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Chapter 11
Chemical RNA Modifications: The Plant Epitranscriptome

Celso Gaspar Litholdo Jr and Cécile Bousquet-Antonelli

Abstract RNA post-transcriptional modifications create an additional layer to control mRNA transcription, fate, and expression. Considering that they are non-genetically encoded, can be of reversible nature, and involved in fine-tuning gene expression, the landscape of RNA modifications has been coined the "RNA epigenome" or "epitranscriptome." Our knowledge of the plant epitranscriptome is so far limited to 3′-uridylation and internal m^6A and m^5C modifications in Arabidopsis. m^6A is the most abundant and well-studied modification on mRNAs, and involves the activities of evolutionarily conserved "writer" (methyltransferase), "reader" (RNA binding proteins), and "eraser" (demethylases) proteins. In Arabidopsis, m^6A is crucial for embryogenesis, post-embryonic growth, development, phase transition, and defense responses. Conversely to animals, our understanding of the roles of m^6A is limited to the finding that it is an mRNA stabilizing mark. Yet likely to exist, its roles in controlling plant mRNA maturation, trafficking, storage, and translation remain unexplored. The m^5C mark is much less abundant on the transcriptome and our knowledge in plants is more limited. Nonetheless, it is also an important epitranscriptomic mark involved in plant development and adaptive response. Here, we explore the current information on m^6A and m^5C marks and report knowledge on their distribution, features, and molecular, cellular, and physiological roles, therefore, uncovering the fundamental importance in plant development and acclimation of RNA epigenetics. Likely to be widespread in the green lineage and given their crucial roles in eukaryotes, the fostering of data and knowledge of epitranscriptome from cultivated plant species is of the utmost importance.

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C. G. LitholdoJr (✉) · C. Bousquet-Antonelli (✉)
Centre National pour la Recherche Scientifique (CNRS) - Laboratoire Génome et Développement des Plantes (LGDP - UMR5096), Perpignan, France

Université de Perpignan Via Domitia (UPVD) - Laboratoire Génome et Développement des Plantes (LGDP - UMR5096), Perpignan, France
e-mail: celso.litholdo@univ-perp.fr; cecile.antonelli@univ-perp.fr

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11.1 Chemical RNA Modifications: A New Layer of Post-transcriptional Regulation

The pattern of gene expression of a cell is what determines its identity and activity. Maintaining its homeostasis is hence crucial for any organism. However, cells must also respond to developmental and environmental stimuli for organisms to develop and grow, or to acclimate to external conditions. In such cases, their pattern of gene expression needs to be adjusted, occasionally very fast. This reprogramming takes place simultaneously at the transcriptional (Kaufmann et al. 2010; de Nadal et al. 2011; Lelli et al. 2012) and post-transcriptional levels (Mata et al. 2005; Zhao et al. 2017; Schaefke et al. 2018). Post-transcriptional regulation is exerted at pre-messenger RNA (pre-mRNA) maturation (including transcription termination/polyadenylation and splicing), mRNA intracellular trafficking (including nucleocytoplasmic and sub-compartment localization), storage, stability, and translation. Regulation of the transcriptome is dependent on the primary genetic code, which provides local structures and short sequences, either for binding of proteins that form with the messenger RNA RiboNucleoProtein (mRNP) complexes or for complementary recognition by microRNAs (miRNAs).

In the last couple of years, the scientific community regained interest in RNA (in particular mRNA) chemical modifications, and recognized that they create an additional layer to the control of mRNA transcription and fate. Considering that RNA modifications are non-genetically encoded, they can display a reversible nature, and fine-tune the fate and expression of transcripts harboring them. The landscape of modifications deposited on the transcriptome (in particular on mRNAs) of a cell has been coined the "RNA epigenome" (He 2010) or "epitranscriptome" (Meyer et al. 2012; Saletore et al. 2012).

In all three domains of life (Archaea, Bacteria, and Eukarya) as well as in viruses, RNAs carry chemical modifications. More than 110 distinct modifications (http://mods.rna.albany.edu/mods/) have been recognized across all domains of life and across all types of RNAs [mRNAs, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), long noncoding RNAs (lnRNA), circular RNAs (circRNAs), and small noncoding RNAs (sRNAs)] but the roles of the vast majority of them remain unknown (Li and Mason 2014). Although highly debated until 2012, the existence of chemical modifications deposited on mRNAs is now well recognized and documented in several eukaryotes, such as yeast, mammals, and plants, as well as recently in bacteria (Deng et al. 2015; Hoernes et al. 2015). In addition to the 5′ cap and 3′-poly(A) tail, eukaryotic mRNA 3′-extremities can be modified by the non-templated addition of uridines (uridylation; de Almeida et al. 2018a) and/or carry internal modifications, which can be of over 15 different types (http://mods.rna.albany.edu/mods/; Song and Yi 2017).

