNA, not necessarily limited to the ones shown to occur in other eukaryotes, await discovery and functional characterization. Some studies do indeed provide evidence for the presence of additional modified nucleotides in plant mRNA. For example,

BOX 1. Historical Context of the Exploding mRNA Modification Field

Chemically modified nucleotides in RNA are not new subjects of research. Soon after the discovery of transfer and ribosomal RNA, chromatographic analyses of hydrolysates of rRNA (18S and 28S) and tRNA (4S) fractions revealed the presence of more nucleotides than adenosine, guanosine, cytidine, and uridine (summarized in Boccaletto et al. (2017)). Similarly, only few years after the discovery of poly(A) tails made purification of mRNA fractions by oligo(dT) affinity chromatography possible, *N6*-methyladenosine (m^6A) was detected in mRNA hydrolysates (Desrosiers et al., 1974; Perry and Kelley, 1974). By the mid-1960s, ribonuclease digests and chromatographic methods were used to determine sequences of nucleotides and identify their modifications in pure tRNAs (Holley et al., 1965). These painstaking methods used mg quantities of pure RNA species, meaning that they could not be applied to site-specific mapping in individual mRNAs. For m^6A, which does not result in nucleotide misincorporation by reverse transcriptase, the lack of mapping and detection methods in mRNA meant that one fundamental problem could not be tackled: which specific mRNAs contained m^6A? Was the modification present at low levels in all mRNAs, indicative of a general role in pre-mRNA processing, or was it present in specific subsets of mRNAs, indicative of a regulatory role? And if regulatory, how the modification would affect mRNA function could not be investigated without knowledge of the modified mRNAs. The game changer was to adapt biochemical methods relying on specific chemical reactivities or binding properties of modified nucleotides to detection by high-throughput sequencing, thereby exploiting its unparalleled sensitivity. The development of the first such method led to the generation of transcriptome-wide maps of m^6A in mammalian mRNA in 2012 (Dominissini et al., 2012; Meyer et al., 2012). This achievement suggested that deposition of m^6A is indeed regulatory rather than a general pre-mRNA processing event, simply because m^6A occurs in a specific set of mRNAs, whose composition is adjusted in response to environmental change (Dominissini et al., 2012) and throughout development (Meyer et al., 2012). In addition, the maps showed that overwhelmingly, m^6A occurs towards 3′-ends of mRNAs and in a specific sequence context, RR[m^6A]CH (R=A/G, H=A/C/U), confirming earlier findings (Wei et al., 1976; Dimock and Stoltzfus, 1977; Schibler et al., 1977; Wei and Moss, 1977; Canaani et al., 1979). These simple, yet groundbreaking, discoveries resulting from acquisition of transcriptome-wide m^6A maps initiated the tremendous acceleration in knowledge on mRNA modifications over the last 6 years.

liquid chromatography-mass spectrometry analysis of total hydrolysates indicates the presence of internal m^7G in mRNA of several plant species (Chu et al., 2018). In addition, a thorough examination of sites of recurrent misincorporation by reverse transcriptase in Arabidopsis (*Arabidopsis thaliana*) RNA-seq data suggested the rare presence of several mRNA modifications with potential to alter Watson-Crick base pairing (Vandivier et al., 2015). These included 3-methylcytidine (m^3C), whose presence in some mRNAs was validated by RNA immunoprecipitation with a m^3C-specific antibody (Vandivier et al., 2015). It is worth noting, however, that the number of misincorporation sites was orders of magnitude higher in the uncapped mRNA pool undergoing degradation than in intact, capped mRNAs. Thus, rather than resulting from regulated modification, many modified nucleotides identified by this approach may be chemically damaged (e.g. by oxidation) such that they accumulate throughout the life of an mRNA until its degradation. It is also important to note that, despite their clear utility, detection methods of modified nucleotides involving total hydrolysis of poly(A^+) fractions such as liquid chromatography-mass spectrometry and thin-layer chromatography may easily include contamination from tRNA and/or rRNA in which the relative, and certainly also the absolute, content of modified nucleotides other than m^6A is manyfold higher than in mRNA (Boccaletto et al., 2018). The difficulty in obtaining pure mRNA is highlighted in a study by Legrand et al. (2017), in which total RNA subjected to

two rounds of poly(A) selection followed by two consecutive rounds of rRNA depletion still contained 7.1% rRNA.

CARTOGRAPHY OF MRNA MODIFICATIONS IN PLANTS

Cartography of mRNA modifications (i.e. their mapping to specific sites in mRNAs) is very much in its infancy, particularly in plants, in which m^6A stands out as the so-far best understood mRNA modification, with only limited knowledge available on Ψ and m^5C. A recent report shows that Ψ exists in hundreds of mRNAs in Arabidopsis (Sun et al., 2019), but the functions of Ψ in mRNA or the identities of pseudouridine synthases responsible for mRNA pseudouridylation remain unknown. Three different studies have mapped hundreds of m^5C sites in Arabidopsis mRNA, but there is discrepancy in assessments of whether m^5C is enriched in coding sequences or in 3′-untranslated regions (UTRs), perhaps as a consequence of the different tissues used or because of actual inconsistencies between results obtained by the two different m^5C mapping methods employed (Cui et al., 2017; David et al., 2017). These studies also showed that the tRNA methyltransferase NSUN2/TRM4B may catalyze C5-cytidine methylation in mRNA, because some mRNA-m^5C sites are lost in *trm4b* mutants (Cui et al., 2017; David et al., 2017). In addition, quantitative differences in the extent of m^5C modification of specific mRNAs between different tissues were noted, perhaps pointing to regulatory properties of this modification (David et al., 2017). In one case, orthogonal methods were used for m^5C mapping, yielding a high-confidence set of m^5C sites (Yang et al., 2019). Interestingly, this report also showed that m^5C facilitates mRNA long-distance transport through the phloem (Yang et al., 2019), but the underlying mechanisms of this important phenomenon, including the identities of possible m^5C-binding proteins, remain unknown. We focus the remainder of this Update on m^6A, simply because, at present, its importance is more clearly established and the molecular mechanisms involved are better studied than for any other mRNA modification.

BIOCHEMICAL FRAMEWORK FOR THE FUNCTION OF M^6A

Occurrence of m^6A in Plant Transcriptomes

In agreement with early biochemical studies of animal, viral, and plant mRNA (Wei et al., 1976; Dimock and Stoltzfus, 1977; Schibler et al., 1977; Wei and Moss, 1977; Canaani et al., 1979; Nichols and Welder, 1981), transcriptome-wide mapping of m^6A has identified RR[m^6A]CH (R = A/G, H = A/C/U) as the most significantly enriched motif in m^6A peaks of all eukaryotes analyzed to date (Dominissini et al., 2012; Meyer et al., 2012; Schwartz et al., 2013; Ke et al., 2015; Linder et al., 2015; Lence et al., 2016; Zhao et al., 2017; Li et al., 2019), including plants (Luo et al., 2014; Wan et al., 2015; Shen et al., 2016; Duan et al., 2017; Wang et al., 2017; Anderson et al., 2018; Miao et al., 2019; Parker et al., 2019). This finding strongly supports the existence of a conserved mechanism of m^6A deposition in eukaryotic mRNA and is in perfect agreement with the fact that the adenosine methyltransferase complex is conserved across eukaryotes but not in prokaryotes, in which m^6A occurs in a completely different sequence context in mRNA (Box 2; Deng et al., 2015). In addition to its presence in a defined consensus motif, m^6A was predominantly found in the 3′-UTR of mRNAs by most of the above-mentioned studies in eukaryotes, again supporting a highly conserved mechanism of adenosine methylation, while it is distributed more or less evenly along prokaryotic transcripts (Deng et al., 2015). Nonetheless, recent reports have raised the question of whether different m^6A motifs exist in plants (Li et al., 2014c; Anderson et al., 2018; Wei et al., 2018b; Luo et al., 2019; Miao et al., 2019; Zhang et al., 2019a), a debate that is detailed in Box 2 and illustrated in Figure 1. It is our judgment that the current evidence most strongly supports predominant use of the pan-eukaryotic RR[m^6A]CH motif. In spite of this, there does appear to be a difference in the functional categories of m^6A-containing mRNAs between plants and cultured animal cells. In plants, many m^6A-containing mRNAs encode ribosomal and photosynthesis-related proteins, mitochondrial electron transport factors, and other basic metabolic enzymes (Luo et al., 2014; Wan et al., 2015; Shen et al., 2016; Wang et al., 2017; Anderson et al., 2018), while such housekeeping factors appear to be depleted from sets of m^6A-containing mRNAs in yeast and mammalian cell cultures (Schwartz et al., 2014b; Ke et al., 2017). It is a question of outstanding importance to define if and how the labeling of these many housekeeping transcripts by m^6A contributes to plant growth and development. Finally, a recent report deposited in bioRxiv shows that primary microRNA transcripts in Arabidopsis also contain m^6A and proposes that the modification regulates microRNA biogenesis (Bhat et al., 2019), as previously proposed in animals (Alarcón et al., 2015; Berulava et al., 2015).

