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The Arabidopsis epitranscriptome

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The most prevalent internal modification of plant messenger RNAs, N^6-methyladenosine (m^6A), was first discovered in the 1970s, then largely forgotten. However, the impact of modifications to eukaryote mRNA, collectively known as the epitranscriptome, has recently attracted renewed attention. mRNA methylation is required for normal Arabidopsis development and the first methylation maps reveal that thousands of Arabidopsis mRNAs are methylated. Arabidopsis is likely to be a model of wide utility in understanding the biological impacts of the epitranscriptome. We review recent progress and look ahead with questions awaiting answers to reveal an entire layer of gene regulation that has until recently been overlooked.

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The Arabidopsis genome: from both sides now?

The landmark sequencing of the *Arabidopsis thaliana* (Arabidopsis) genome in 2000, still leaves us asking what the genome really encodes? Subsequent sequencing of Arabidopsis RNAs reveals alternative transcription start sites, alternative splicing and alternative sites of cleavage and polyadenylation of RNAs transcribed from the same locus. The more we sequence in different genotypes, cell-types and situations, the more evidence of alternative processing we detect, and the more non-protein-coding RNAs of unknown function we find. But that's not all. Although we have sequenced from both sides now, DNA and RNA, the modifications of mRNA, known as the epitranscriptome, have been almost completely overlooked. Yet we know mRNA methylation is essential for Arabidopsis embryogenesis [1], and crucial to proper development [2]. Consequently, the control and impact of an entire layer of gene regulation awaits discovery.

Methylating mRNA m^6A

The most prevalent internal modification of eukaryotic mRNA is methylation of adenosine at the N^6 position (m^6A). Although first discovered in mammalian [3,4] and plant [5,6] mRNAs in the 1970s, it is only recently that m^6A has been mapped transcriptome-wide and that functions for m^6A have been uncovered [7•,8•].

Mapping m^6A modifications

Base-specific identification of m^6A is technically challenging and not yet feasible transcriptome-wide. Instead, the current state-of-the-art involves using antibodies that recognize m^6A to immunoprecipitate and then sequence RNAs carrying this modification in a procedure known as MeRIP-Seq [9]. Because the specificity of anti-m^6A antibodies is variable, such experiments should ideally be controlled for by sequencing genetic backgrounds defective in mRNA methylation [10•], and possibly in conjunction with cross-linking of the methylating enzymes to reference the directness of the reactions involved.

m^6A is mostly associated with the 3′ end of Arabidopsis mRNA transcripts (within 150 nt of the poly(A) tail) [2]. Subsequent MeRIP-Seq transcriptome-wide mapping studies with yeast, human and Arabidopsis mRNA confirm this phenomenon, revealing a peak of methylation in 3′UTRs and over the stop codon [10•,11,12••]. A consensus methylation target sequence closely related to that derived for human data of (G/A)(G/A)**A**CU, is found in different eukaryotes and is consistent with a previously established methylation motif identified by *in vitro* enzyme activity, RNAse fragmentation and labelling studies [13], and by direct biochemical mapping of sites in Rous sarcoma virus and bovine prolactin transcripts [14,15].

Although enriched in 3′UTRs, m^6A is found throughout mRNAs, including intronic sequences, indicating the modification may be added co-transcriptionally [16]. It is not yet known whether nascent transcripts are more widely methylated and then subsequently demethylated in specific regions. In addition to the 3′ enrichment, Arabidopsis MeRIP-Seq data indicates an increased abundance of m^6A at the 5′ end of some transcripts. However, an association of m^6A with long exons that has been found in mammalian mRNA was not reported in the first Arabidopsis MeRIP-Seq data [12••].