The most common of the internal nucleotide modifications consists in the addition of a methyl group to the 2′-O position of the ribose moiety. In addition, up-to-date transcriptome-wide mapping on mRNAs and functional data are available on transcripts that can be edited by deamination of adenosine to inosine (A-to-I editing;

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Yablonovitch et al. 2017; Sinigaglia et al. 2018) or carry N^1-methyladenosine (m^1A; Dominissini et al. 2016), N^6-methyladenosine (m^6A; Dominissini et al. 2013), 5-methylcytosine (m^5C; Squires and Preiss 2010), N^4-acetylcytidine (ac^4C; Arango et al. 2018), pseudouridine (Ψ; Schwartz et al. 2014a; Carlile et al. 2014), or hydroxymethylcytosine (h^5mC) (Fig. 11.1a, b). Additional modifications include the N^6-2′-O-dimethyladenosine (m^6Am) and 5-hydroxymethylcytosine (h^5mC)

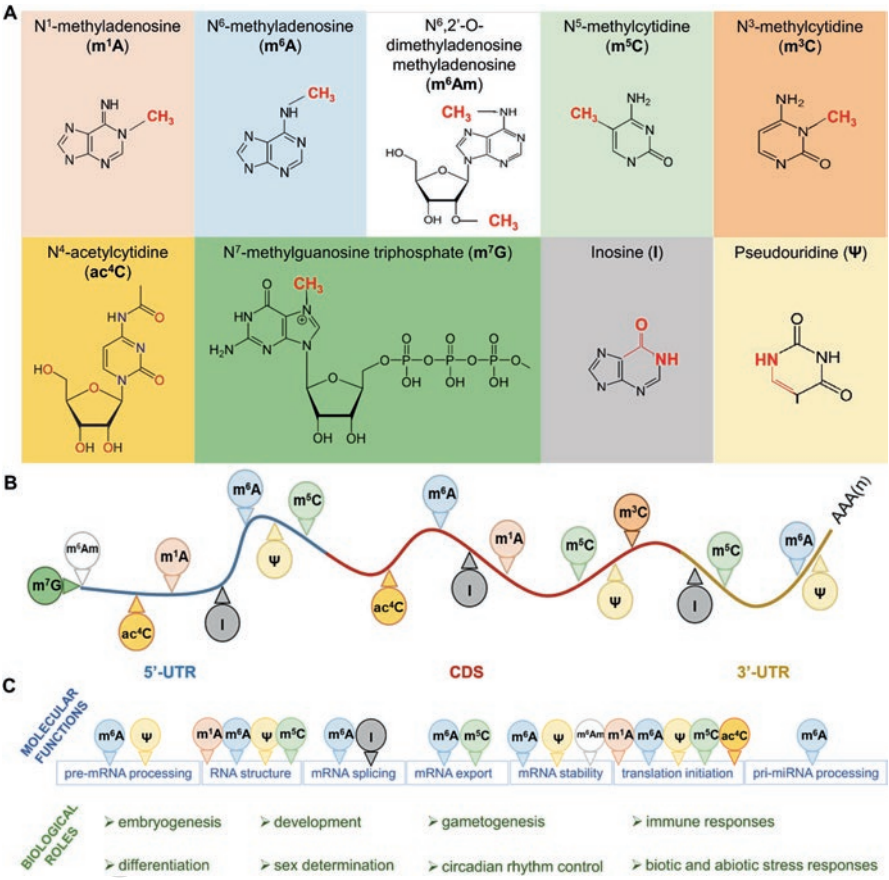

Fig. 11.1 The epitranscriptome landscape. (**a**) The major post-transcriptional modifications deposited on the transcriptome of mammalian cells are N^1-methyladenosine (m^1A), N^6-methyladenosine (m^6A), N^6,2′-O-dimethyladenosine methyladenosine (m^6Am), N^5-methylcytosine (m^5C), N^3-methylcytosine (m^3C), N^4-acetylcytidine (ac^4C), N^7-methylguanosine triphosphate (m^7G), inosine (I), and pseudouridine (Ψ). (**b**) An RNA polymerase II transcribed RNA is represented, including the 5′-cap structure, which is a modified 7-methylguanosine (m^7G) linked via an unusual 5′ to 5′ triphosphate linkage to mRNA, and the 3′-end poly(A) tail (AAAn). For each particular RNA chemical modification, a representation is shown in relation to mRNA position (5′ UTR, blue; CDS, red or 3′ UTR, yellow). (**c**) The molecular consequences of each RNA marks and the biological roles of these modifications are also represented. It is important to mention that only m^6A and m^5C modifications have been identified so far in the plant epitranscriptome AU3