Writing m^6A

mRNA-modifying enzymes are often referred to as writers, by analogy with signal transduction systems in which writers catalyze the formation of a regulatory posttranslational modification, readers act as effectors by binding to the modified amino acid, and erasers remove the modification to reset signaling (Lim and Pawson, 2010). We adopt this nomenclature here but note that its appropriateness is intensely debated, because it is difficult to obtain direct proof that the same mRNA molecules undergo reversible cycles of methylation and demethylation (Ke et al., 2017; Mauer et al.,

BOX 2. The RRACH Motif and More

In 1981, ribonuclease mapping of m^{6}A in poly(A)+ RNA fractions from maize showed that it occurs in the R[m^{6}A]C (R=A/G) sequence context (Nichols and Welder, 1981), in agreement with earlier studies on animal and viral RNA (Wei et al., 1976; Dimock and Stoltzfus, 1977; Schibler et al., 1977; Wei and Moss, 1977; Canaani et al., 1979). The extended consensus motif RR[m^{6}A]CH (R=A/G, H=A/C/U) (Schibler et al., 1977) has now emerged as enriched around m^{6}A sites in transcriptome-wide m^{6}A maps from vertebrates (Dominissini et al., 2012; Meyer et al., 2012; Zhao et al., 2017), insects (Lence et al., 2016; Li et al., 2019), yeast (Schwartz et al., 2013), and plants (Luo et al., 2014; Wan et al., 2015; Shen et al., 2016; Duan et al., 2017; Wang et al., 2017; Anderson et al., 2018; Miao et al., 2019; Parker et al., 2019). The existence of a pan-eukaryotic m^{6}A motif is in agreement with the high degree of conservation of the eukaryotic m^{6}A methyltransferase complex (Balacco and Soller, 2019). Conversely, m^{6}A in prokaryotic mRNA occurs in a different sequence context (GCC[m^{6}A]U), consistent with the lack of bacterial orthologs of eukaryotic m^{6}A writer components (Deng et al., 2015).

Nevertheless, motifs in addition to RRACH may also be enriched around m^{6}A sites in eukaryotes: tissue-specific m^{6}A peaks in rice are enriched for UGWAMH, UGUAMM, and RAGRAG (R=A/G, H=A/C/U, W=A/U, M=A/C), perhaps suggesting that additional RNA binding proteins may influence m^{6}A deposition or removal (Li et al., 2014c; Zhang et al., 2019a). m^{6}A-proximal U-rich motifs that serve as binding sites for subunits of the m^{6}A-methylation complex do indeed exist in animals (Patil et al., 2016).

Furthermore, GGAU and U-rich motifs are associated with endonucleolytic cleavage 4-5 nt upstream of m^{6}A peaks in Arabidopsis (Anderson et al., 2018). The occurrence of additional motifs is in line with motif enrichment analysis around maize m^{6}A sites reported by Miao et al. (2019) and the re-analysis of Arabidopsis m^{6}A data (Luo et al., 2014; Shen et al., 2016; Anderson et al., 2018) performed by the same authors (Miao et al., 2019). In all cases, both the canonical RR[m^{6}A]CH motif (91.2% of the m^{6}A peaks in maize) and a second URUAY (R=A/G, Y=C/U) motif were enriched. However, only UGUAMM is found around m^{6}A peaks in maize seedlings by Luo et al. (2019), and a study on the Arabidopsis m^{6}A reader ECT2 also reported URUAY as its only enriched binding motif (Weiet al., 2018b). Accordingly, the authors observed more efficient *in vitro* methylation of UGUA (URUAY)-than of GGACU (RRACH)-containing oligonucleotides using Arabidopsis nuclear extracts.

A definitive answer as to whether GGAU and URUAY are plant-specific m^{6}A motifs (as suggested by Anderson et al. (2018) and Wei et al. (2018b) respectively) or whether they co-occur in close vicinity with m^{6}A-peaks, must await publication of m^{6}A maps with single-nucleotide resolution. Interestingly, a study recently posted on bioRXiv reported on Arabidopsis single-nucleotide resolution m^{6}A maps using miCLIP (Linder et al., 2015) and direct RNA Nanopore sequencing (Garalde et al., 2018). In this analysis, only the canonical RR[m^{6}A]CH motif was enriched at m^{6}A sites (Parker et al., 2019).

2017, 2019; Meyer and Jaffrey, 2017; Rosa-Mercado et al., 2017; Darnell et al., 2018; Wei et al., 2018a; Shi et al., 2019). Despite the simple biochemistry of the m^{6}A writer reaction, nucleophilic substitution resulting in transfer of the methyl group from *S*-adenosylmethionine (SAM) to the *N*^{6}-amine of adenosine, the major eukaryotic mRNA adenosine methyltransferase turns out to be extraordinarily complicated. Initial attempts at its purification from mammalian cells resulted in the definition of two subcomplexes, ~200-kD MT-A and ~875-kD MT-B, both required for full methyltransferase activity, even in vitro (Bokar et al., 1994). While we now know that MT-A contained the catalytic core consisting of a dimer of the two methyltransferase-like proteins METTL3 (Bokar et al., 1997) and METTL14 (Liu et al., 2014), the exact composition of the originally defined MT-B complex is still unclear. Both MT-A subunits have direct homologs in plants: METTL3 corresponds to MTA (Zhong et al., 2008) and METTL14 corresponds to MTB (Růžička et al., 2017). Several additional factors