Figure 1
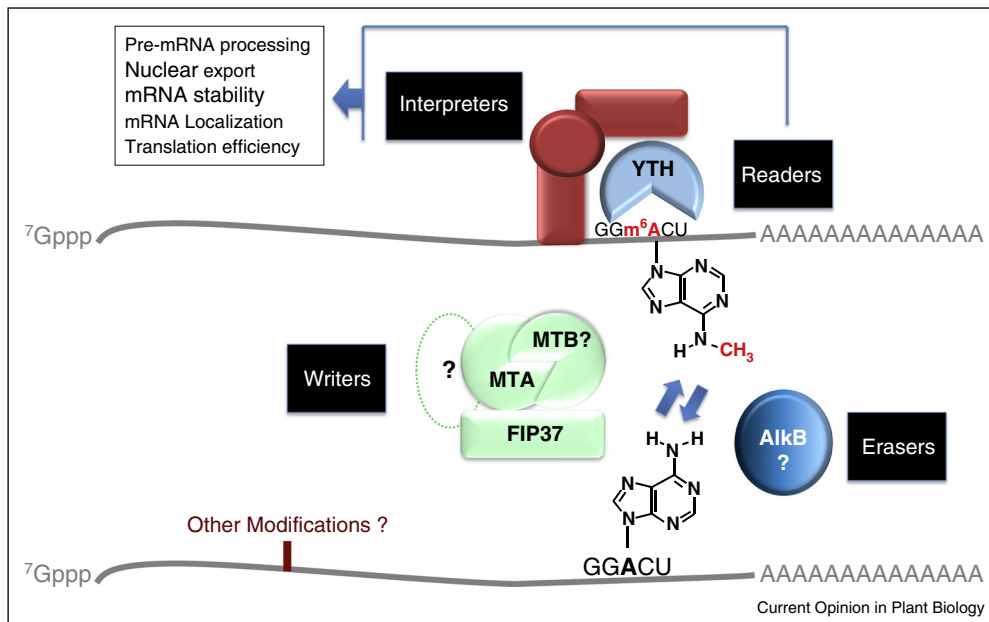
The Arabidopsis epitranscriptome. The most prevalent internal modification of eukaryote mRNA is methylation of adenosine at the N^6 position. Although m^6A is found throughout mRNA it is enriched towards the 3′ end of Arabidopsis mRNAs. This code is written by a writer complex comprised of MTA, FIP37 and probably other proteins that likely include a protein closely related to MTA called MTB. In humans this modification is apparently reversible through the action of AlkB family proteins. Although related AlkB family proteins exist in Arabidopsis, there is no evidence yet that they are involved in this process. m^6A is read by YTH domain proteins, but the combination of direct and indirect influences on RNP composition and structure determine mRNA fate. Other modifications to Arabidopsis mRNA likely remain to be discovered.

It is clear that mRNA transcribed from thousands of Arabidopsis genes is methylated, but due to the limitations of MeRIP-Seq, the best peak-calling data correspond to the most abundant RNA transcripts. Further Arabidopsis MeRIP-Seq studies and alternative validation approaches will be needed to help clarify the RNA methylome.

Writing m^6A

The Arabidopsis enzyme MTA (TAIR: At4g10760) is required for mRNA methylation [1]. Null mutant alleles are embryo lethal, indicating that mRNA m^6A is essential for plant survival [1]. Expressing MTA under the control of the largely embryo-specific *ABI3* promoter rescues lethality of null *mta* mutants and the plants go on to complete seed-set [2]. m^6A levels in these plants are reduced to 5–15% levels of wild-type, confirming the requirement for this enzyme to methylate mRNA m^6A.

It seems likely that a conserved complex of proteins mediates m^6A mRNA methylation in different eukaryotes. MTA interacts with FIP37 in plants [1], and subsequently, the interaction of the human (METTL3 and Wilm's Tumor Associated Protein, WTAP, respectively) and yeast (IME4 and Mum2 respectively) homologs have been shown to be integral to the formation of functional methylation complexes [17–19]. MTA and FIP37 are predominantly nuclear localized [1], indicating that m^6A modification takes place in the nucleus. However, the possibility that some methylation of mRNA might occur in the cytoplasm cannot be ruled out at this stage. The exact composition of the writer complexes, their regulation and the degree of conservation remains to be determined. It is clear that not all mRNAs are methylated and not all potential consensus target sites are methylated either. However, the mechanistic or regulatory basis of m^6A selectivity is unknown.