(Song and Yi 2017; Frye et al. 2018). These modifications can regulate all steps of an mRNA life (Fig. 11.1c) and can even recode open reading frames (Powers and Brar 2018). Several were proposed to be of a dynamic nature (i.e., they can be erased) and their profiles found to be distinct across development or in response to stress exposure. At the organism level, RNA modifications are required for differentiation, development, gametogenesis, sex determination, embryogenesis, circadian rhythm control, immune response, biotic and abiotic stress responses (Fig. 11.1c, Sinigaglia et al. 2018; Song et al. 2018).

Except for the 5′-cap and poly(A)-tail, our knowledge of the plant epitranscriptome is so far limited to uridylation (de Almeida et al. 2018a, b), m^6A (Luo et al. 2014; Li et al. 2014c, 2018), and m^5C (Cui et al. 2017; David et al. 2017). Plant mRNAs are likely to carry other types of modifications but their existence and roles remain to be explored. A-to-I editing though is absent from the plant nuclear transcriptome but organelle transcripts (chloroplast and mitochondria) carry C to U edited bases, and in ferns and mosses also U-to-C changes (Takenaka et al. 2013). Excellent reviews have recently been published on the synthesis, molecular, cellular, and physiological roles of uridylation (de Almeida et al. 2018a, b), and organelle editing (Takenaka et al. 2013). We will hence focus the present chapter on the features and functions of the internal m^6A and m^5C modification of messenger RNAs in plants.

11.2 Roles and Features of the m^6A Mark in Plants

11.2.1 General Features of the m^6A Mark

The m^6A mark is the most abundant and widespread of mRNA modifications. It has been profiled on the polyadenylated transcriptome of the yeast *Saccharomyces cerevisiae* (Schwartz et al. 2013) and of various human and mouse cell lines and tissues (Dominissini et al. 2012; Meyer et al. 2012; Fustin et al. 2013; Schwartz et al. 2014b; Wang et al. 2014; Chen et al. 2015). In higher plants, it has been mapped on rice callus and leaves (Li et al. 2014c), in two distinct ecotypes of *Arabidopsis thaliana* (Luo et al. 2014), and in mature leaves (Anderson et al. 2018), 5- and 14-day old seedlings (Shen et al. 2016; Duan et al. 2017) and across several organs (leaves, flowers, and roots; Wan et al. 2015) of Arabidopsis Columbia-0 ecotype. Consistent with the evolutionarily conserved nature of the m^6A mark, several of its features were found to be conserved across organisms and tissues. Transcriptome-wide, m^6A represents 1–1.5% of the total number of adenosines on polyadenylated transcripts. It mostly localizes in the 3′-UTRs, following the stop codon and in the last exons of transcripts (Ke et al. 2015). A nucleotide sequence context around m^6A is shared across eukaryotes. Indeed, m^6A is mainly confined at the consensus RRACH (where R = A/G and H = U > A > C) and found in 70% of the cases at GAC. In mammals at least, the m^6A mark was detected on most, if not all, polymerase II transcribed RNAs, including

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primary transcripts of miRNAs (Alarcon et al. 2015), lnRNAs, circRNAs, and mRNAs (Meyer et al. 2012; Dominissini et al. 2013; Schwartz et al. 2013).