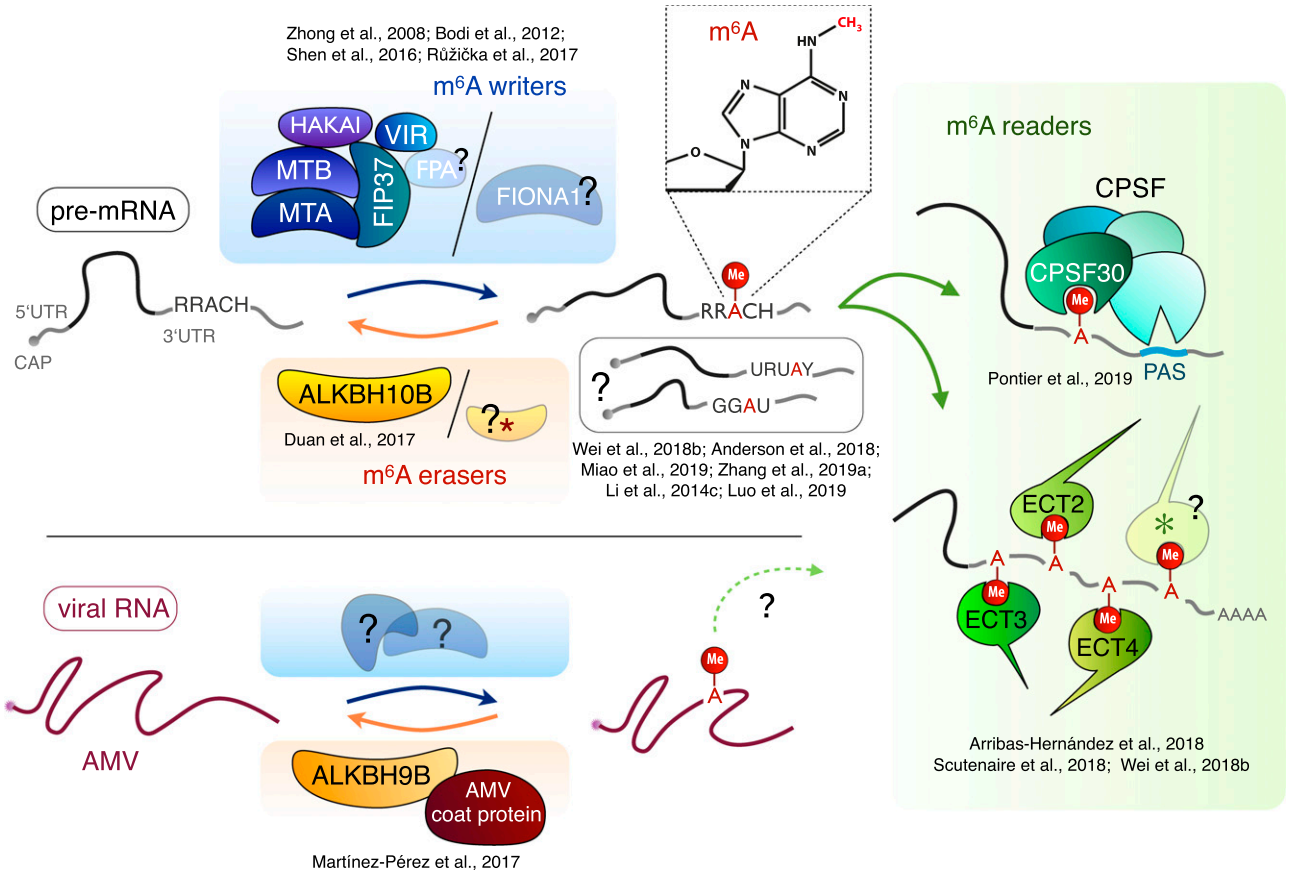

Figure 1. Schematic representation of the m^{6}A pathway and the functions of its characterized components. m^{6}A writers are depicted on a blue field, readers on green, and erasers on orange. The canonical m^{6}A consensus motif (RRACH) is chosen for the general representation, but alternative motifs (UGUAY and GGAU) recently proposed as plant specific are indicated in a separate box (see Box 2 for details). An endogenous m^{6}A target is depicted as a pre-mRNA to highlight the connection of m^{6}A writing to transcription. YTHDF m^{6}A readers (ECT2, ECT3, ECT4, and probably additional ECTs) are represented as binding to the same transcript for convenience, but there are still no data clarifying whether different ECTs can bind in cis or not. If so, they could compete or have synergistic effects, perhaps interacting with each other as proposed for animal YTHDFs (Shi et al., 2017). Asterisks represent putative additional readers/erasers (listed in the gray boxes of Fig. 2), as many homologs in the plant YTH and ALKBH families remain uncharacterized. AMV, *Alfalfa mosaic virus*; CPSF, cleavage and polyadenylation specificity factor. PAS, polyadenylation signal.

either fully or partly required for m^{6}A deposition in vivo have been identified. Many of these factors are also conserved and include the following proteins, denoted with plant name first and mammalian homolog following a slash (see Figs. 1 and 2 for additional details): the splicing factor FKBP12 Interacting Protein37 (FIP37/WTAP; Vespa et al., 2004; Zhong et al., 2008; Liu et al., 2014; Ping et al., 2014; Schwartz et al., 2014b; Shen et al., 2016; Růžička et al., 2017); the protein VIRILIZER (VIR/KIAA1429), originally identified in *Drosophila melanogaster* (Niessen et al., 2001; Ortega et al., 2003; Schwartz et al., 2014b; Růžička et al., 2017); the putative ubiquitin E3 ligase HAKAI/CBLL1 (Horiuchi et al., 2013; Růžička et al., 2017); the Zn finger protein ZC3H13 (Guo et al., 2018; Knuckles et al., 2018; Wen et al., 2018) without orthologs in plants (Balacco and Soller, 2019); and the RNA-binding proteins RBM15A/B (Horiuchi et al., 2013; Yan and Perrimon, 2015; Lence et al., 2016; Patil et al., 2016). Interestingly, the closest plant homolog of RBM15A/B is FLOWERING LOCUS PA (FPA), an RNA-binding protein required for the control of flowering time (Schomburg et al., 2001). However, the homology between FPA and RBM15A/B is confined to the RNA recognition motifs and the overall degree of identity is low. Although the possible involvement of FPA in m^{6}A deposition remains uninvestigated, mutants in *FPA* and m^{6}A pathway components share at least one molecular phenotype: partial loss of function causes transcriptional read-through and chimeric RNA formation (Hornyik et al., 2010; Duc et al., 2013; Pontier et al., 2019). Nonetheless, it is clear that the requirement of FPA for m^{6}A writer function, if any, cannot be absolute, because in contrast to most knockout mutants in m^{6}A writer components (Vespa et al., 2004; Zhong et al., 2008; Růžička et al., 2017), null

	Arabidopsis	Mammals	Associated phenotypes (biological function) in Arabidopsis		Ref.
			Allele / Transgenic line	Phenotype	
m^{6}A WRITERS	**MTA** (At4g10760)	METTL3	*mta-1* (SALK_074069)	Embryo lethality (arrested at globular stage).	1
			mta-1 ABI3:MTA	Reduced apical dominance. Abnormal organ definition. Increased number of trichome branches.	2
				Reduced root growth. Defects in protoxylem and gravitropism.	3
				Abnormal transcription termination.	15
			AmiR-MTA	SAM overproliferation. Delayed growth. Abnormal leaf shape.	4
	MTB (At4g09980)	METTL14	*emb1691*	Embryo lethality.	3
			MTB RNAi	Reduction in m^{6}A/A ratio to 50%. Delayed growth. Defects in apical dominance, protoxylem, root growth and gravitropism.	
	FIP37 (At3g54170)	WTAP	*fip37-1, fip37-2, fip37-3* (SALK_029377)	Delay in the pace of the cell cycle in embryo and endosperm. Embryo arrest at midglobular stage.	5
			35S:FIP37	More numerous, highly branched trichomes.	
			fip37-4 (SALK_018636)	*fip37-4/+* produced 1/4 of white arrested seeds and failed to produce viable homozygous seeds.	4
				Viable with stunted growth. Reduction in m^{6}A/A ratio to 5-15%. Defects in apical dominance, protoxylem, and gravitropism.	3
			fip37-4 LEC1:FIP37	SAM overproliferation. Delayed growth. Abnormal leaf shape.	4
				Abnormal transcription termination.	15
	VIR (At3g05680)	KIAA1429	*emb2016*	Embryo lethality.	3
			vir1-1	Reduction in m^{6}A/A ratio to 5-15%. Stunted growth. Defects in apical dominance, protoxylem, lateral root and gravitropism.	
				Lengthening of the circadian period. Altered PAS usage.	6
	HAKAI (At5g01160)	CBLL1	*hakai-1* (GABI_217A12) *hakai-2* (CRISPR/Cas9)	Reduction in m^{6}A levels to 65%. Viable, morphologically wild type phenotype.	3
	***FPA** (At2g43410)	RBM15	*fpa-6*	Delayed flowering.	7
	***FIONA1** (At2g21070)	METTL16	*fio1-1*	Longer circadian period.	8
m^{6}A ERASERS	**ALKBH10B** (At4g02940)	ALKBH5	*alkbh10b-1* (SALK_004215C)	Elevated m^{6}A/A ratio in total mRNA. Late flowering.	9
			35S:ALKBH10B	Reduced m^{6}A/A ratio in total mRNA. Early flowering.	
	ALKBH9B (At2g17970)	ALKBH5	*alkbh9b-1* (SALK_055591C) *alkbh9b-2* (SALK_111811C)	No significant changes in m^{6}A/A ratio of endogenous mRNAs.	9
			alkbh9b-3 (SALK_015591)	Increased resistance to Alfalfa Mosaic Virus (AMV). Elevated m^{6}A/A ratio in viral RNA of AMV-infected plants.	10
	*__ALKBH1A__ (At1g11780), *__ALKBH1B__ (At3g14140), *__ALKBH1C__ (At3g14160), *__ALKBH1D__ (At5g01780), *__ALKBH2__ (At2g22260), *__ALKBH6__ (At4g20350), *__ALKBH8A__ (At1g31600), *__ALKBH8B__ (At4g02485), *__ALKBH9A__ (At1g48980), *__ALKBH9C__ (At4g36090), *__ALKBH10A__ (At2g48080)				11
m^{6}A READERS	**ECT2** (At3g13460)	YTHDF1-3	*ect2-1* (SALK_002225)	Stochastic increase of trichome branches.	12, 13, 14
			ect2-2 (SAIL_11_D07)		13, 14
			ect2-3 (GK_132_F02)		12
	ECT3 (At5g61020)	YTHDF1-3	*ect3-1* (SALK_077502) *ect3-2* (GABIseq487H12.1)	Stochastic increase of trichome branches.	12
			ect2-1/ect3-1 *ect2-3/ect3-2*	Additive effect on the increase of trichome branches. Delayed growth. Aberrant leaf morphology.	12
	ECT4 (At1g55500)	YTHDF1-3	*ect4-2* (GK-241H02)	No described phenotype in the single mutant, or in combination with *ect2* or *ect3.*	12
			ect2-1/ect3-1/ect4-2	Strong enhancement of the delayed growth and aberrant leaf morphology of *ect2/ect3* double mutants.	12
	CPSF30 (At1g30460)		*cpsf30-1 (oxt6)* *cpsf30-3* (GABI_477H04)	Abnormal transcription termination.	15
	*__ECT1__ (At3g03950), *__ECT5__ (At3g13060), *__ECT6__ (At3g17330), *__ECT7__ (At1g48110), *__ECT8__ (At1g79270), *__ECT9__ (At1g27960), *__ECT10__ (At5g58190), *__ECT11__ (At1g09810), *__At4g11970__				16, 13