Reading and interpreting m^6A
m^6A can directly influence the stability or conformation of RNA in the absence of RNA binding proteins [20]. In addition, m^6A can be 'read' directly by YTH domain containing proteins that bind this modification specifically [21•]. There are 13 Arabidopsis genes predicted to encode YTH domain containing proteins, but their functions are almost wholly uncharacterized [22,23]. Structural analyses indicate that a cage of aromatic amino acids in the YTH domain binds m^6A [24,25]. Notably, the corresponding aromatic residues are conserved in each predicted Arabidopsis YTH domain protein, suggesting that they have the potential to bind m^6A. Transcripts

encoding Arabidopsis YTH domain proteins show distinct developmental expression patterns and responses to abiotic and biotic stresses [23].

One Arabidopsis YTH domain protein is relatively well characterised: the Arabidopsis homologue of mRNA Cleavage and Polyadenylation Specificity Factor 30 (CPSF30) [26•]. It has recently been shown in mammalian cells that CPSF30 plays a crucial role in cleavage and polyadenylation because together with WDR33 it appears to be involved in binding to and selecting the poly(A) signal AAUAAA [27,28]. Remarkably, although CPSF30 is highly conserved, the YTH domain appears to be restricted to plant CPSF30 [29]. Moreover, the inclusion of the YTH domain depends upon alternative polyadenylation of *CPSF30* pre-mRNA [30]. Expression of the proximally polyadenylated CPSF30 isoform lacking the YTH domain in *oxt6* mutants defective in CPSF30 function complements some defects in RNA 3′ end formation, while expression of the full-length CPSF30 isoform that includes the YTH domain complements other 3′ end defects [30]. Together, these findings raise the possibility that 3′ end formation in plants may be particularly sensitive to m^{6}A methylation.

m^{6}A and also its recognition by YTH domain proteins likely alters the structural and ribonucleoprotein landscape of mRNA such that the accessibility of other RNA binding proteins is affected [31]. In this sense, mRNA methylation is not only read, but interpreted in terms of the impact that it has on subsequent, specific processing events. For example, it is possible that there are RNA binding proteins whose affinity for RNA is diminished by the presence of m^{6}A within their preferred binding sites. Such 'anti-readers' could have an equally important role in determining m^{6}A-dependent phenotypes.

Erasing m^{6}A
An important aspect of the concept of a regulated epitranscriptome is that m^{6}A mRNA methylation is reversible. Two human enzymes of the AlkB family (nonheme Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases), FTO and ALKBH5, have been shown to demethylate mRNA m^{6}A [32,33]. Thirteen proteins of the AlkB family have been reported to be encoded in the Arabidopsis genome [34], but it is not yet known if any function to demethylate m^{6}A. AlkB proteins were originally characterised as dioxygenases required for the repair of glycosidic bonds introduced into DNA as a result of exposure to alkylating agents. However, some AlkB family members have much wider roles, including demethylation of DNA by the stepwise oxidation of 5 methylcytosine [35]. Consequently, the specificity of these enzymes on different substrates *in vitro* needs to be tested, and evidence of increased m^{6}A levels associated with disruption of the corresponding gene obtained before they can be considered as m^{6}A demethylases.

Interestingly AlkB proteins with a demonstrated preference for RNA substrates are encoded in some viruses that infect plants [36]. Although these enzymes may function to repair the RNA genomes of these viruses, an untested possibility is that they may target host mRNAs during infection. The interplay between the epitranscriptome and pathogen interactions is unexplored (Figure 1).

The dynamic nature of m^{6}A methylation
If m^{6}A is regulatory, one might expect it to be dynamic. There is so far insufficient data to know if this is the case in Arabidopsis. However, the global levels of m^{6}A is different in specific Arabidopsis tissues indicating it may be under some level of control [2].