In plants, a thin layer chromatography analysis of the m^6A/A ratio on the polyadenylated transcriptome of Arabidopsis shows that it ranges from 0.9% in roots and leaves to 1.4% in flowers (Zhong et al. 2008) and that it is not randomly distributed, but mostly enriched at the 3′-end of transcripts (Bodi et al. 2012). Subsequently, next generation sequencing (NGS) profiling of the polyadenylated transcriptome found, both in rice and Arabidopsis, that the vast majority of the m^6As peaks occur in the 3′-UTRs or overlap the stop codon (Li et al. 2014c; Luo et al. 2014; Anderson et al. 2018; Shen et al. 2016; Wan et al. 2015). These studies in rice and Arabidopsis also found that 10–15% of the detected m^6A peaks are located around the start codon (Li et al. 2014c; Luo et al. 2014; Shen et al. 2016). The presence of some m^6A marks around the start codon and in 5′-UTR is not restricted to plants, for instance, this has been observed in certain mammalian cells types and growth conditions (Domnissini et al. 2012; Zhou et al. 2015). Most of the m^6A peaks were found to carry the RRACH consensus suggesting that this sequence motif is necessary also in plants for the deposition of the mark. However, recent findings support the idea that m^6A sites could occur in sequence contexts other than RRACH [such as "GGAU" or URUAY (R = G > A, Y = U > A)] in Arabidopsis (Luo et al. 2014; Anderson et al. 2018; Shen et al. 2016; Wei et al. 2018) and rice (Li et al. 2014c). Whether other types of plant RNA polymerase II transcripts (such as pre-miRNAs, lnRNAs, and sRNA) are modified with m^6A remains to be explored.

In mammals and flies at least, the m^6A mark is deposited co-transcriptionally by a conserved heteromultimeric complex called the "writer" complex and can be reverted to unmodified adenines by demethylases tagged as "erasers" (see Sect. 11.2.2; Fig. 11.2). At the molecular level, the most prevalent role of m^6As is to influence the binding of proteins to their RNA targets. They can either act to repel or attract RNA binding proteins (RBPs), the latter of which are known as "m^6A readers" (Arguello et al. 2017; Edupuganti et al. 2017). Readers convey the m^6A signal by directly controlling the fate of their RNA target and/or by recruiting effector proteins. The m^6A mark recruits readers by two main processes. First, the reader may carry a YTH domain, an evolutionarily conserved RNA binding motifs whose folding forms a pocket that tightly accommodates the m^6A residue (see Sect. 11.2.4; Fig. 11.2). Alternatively, the presence of m^6A may positively influence the recruitment of RBPs by: (1) increasing their affinity for their RNA binding region, or (2) acting through alteration of RNA structures in a mechanism called "m^6A-switch" (Zhou et al. 2016; Roost et al. 2015; Liu et al. 2015).

11.2.2 The Plant Writer and Eraser Systems

In 1994, Bokar and colleagues characterized and partially purified an mRNA N^6-methyltransferase from HeLa cell nuclei. They found that it comprises a multisubunit complex composed of two fractionable subcomplexes: MT-A (200 kDa) and

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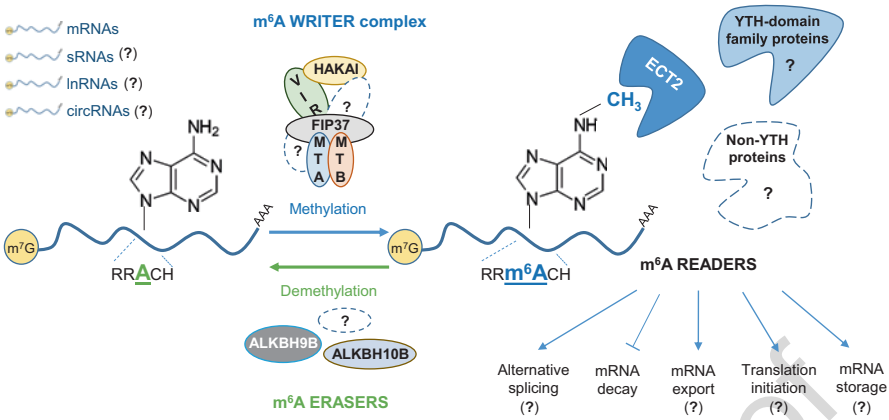

Fig. 11.2 The m^{6}A modification regulatory system. The m^{6}A mark is found in most, if not all, RNA polymerase II transcribed RNAs, including messenger RNAs (mRNAs), small RNAs (sRNAs), long noncoding RNAs (lnRNAs), and circular RNAs (circRNAs); except the latter, all contain the modified m^{7}G nucleotide at the 5′-end and poly(A) tail at the 3′-end. A nucleotide consensus sequence RRACH (R = A/G, H = U > A > C) is mainly the site for the m^{6}A writer complex, which includes the subunit methyltransferase proteins MTA, MTB, FIP37, VIR, and HAKAI. m^{6}A-RNA pol II RNA demethylation is carried by two eraser enzymes, ALKBH9B and ALHBH10B. So far only m^{6}A readers carrying a YTH-RNA binding domain have been identified in plants, which include the recently characterized ECT2 protein. The molecular role of m^{6}A mark depends on the reader protein that binds to the modified nucleotide, generally in animals, directing the RNA to alternative splicing, mRNA decay, mRNA export, translation initiation, or mRNA storage. Question mark (?) indicates the unknown features of the plant m^{6}A regulatory system