Figure 2. Biological functions of the m^{6}A pathway in plants as inferred by the mutant phenotype of its characterized components in Arabidopsis. The mammalian homologs of each factor is indicated. Arabidopsis bona fide writers are shaded in blue, erasers in orange, and readers in green. Arabidopsis genes marked with asterisks (as also labeled in Fig. 1) inside gray-shaded boxes are orthologs of m^{6}A pathway components in other organisms or paralogs of such genes in Arabidopsis, but their possible or expected

alleles of *fpa* complete embryogenesis (Schomburg et al., 2001).

Although the MTA/MTB methyltransferase accounts for the vast majority of m^{6}A sites in Arabidopsis (Zhong et al., 2008; Shen et al., 2016; Růžička et al., 2017; Anderson et al., 2018), additional m^{6}A methyltransferases exist. The essential mammalian enzyme METTL16 methylates structured RNAs containing the nonamer UAC(A)GAGAA (Pendleton et al., 2017). Its few identified targets include the MAT2A mRNA encoding SAM synthetase, and the embryo-lethal phenotype of *mettl16* mutants may indeed result from disrupted SAM homeostasis (Pendleton et al., 2017; Warda et al., 2017; Mendel et al., 2018). Interestingly, a clear METTL16 homolog, *FIONA1* (*FIO1*), exists in plants. *fio1* loss-of-function mutants are viable and fertile but exhibit increased period length in the circadian clock (Kim et al., 2008), a phenotype shared with mutants homozygous for the hypomorphic *vir-1* allele (Růžička et al., 2017; Parker et al., 2019). The biochemical functions of plant FIO1/METTL16, including as an m^{6}A methyltransferase, remain unexplored, however.

It is a relevant question how cells avoid that the specificity of the m^{6}A methyltransferase be undercut by direct incorporation of m^{6}ATP during transcription. After all, degradation of m^{6}A-containing RNA would result in m^{6}AMP that could be converted into an RNA Polymerase II substrate (m^{6}ATP) through salvage pathways. The answer appears to lie in a combination of two factors (Chen et al., 2018): first, the existence of a conserved enzyme with specific m^{6}AMP deaminase activity; second, the fact that adenylate kinase has poor activity toward m^{6}AMP, resulting in poor conversion of m^{6}AMP into m^{6}ADP. Interestingly, knockout of the Arabidopsis m^{6}AMP deaminase MAPDA1 leads to reduced root growth, suggesting that avoidance of m^{6}A recycling may be of importance in vivo (Chen et al., 2018).

Erasing m^{6}A

One of the spectacular early findings in the m^{6}A field was the discovery that enzymes in the ALKBH family have m^{6}A demethylase activity (Jia et al., 2011; Zheng et al., 2013), suggesting the regulatory capacity of the modification for the first time. Several of these enzymes catalyze oxidative dealkylation of *N*-methylated nucleotides (Ougland et al., 2015). The Arabidopsis genome encodes 13 members of the ALKBH family of oxidases (Mielecki et al., 2012), two of which (ALKBH9B and ALKBH10B) have been shown to have m^{6}A demethylase activity in vitro and have m^{6}A-related functions in vivo (Figs. 1 and 2; Duan et al., 2017; Martínez-Pérez et al., 2017). While such demethylases are important for the control of flowering time (ALKBH10B) and susceptibility to viruses (ALKBH9B) and have an impact on m^{6}A/A ratios in viral RNA (ALKBH9B) and, more subtly, in endogenous mRNA (ALBH10B; Duan et al., 2017; Martínez-Pérez et al., 2017), their exact functions in vivo remain ill defined.

Reading m^{6}A

At least two different properties of RNA may change upon chemical modification of nucleotides. (1) The structure may change as a consequence of altered base pairing properties or rigidity of structure. In turn, this may create or influence the accessibility of binding sites for proteins, or even small molecules, in nearby sequences. (2) The modification itself may create a binding site for an RNA-binding protein with specific affinity for the modified nucleotide.

For m^{6}A, there is evidence for both types of function in mammalian cells (Wang et al., 2014; Liu et al., 2015), although category 2 appears to be more prevalent (Patil et al., 2018). The best studied proteins with specific m^{6}A-binding capacity contain a so-called YT521-B Homology (YTH) domain of ~140 amino acids (Imai et al., 1998; Hartmann et al., 1999; Stoilov et al., 2002; Zhang et al., 2010) that recognizes the N^6-methyl group on adenosine via a highly conserved hydrophobic binding pocket containing three aromatic side chains surrounding the methyl group (the aromatic cage; Li et al., 2014b; Luo and Tong, 2014; Theler et al., 2014; Xu et al., 2014; Zhu et al., 2014). Two phylogenetic classes of YTH domains can be defined, YTHDF and YTHDC. In mammals, YTHDF-containing proteins are predominantly cytoplasmic, bind to all m^{6}A sites in mRNA, and the three paralogs (YTHDF1–YTHDF3) share high amino acid similarity throughout their entire length, including in their long N-terminal intrinsically disordered regions (IDRs). On the contrary, the YTHDC domain is found in

Figure 2. (*Continued.*)
roles as m^{6}A players in plants have not been verified experimentally. For this reason, columns 4 and 5 are omitted in the gray boxes corresponding to putative erasers and readers. Column 3 is also omitted for simplicity as it can be summarized as follows: AtALKBH proteins are homologs of the mammalian ALKBH1 to ALKBH8 and FTO family (Mielecki et al., 2012); all ECTs belong to the YTHDF clade (YTHDF1–YTHDF3 in mammals), while At4g11970 presents homology with the YTHDC clade (YTHDC1 and YTHDC2 in mammals; Scutenaire et al., 2018). In addition to knockout, knockdown, and overexpression lines, transgenic plants expressing point mutants of *ECT2* (12–14), *ECT3* (12), *ECT4* (12), and *CPSF30* (15) with impaired ability to bind m^{6}A, or a catalytically inactive *ALKBH10b* (9), are also described in the indicated references and behave like null mutants for the phenotypes described in all cases. References are as follows: 1, Zhong et al., 2008; 2, Bodi et al., 2012; 3, Růžička et al., 2017; 4, Shen et al., 2016; 5, Vespa et al., 2004; 6, Parker et al., 2019; 7, Schomburg et al., 2001; 8, Kim et al., 2008; 9, Duan et al., 2017; 10, Martínez-Pérez et al., 2017; 11, Mielecki et al., 2012; 12, Arribas-Hernández et al., 2018; 13, Scutenaire et al., 2018; 14, Wei et al., 2018b; 15, Pontier et al., 2019; 16, Li et al., 2014a.