MeRIP-seq data exists for two different Arabidopsis accessions. Although many of the methylated transcripts are common, strain-specific differences were also detected [12••]. A comparison of differential expression between the two strains indicated that highly expressed transcripts were more likely to be methylated. This finding apparently differs from a role for m^{6}A in targeting human mRNAs for degradation [21•]. However, this distinction appears to be associated with m^{6}A modifications enriched at the 5′ end of Arabidopsis transcripts where the target consensus sequence may also be different [12••]. Arabidopsis may therefore exhibit plant-specific features and consequences of m^{6}A methylation.

Is that all there is? Other mRNA modifications
In addition to N^6 methyladenosine, around 150 modifications of RNA exist and other modified mRNA nucleotides are known in eukaryotes. Some of these are likely specific to metazoans, but it seems probable that others will also be found in plants.

For example, most eukaryote mRNAs are modified at their 5′ end by the addition of a 7-methyl guanosine (m^{7}G) cap in a 5′ to 5′ pyrophosphate linkage to the first nucleoside of the nascent transcript. If no additional modifications are made to the cap-adjacent nucleotides, the structure is referred to as a cap 0. In metazoans, the first or first and second nucleotides following the cap are frequently modified by methylation on the ribose in the 2′ position to form cap 1 or cap 2 structures [37]. However, homologues of the animal cap 1 and cap 2 methylases are not found in Arabidopsis [38] and only cap 0 mRNAs are found. Likewise, inosine has been reported in the mRNAs of many metazoans. Since inosine is read as a guanosine upon reverse transcription, it can be mapped in RNA sequencing data. Inosine is formed post transcriptionally by the action of adenosine deaminases (ADARs) acting on the N^6 amino group of adenosine. However, Arabidopsis lacks homologues of these ADARs, and inosine has not been reported in plant mRNAs.

It is an open question whether other modifications are found in Arabidopsis mRNAs. Pseudouridine [39,40] and 5-methylcytosine [41,42] have recently been reported in yeast and mammalian mRNAs. Proteins related to the NSun family of methyltransferases implicated in cytosine methylation in mammals [43] are encoded by the Arabidopsis genome. $2'O$ methylation is a common modification in rRNA and tRNA that has also been reported within the body of mRNAs. Indeed, the first biochemical mapping of m[6]A within bovine prolactin identified a $2'O$ methylated uridine just 42 nucleotides upstream of the m[6]A site [15]. Analysis of a partially characterised non-standard nucleotide detected at low levels within the poly(A) tails of maize mRNA transcripts [44] could perhaps be explained by misincorporation of deoxy adenosine by poly(A) polymerase.

Future directions — more questions than answers

The epitranscriptome is a field wide open to progress. The first maps of m[6]A have appeared, but a method that allows site-specific transcript mapping is needed to allow quantitative analyses.

We know some of the enzymes involved in writing m[6]A, but almost nothing of the wider complex, its interactions or its regulation. We still do not know if any of the conserved reader YTH domains or related eraser AlkB proteins carry out epitranscriptome related functions in plants. If they do, then how are the functions of the 13 different Arabidopsis YTH domain proteins specialised? Do all of the YTH proteins bind to m[6]A with the same affinity and do they have complimentary, synergistic or competitive functions? If YTH proteins recognize the m[6]A, do the different YTH readers interact with different protein 'interpreters'?

In what situations is the epitranscriptome dynamically controlled? Does the reprogramming of gene expression during development or stress responses depend upon methylated transcripts? And what are the consequences of disrupting mRNA methylation on pre-mRNA processing, export, translation and stability?

We do not yet know if any other modifications are found in Arabidopsis mRNA. Beyond 5-methylcytosine and pseudouridine (already identified in yeast and mammalian mRNA), there are around 150 known RNA modifications. However, the recent development of the 'HAMR' software by Li-Sang Wang, Brian Gregory and colleagues enables the identification in RNA-Seq data of modifications that affect reverse-transcriptase activity [45••]. Consequently, we may soon have maps of transcriptome features we never knew we were missing. Revealing the epitranscriptome landscape and defining its functional impact will be essential for us to understand what plant genomes really encode.

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