two very different proteins in mammals: YTHDC1 is nuclear and binds to some sites in mRNAs and nuclear non-coding RNAs, whereas YTHDC2 is enriched in perinuclear regions of the cytoplasm and its mRNA-binding profile shows little overlap with m^{6}A sites (Patil et al., 2016; Hsu et al., 2017; Kretschmer et al., 2018; Zaccara et al., 2019). YTHDC2 is specific to mammals and contains several other folded domains in addition to the YTHDC domain (Bailey et al., 2017; Wojtas et al., 2017; Kretschmer et al., 2018), while YTHDC1 has long N- and C-terminal IDRs (Patil et al., 2016). YTHDF- and YTHDC1-type proteins are found in many eukaryotes (Balacco and Soller, 2019), including plants, whose genomes encode more YTH domain proteins than other organisms (Li et al., 2014a; Scutenaire et al., 2018). For example, 13 YTH domain-containing proteins are encoded in Arabidopsis (Fig. 2) compared with five in humans. The 13 Arabidopsis YTH-domain proteins can be divided into 11 YTHDF proteins called EVOLUTIONARILY CONSERVED C-TERMINAL REGION1 (ECT1) to ECT11, one classical YTHDC1-type protein (At4g11970), and one YTHDC protein, unusual in that it also contains additional folded domains: it is CPSF30, the 30-kD subunit of the cleavage and polyadenylation specificity factor involved in pre-mRNA cleavage and 3′-end formation (Zhang et al., 2008; Thomas et al., 2012; Bruggeman et al., 2014; Pontier et al., 2019).

Several lines of evidence suggest that while m^{6}A-binding specificity resides in the YTH domain, the effector function, at least of YTHDF proteins, resides in the IDR. For example, tethering the IDR of YTHDF2 to reporter mRNAs is sufficient to cause their localization to P-bodies in mammalian cells (Wang et al., 2014). The IDR of YTHDF2 also interacts with the deadenylase complex CCR4-NOT (Du et al., 2016) and, via the adaptor protein HRSP12, with the endoribonuclease RNase P/MRP (Park et al., 2019). However, since the affinity of isolated YTH domains for m^{6}A-modified RNA is modest (0.1–5 μM; Li et al., 2014b; Luo and Tong, 2014; Theler et al., 2014; Xu et al., 2014, 2015; Zhu et al., 2014), it is possible that the IDR participates in RNA binding in vivo, as suggested by the loss of mRNA binding in vivo of a mutant in human YTHDF3 containing a deletion in the IDR (Zhang et al., 2019c). In this regard, it is noteworthy that three Arabidopsis YTHDF proteins (ECT5, ECT9, and ECT10) contain an amino acid substitution expected to result in 10-fold higher affinity toward m^{6}A RNA than other YTH domains, based on structural and biochemical analyses of analogous mutants in human YTHDF1 (Xu et al., 2015; Scutenaire et al., 2018). These proteins may, therefore, differ in requirements for their IDRs for RNA interactions compared with other YTH proteins, or they may simply bind with higher affinity. It is an interesting property of both mammalian YTHDFs and plant ECTs that they are able to undergo phase transition in vitro to a condensed liquid or gel-like phase (Arribas-Hernández et al., 2018; Fu and Zhuang, 2019; Gao et al., 2019; Ries et al., 2019). It is of considerable interest to determine whether such phase separation properties underlie biological functions and the subcellular localization of YTHDF proteins. To date, YTH domain proteins are the only characterized class of m^{6}A readers in plants (Figs. 1 and 2). In mammalian cells, several other RNA-binding proteins have been proposed to function as m^{6}A readers (Balacco and Soller, 2019), including the translation initiation factor eIF3 that is recruited to m^{6}A-containing 5′-UTRs to stimulate cap-independent translation initiation (Meyer et al., 2015).

MOLECULAR ROLES OF M^{6}A IN PLANTS

Cytoplasmic Roles

What effect does the presence of m^{6}A in an mRNA have on its stability and translatability? In yeast and mammalian cell culture, the evidence is strong that it accelerates mRNA decay (see e.g. Herzog et al. [2017] or Ke et al. [2017] for animals, Bushkin et al. [2019] for yeast, or Zaccara et al. [2019] for the most recent review), as proposed in early studies (Sommer et al., 1978). Accelerated mRNA decay in mammals involves YTHDF proteins, perhaps in particular YTHDF2 (Wang et al., 2014; Ivanova et al., 2017; Zhang et al., 2017; Zhao et al., 2017; Paris et al., 2019), but whether the mechanism relies mostly on CCR4-NOT deadenylase recruitment (Du et al., 2016), perhaps concurrent with sorting to P-bodies (Wang et al., 2014), enhanced endonucleolysis (Park et al., 2019), or a combination remains unclear. m^{6}A may also stimulate translation, in this case via other YTHDF proteins (Wang et al., 2015; Li et al., 2017a; Shi et al., 2017). In particular, YTHDF1 promotes the translation of m^{6}A targets via interaction with the eukaryotic initiation factor eIF3 (Wang et al., 2015) in a process that may be of particular biological relevance in neurons (Shi et al., 2018; Weng et al., 2018). A cytoplasmic role of METTL3 itself has also been proposed in the activation of translation, also via eIF3 recruitment (Lin et al., 2016; Choe et al., 2018).

In plants, transcriptomic analyses have also been applied to analyze the effect of m^{6}A on mRNA abundance (Fig. 3). Since knockout of m^{6}A writer subunits is embryonically lethal (see "Functions in Development" below; Zhong et al., 2008), plants expressing either MTA or FIP37 from embryo-specific promoters in the respective knockout backgrounds have been used to obtain postembryonic tissues with 80% to 90% loss of m^{6}A (Bodi et al., 2012; Shen et al., 2016). Studies using either these plants or the partial loss-of-function *vir-1* allele (Růžička et al., 2017) showed a slight tendency for m^{6}A targets to be less abundant in tissues of plants with reduced levels of m^{6}A (Shen et al., 2016; Anderson et al., 2018; Parker et al., 2019). The same tendency was observed in knockouts of the m^{6}A reader *ect2* (Wei et al., 2018b). Altogether, those observations suggest that m^{6}A may stabilize mRNAs, potentially by protection against endonucleolytic cleavage that occurs in m^{6}A-depleted plants around sites of m^{6}A deposition (Anderson et al., 2018). On the other hand,

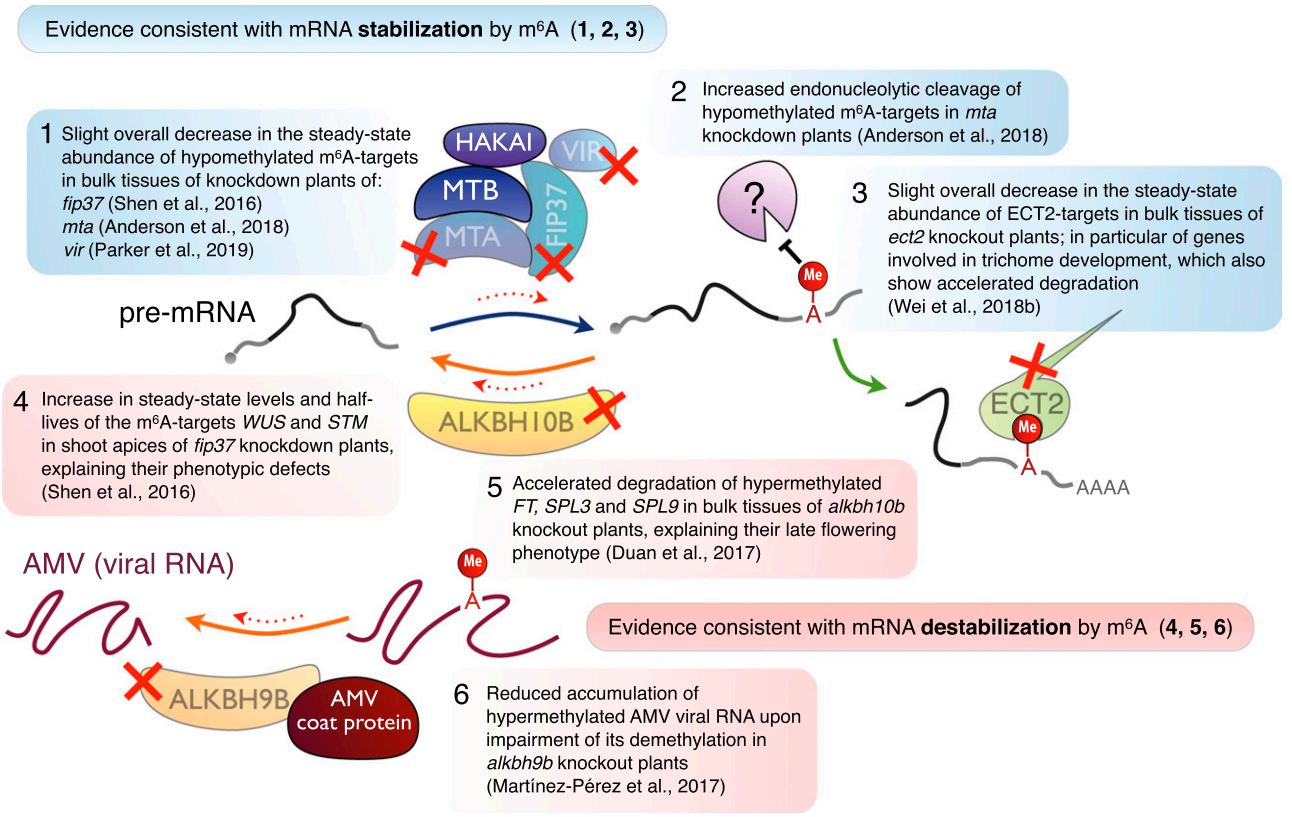

Figure 3. Plant studies reporting on effects of m^{6}A on target RNA accumulation and, in a few cases, decay rates upon global inhibition of transcription.

overaccumulation of m^{6}A-containing mRNAs that encode important developmental regulators has also been found in m^{6}A-depleted mutants (Shen et al., 2016). Moreover, ALKBH10B-mediated m^{6}A demethylation of transcripts encoding key flowering-related genes is associated with their increased abundance and stability (Duan et al., 2017). Thus, the existence of m^{6}A-induced mRNA destabilization in plants should not be ruled out.

We note that the interpretation of the transcriptome profiles reported of wild-type and m^{6}A-deficient plants (Fig. 3) suffers from limitations that may preclude clear conclusions to be drawn on whether m^{6}A actually stabilizes or destabilizes mRNA. First, they use mRNA isolated from all cells of seedlings, while analysis of activity of the MTA promoter suggests that m^{6}A deposition may not be active in all cells (Zhong et al., 2008). Thus, in total extracts, the fraction of a particular mRNA target whose abundance can be directly influenced by the loss of methyltransferase activity is diluted by contributions from cells that do not even express the methyltransferase. That is especially relevant for assessing the overall abundance of m^{6}A targets in plants, as they predominantly include ubiquitously expressed housekeeping genes (Anderson et al., 2018). Furthermore, as knockdown of *MTA*, *FIP37*, or *VIR* causes global changes in plant morphogenesis that may affect the expression domains of m^{6}A-containing mRNAs, it compromises the ability of such experiments to yield conclusive results. Second, steady-state measurements comparing a stable mutant with a wild type do not provide kinetic information, as degradation and synthesis rates will inevitably be confounded. In conclusion, it is at present not clear if mRNAs are stabilized or destabilized by m^{6}A (Fig. 3). There is limited direct evidence for either outcome, and the possibility of target- and/or cell-specific outcomes of mRNA methylation simply has not yet been investigated. Ideally, transcriptome-wide pulse-chase analyses that do not disrupt global transcription [e.g. using thiol(SH)-linked alkylation for the metabolic sequencing of RNA; Herzog et al., 2017] should be employed in the relevant cells rather than bulk tissue of the wild type versus *mta/mtb/fip37/vir* knockdowns or *ect* knockouts to reveal the effect of m^{6}A deposition and ECT binding on transcript stability. Finally, the possible effects of m^{6}A on translational control in plants remain entirely unexplored.

Nuclear Roles

In addition to the intensely studied effects on mature mRNA, roles of m^{6}A in pre-mRNA processing have

also been reported. For example, m^{6}A is required for sex determination in flies, because the sex determination pathway relies on alternative splicing of the *sex-lethal* (*sxl*) pre-mRNA, a process that in turn depends critically on m^{6}A deposition around the alternatively spliced *sxl* exon (Haussmann et al., 2016; Lence et al., 2016). In plants, a recent study has uncovered a special role of m^{6}A in mRNA 3′-end formation and transcription termination at recently duplicated genes: at such loci, read-through transcription into the downstream gene is observed in m^{6}A-deficient plants and, interestingly, in plants containing point mutations to abrogate the aromatic cage in the YTH domain of CPSF30 (Pontier et al., 2019). It is possible that m^{6}A-directed CPSF30 function stimulates pre-mRNA cleavage and RNA Polymerase II termination more generally but that the effect of losing this reinforcement only becomes visible at loci with recent gene duplications (Pontier et al., 2019). In agreement with the idea of m^{6}A-dependent 3′-processing in plants, Parker et al. (2019) recently reported that lack of m^{6}A is associated with a shift to usage of more proximal poly(A) sites and transcriptional read-through, and Luo et al. (2019) observed correlation between the presence of m^{6}A and alternative polyadenylation site usage in maize (*Zea mays*).

BIOLOGICAL ROLES OF M^{6}A IN PLANTS

Functions in Development

The study of plants provided one of the key discoveries in the development of the mRNA modification field: using Arabidopsis, Fray and coworkers (Zhong et al., 2008) were able to prove in 2008 that m^{6}A is crucial for ontogenesis in a multicellular eukaryote, as embryos defective in the homolog of the m^{6}A methyltransferase identified in yeast and mammals (MTA) arrested at the globular stage. Subsequent phenotypic analyses of hypomorphic alleles or postembryonic knockdowns of *mta*, *mtb*, *fip37*, and *vir* showed that reduction of m^{6}A results in slower growth, abnormal organ definition, loss of apical dominance, increased number of trichome branches, defective gravitropic responses, and aberrant development of lateral roots and vasculature (Bodi et al., 2012; Růžička et al., 2017; see Fig. 2 for a comprehensive description of knockout and knockdown lines). Crucial additional insights were gained from the work of Shen et al. (2016), who showed that stem cell differentiation in the shoot apical meristem depends on m^{6}A: in plants depleted of FIP37 postembryonically, the shoot apical meristem dramatically increases in size and forms aberrant leaf primordia with a significant delay compared with the wild type. Interestingly, these strong differentiation phenotypes are associated with expansion of the organizing center of the meristem expressing the key transcription factor *WUSCHEL* (At2g17950), whose mRNA is modified by m^{6}A (Shen et al., 2016). Another m^{6}A target, the mRNA of the meristematic transcription factor *SHOOT MERISTEMLESS* (At1g62360), also overaccumulates in *fip37* knockdown plants, apparently as a consequence of increased mRNA stability (Shen et al., 2016). Thus, m^{6}A in plants is clearly required for meristem function, perhaps in particular for the step of stem cell differentiation, reminiscent of the requirement for m^{6}A for embryonic stem cell differentiation in mammals (Batista et al., 2014; Geula et al., 2015). m^{6}A is also involved in the maintenance of circadian and seasonal plant rhythms, as mutants defective in m^{6}A deposition or removal exhibit lengthening of the circadian period (Parker et al., 2019) and late flowering (Duan et al., 2017), respectively. Finally, recent phenotypic and molecular studies of plants lacking one or several m^{6}A readers provide additional mechanistic insights into the role of m^{6}A in development (Arribas-Hernández et al., 2018; Scutenaire et al., 2018; Wei et al., 2018b). Such advances are discussed in detail below.

Functions in Stress Adaptation

Many mechanisms of posttranscriptional gene regulation, perhaps most prominently small RNA-based gene regulation, are employed to mediate rapid changes in gene expression required for stress adaptation (Sunkar et al., 2007), and it would, therefore, not be surprising to find such roles of the m^{6}A-YTH system. Although early studies based on publicly available microarray data on Arabidopsis and rice (*Oryza sativa*) YTH domain proteins pointed to their induction by various abiotic and biotic stresses (Li et al., 2014a), relatively little has been done to test their functional implication in such responses. Scutenaire et al. (2018) found that ECT2 relocalizes to cytoplasmic stress granules upon heat stress. Similarly, Arribas-Hernández et al. (2018) observed that ECT2 and ECT4, and to a lesser degree ECT3, relocalize to cytoplasmic foci distinct from P-bodies upon osmotic stress. These studies show that reader proteins respond to stress, but they do not establish whether this is inconsequential or important for the adaptive response. Similar correlative evidence between m^{6}A levels and a biotic stress response in tobacco (*Nicotiana tabacum*) plants has been reported. In response to tobacco mosaic virus infection, m^{6}A/G ratios and expression of m^{6}A writers decreased whereas mRNA levels of one potential demethylase increased (Li et al., 2018). The available evidence does not, however, allow a distinction between whether such changes are brought about by the virus to promote infection or by the host to limit infection, or even whether they are relevant for the host-virus interaction at all. In animals, examples of both positive and negative regulation of innate and adaptive immunity by m^{6}A have been reported (see Williams et al. [2019] for review), and similar complexity might be expected for different pathogens or cell types in plants.

A role of m^{6}A in selective stabilization of mRNAs responsive to salt stress has recently been suggested (Anderson et al., 2018). This study showed that transcripts

involved in salt and osmotic stress were hypermethylated under salt treatment and more highly expressed in treated versus nontreated samples. On the contrary, mRNAs with a tendency to lose m^{6}A marks in response to the treatment were related to more general processes like photosynthesis, and their abundance decreased upon salt stress. Nevertheless, normalization of relative m^{6}A contents in treated versus untreated samples is not trivial when considering on/off genes such as salt stress-response genes. In addition, since m^{6}A peaks are more easily mapped in abundant transcripts with antibody-based mapping approaches (Shen et al., 2016; Garcia-Campos et al., 2019), a bias toward the detection of de novo methylation of salt-responsive transcripts highly expressed after treatment cannot be excluded. Thus, orthogonal mapping approaches with the ability to accurately quantify m^{6}A stoichiometry (e.g. MAZTER-seq; Garcia-Campos et al., 2019) will be valuable to address the dynamics of m^{6}A marks in response to stress. In addition, genetic studies, ideally using sophisticated conditional loss-of-function systems of m^{6}A writers and readers, will be required to evaluate the physiological relevance of the m^{6}A regulatory pathway in stress adaptation.

Methylation of Viral RNA as an Immune Response in Plants

Modification of viral RNA by m^{6}A can modulate the outcome of host-virus interactions in mammals. In some examples, such as the cytoplasmic (+)-strand RNA virus Zika, m^{6}A attenuates viral infectivity (Gokhale et al., 2016; Lichinchi et al., 2016). In other cases, including some m^{6}A sites in HIV, the presence of m^{6}A on viral RNA enhances viral gene expression (Kennedy et al., 2017), making it clear that there is no general outcome of N^6-adenosine methylation in host-virus interactions (Williams et al., 2019). In plants, RNA of *Alfalfa mosaic virus* (AMV) has been shown to contain m^{6}A (Martínez-Pérez et al., 2017). In this case, the multifunctional AMV coat protein recruits the m^{6}A eraser ALKBH9B, resulting in decreased m^{6}A levels in viral RNA (Fig. 1). This m^{6}A erasure dramatically increases systemic infection, indicating that m^{6}A has strong antiviral effects in the case of AMV (Martínez-Pérez et al., 2017). It is not yet clear at which precise points in the infection cycle m^{6}A acts (e.g. initial translation of viral RNA, transcription, cell-to-cell movement, or stability of viral RNA), and the molecular mechanisms underlying its clear antiviral effect remain unexplored. This includes important questions concerning possible links between nonself RNA recognition and N^6-adenosine methylation, the identity of the m^{6}A writer, whether reader proteins are involved, and if so how.

MOLECULAR MECHANISMS UNDERLYING BIOLOGICAL ROLES OF M^{6}A IN PLANTS

The clear biological importance of m^{6}A in plants immediately begs mechanistic questions. Given the biochemical framework established for at least some m^{6}A-dependent functions, we can now ask these questions in more precise terms. These include the following. (1) How much of the observed m^{6}A effects is mediated by readers? (2) How many readers are involved? (3) How much is explained by YTH domain proteins? (4) Which mRNAs are targeted, and what are the effects of reader binding to the relevant targets?

A few recent studies have started to wrestle with the considerable problem of doing genetics with 11 different, potentially redundant, ECT/YTHDF proteins (Li et al., 2014a). Two studies showed that single knockouts of *ECT2* led to weak and stochastic increases in trichome branching (Scutenaire et al., 2018; Wei et al., 2018b), resembling one of the defects previously described in postembryonic *mta* knockdown mutants

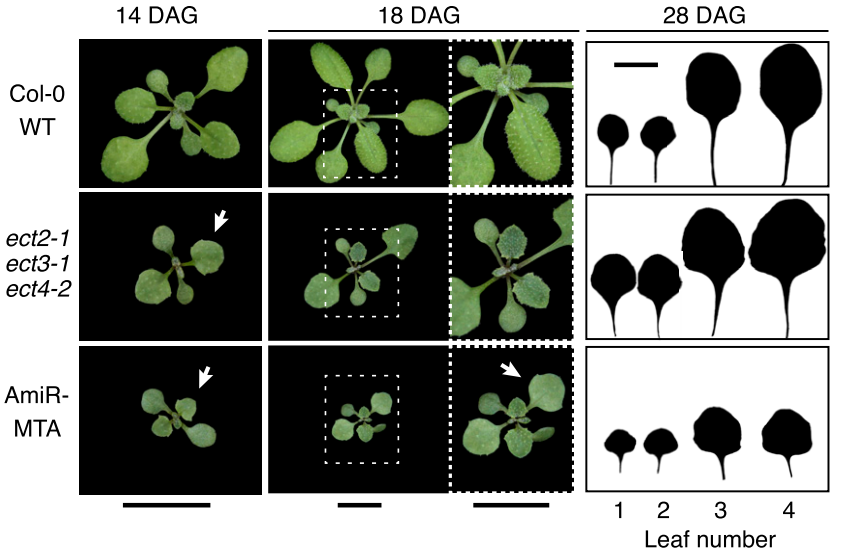

Figure 4. Phenotypic comparison between *mta* knockdown plants with low levels of m^{6}A (AmiR-MTA; Shen et al., 2016) and the triple mutant *ect2/ect3/ect4* defective in m^{6}A reader function (Arribas-Hernández et al., 2018). Notice the similarity in the developmental delay (number of leaves at each stage), reduced stature (although aggravated in AmiR-MTA plants), and identical leaf shape (white arrows on photographs and silhouettes of the first true four leaves): triangular blade with more serrations than in wild-type (Col-0 WT) leaves. Plants were grown side by side in Percival growth chambers at 21°C/18°C (day/night) and with a long-day (16 h) light regime. DAG, Days after germination. Bars = 1 cm.

BOX 3. Molecular basis of trichome phenotypes in *ect2* knockouts

The mRNAs encoding the trichome morphogenesis factors *TTG1*, *ITB1*, and *DIS2* were identified by ECT2 formaldehyde crosslinking-immunoprecipitation as targets of ECT2. Those mRNAs were also found to be less abundant in *ect2* mutant seedlings and to turn over more rapidly than in wild type, prompting the model that the defects in stabilizing these mRNAs underlie the trichome branching phenotype of *ect2* (Wei et al., 2018b). Phenotypic similarity between the trichomes of *ect2*, *ttg1*, *itb1*, and *dis2* mutant plants would be expected for this model to be true, but an examination of the available literature shows that this criterion is not fulfilled:

TTG1 is required for specification of epidermal hair fate, such that *ttg1* mutants fail to produce trichomes altogether (Koornneeff et al., 1982), but no available evidence links *TTG1* function to number of trichome branches (Pattanaik et al., 2014). *itb1* and *dis2* mutant plants produce distorted trichomes (Saedler et al., 2004; Zhang et al., 2005), but their defects differ from those in m^{6}A-pathway mutants, and so does the molecular bases of the deficient morphogenesis: trichome branches of *dis2* and *itb1* mutant plants expand unevenly and do not converge to a single spot on the stalk, often adopting a spike-like appearance.

However, at least for *itb1* plants, the number of branches is not significantly different than those of wild type plants, consistent with the proposed functions in cytoskeleton organization for *ITB1* and *DIS2* (Hülskamp, 2004; Saedler et al., 2004; Zhang et al., 2005). In contrast, the aberrant trichomes described for *ect2*, *ect3*, *ect2/ect3*, and post-embryonic *mta* knockdown possess well-extended branches and do not present morphological defects other than the appearance of supernumerary ramifications (Bodi et al., 2012; Arribas-Hernández et al., 2018; Scutenaire et al., 2018; Wei et al., 2018b). This phenotype is characteristic of increased levels of ploidy due to additional endoreduplication cycles (Hülskamp, 2004). Indeed, Scutenaire et al. (2018) showed that the DNA content of 4-spiked trichomes in *ect2* mutants is about 2-fold higher than that of 3-spiked trichomes of wild type plants, suggesting that exit from endoreduplication in developing trichomes is delayed in *ect2* mutants. Hence, a coherent model for control of trichome development via m^{6}A-ECT2/ECT3 must include regulation of targets with the capacity to control the timing of cell cycle exit.

(Bodi et al., 2012). No other developmental phenotypes could be observed, suggesting that YTHDF reader function is partly involved in specifying branch numbers on trichomes but failing to make a case for reader function in the processes of clear biological importance unveiled by the studies of m^{6}A-deficient plants (Fig 2; Vespa et al., 2004; Zhong et al., 2008; Bodi et al., 2012; Shen et al., 2016; Růžička et al., 2017). In contrast, the simultaneous knockout of three YTHDF proteins, ECT2, ECT3, and ECT4, offered substantial additional insights (Arribas-Hernández et al., 2018). This work demonstrated that the slow development and aberrant leaf morphology in postembryonic *mta* knockdown plants can be largely recapitulated upon knockout or inactivation of m^{6}A binding of ECT2/3/4, strongly suggesting that the control of these important developmental processes by m^{6}A requires this specific set of YTH domain proteins (Figs. 2 and 4). This study also revealed that both ECT2 and ECT3 play similar roles in the definition of trichome branch numbers and that the combined *ect2/ect3* knockout has a much stronger defect than either single mutant, even exceeding the severity observed upon knockdown of m^{6}A (Bodi et al., 2012; Arribas-Hernández et al., 2018). Thus, at least for the phenotypes of slow postembryonic growth, defective leaf morphogenesis, and definition of trichome branch numbers, the YTHDF proteins ECT2 and ECT3, in some cases also ECT4, are responsible for a considerable part of the effects dictated by m^{6}A deposition. For the effects of m^{6}A in other developmental processes (see Fig. 2 for a compilation), we do not have the answers yet.

Only a single study has attempted to answer our question 4. Wei et al. (2018b) used formaldehyde crosslinking followed by ECT2 immunoprecipitation and sequencing to identify ECT2 mRNA targets. Nearly one-third of all expressed genes were identified as mRNA targets using this approach. These targets also

included the known trichome regulators *TRANSPARENT TESTA GLABRA1* (At5g24520), *IRREGULAR TRICHOME BRANCH1* (At2g38440), and *DISTORTED TRICHOME2* (At1g30825), whose mRNAs were less stable in *ect2* mutants than in the wild type, perhaps accounting for their ~0.6 to 0.8 times lower levels, as measured by quantitative reverse transcription PCR from RNA of whole seedlings. Hence, a model was proposed in which ECT2 stabilizes these mRNAs and their decreased expression in *ect2* mutants underlies the increased trichome branch number phenotype (Wei et al., 2018b). As appealing as this model may seem at first sight, some problems are apparent with the evidence supporting it. First, the study did not recover the consensus RR[m^{6}A]CH motif identified in multiple m^{6}A-seq studies (Box 2) as enriched around ECT2 cross-linking sites. This question is more pertinent because formaldehyde, not UV light, was used as a cross-linker. Upon reaction with Lys side chains, formaldehyde forms a Schiff base intermediate that readily reacts with nucleophiles in either proteins or RNA, thereby facilitating indirect cross-links to RNA via other proteins (Hoffman et al., 2015). Thus, many targets identified by formaldehyde-CLIP may not be direct. Second, the transcriptomic analysis measuring decay rates and steady-state abundance were done with entire seedlings, not necessarily informative for gene expression changes in the few cells that matter for the described phenotype: trichomes at an early stage of development. Third, the function of the genes whose decreased abundance in *ect2* mutants is proposed to underlie the stochastic increase in trichome branches does not match with the morphological and molecular phenotypes observed (Box 3; Koornneeff et al., 1982; Hülskamp, 2004; Saedler et al., 2004; Zhang et al., 2005; Pattanaik et al., 2014; Arribas-Hernández et al., 2018; Scutenaire et al., 2018; Wei et al., 2018b). For these reasons, we consider it fair to conclude that the evidence is strong that the YTHDF proteins ECT2, ECT3, and ECT4 mediate some m^{6}A-dependent effects on growth and development in a manner that requires m^{6}A binding. On the other hand, how precisely they do so remains much less well defined at present.

CONCLUDING REMARKS

We are just starting to elucidate how mRNA modifications and m^{6}A in particular are at the core of plant development (see Advances Box), response to abiotic stress, and antiviral defense. Although it has not been addressed yet, modulation of growth in response to other phytopathogens and herbivory is likely to be another function. With m^{6}A-YTH axes as the fundamental regulatory units, we now have the guidelines and the tools to find precise answers to how m^{6}A exerts its regulatory functions. Plants might be particularly adept at exploiting the regulatory capacity of m^{6}A, since they grow and develop throughout their life span in the face of a changing environment. They adapt their growth pace in different organs to shape their bodies in unique ways according to environmental cues: root- and stem-branching patterns, leaf shape and size, number of reproductive organs, and overall architecture are dynamically remodeled according to light, water, and nutrient availability, herbivory, and pathogen attack. The expanded families of YTHDF and ALKBH members in plants (Fig. 2) may reflect this necessity (see Outstanding Questions Box). By tissue- or stimulus-dependent expression of these growth regulators, plants may be able to balance the growth rate and final size of different organs in response to different conditions. Potentially, additional mRNA modifications could be combined with m^{6}A to establish a sophisticated network for growth control (see Outstanding Questions Box). A challenge of the next few years will be to clearly define the molecular workings of these potent gene regulatory systems to enable precise tests of this hypothesis of dynamic plant growth control via mRNA modification systems.

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OUTSTANDING QUESTIONS

- How many mRNA modifications exist in plant mRNAs; for what, if anything, are they important; which mRNAs are modified; and what are the molecular mechanisms underlying the functions of these modifications?
- In particular, are RNA modifications in plants implicated in self/non-self distinction of RNA, and how do RNA modifications on viral RNA influence outcomes of plant-virus interactions?
- Which molecular determinants in addition to occurrence of the RRACH motif lead to co-transcriptional methylation of specific pre-mRNAs by the m^{6}A methyltransferase in plants?
- How large a fraction of m^{6}A-dependent gene control is accounted for by the action of YTH domain proteins in plants, and in eukaryotes in general? Which other mechanisms are used?
- How do YTH domain proteins affect stability and translatability of mRNA?

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