MT-B (875 kDa) containing the S-adenosyl-methionine-binding site and the RNA binding site, respectively (Bokar et al. 1994). The MT-A subcomplex carries on a 70 kDa component, the methyltransferase player that was identified and named MT-A70 (Bokar et al. 1997). MT-A70 is conserved across eukaryotes and is known as METTL3 in mammals (Liu et al. 2014), IME-4 (Inducer of Meiosis-4) in *S. cerevisiae* (Yadav and Rajasekharan 2017) and *Drosophila melanogaster* (Lence et al. 2016), and MTA70 in *A. thaliana* (Zhong et al. 2008).

Purification of the writer complex from animals (human and fly) confirmed that it is an heteromultimeric complex, whose catalytic core is composed of two RNA methyltransferases (METTL3 and METLL14) and the cofactor WTAP (fly Fl(2)d). METTL3 and 14 physically interact with each other and their association has a synergetic effect on the complex catalytic activity (Liu et al. 2014). METTL3 is the catalytically active component while METTL14, which has a degenerate methyltransferase site, plays a scaffolding role that is critical for substrate recognition (Wang et al. 2016; Śledź and Jinek 2016). The animal writer complex contains other subunits: VIRMA (fly Virilizer), RBM15/RBM15B (fly spenito), Z3CeH13 (fly Xio/Flacc), and HAKAI (Ping et al. 2014; Yue et al. 2018; Haussmann et al. 2016; Lence et al. 2016; Guo et al. 2018; Knuckles et al. 2018; Patil et al. 2016). Zc3H13 bridges the mRNA binding factor RBM15 to WTAP (Knuckles et al. 2018) and

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VIRMA mediates preferential methylation by recruiting the METTL3/METTL4/WTAP core complex to 3′-UTRs and near the stop codons (Yue et al. 2018).

Up to now, data on the plant writer complex comes from *A. thaliana* (Table 11.1; Zhong et al. 2008; Bodi et al. 2012; Ruzicka et al. 2017). Following the discovery by Bokar et al. (1997) that the methyltransferase activity of the writer complex was carried by METTL3, further characterization of the complex remained incomplete. It is in 2008 that the team of Rupert Fray ran the first functional study of an MTA70 protein and also identified FIP37 (the Arabidopsis homolog of WTAP) as a component of the writer complex (Zhong et al. 2008). Further biochemical characterization of the Arabidopsis writer complex showed that it also contains MTB (the plant homolog of METTL14), VIRILIZER, and HAKAI (Ruzicka et al. 2017). The Arabidopsis writer complex hence closely resembles the animal complex, but, whether it contains additional factors in particular homologs of RBM15 and Z3CeH13 remains to be explored. Every component of the Arabidopsis complex is found in the nucleoplasm. However, their nucleoplasmic distribution changes between root meristematic cells and cells in the root elongation zone. While showing a nucleoplasmic diffuse pattern in non-differentiated cells, they localize to nuclear speckles in dividing cells (Ruzicka et al. 2017). These observations support the idea that m^6A deposition is likely co-transcriptional in plants, as in animals, and that the activity of the writer complex might be regulated. Total or partial loss of any of the five components, except for HAKAI, of the Arabidopsis writer complex drastically decreases the total levels of m^6A in polyadenylated transcripts (Zhong et al. 2008; Ruzicka et al. 2017). HAKAI is not required for plant viability (see Sect. 11.2.3) and shows only a 35% reduction of m^6A levels in loss-of-function mutants. Except for MTA70, which based on evolutionary analyses (Bujnicki et al. 2002) is a *bona fide* methyltransferase and homolog to METTL13, the molecular roles that other components carry out inside the writer complex remain to be uncovered in plants.

The m^6A epitranscriptomic mark was proposed to be dynamic following two reports that identified mammalian FTO (fat mass and obesity) and ALKBH5 (the alkylation repair homolog protein) as specific RNA m^6A demethylases, both *in vitro* and *in vivo* (Jia et al. 2011; Zheng et al. 2013). They both belong to the AlkB subfamily of Fe(II)/ α-Ketoglutarate-dependent dioxygenases superfamily that has 9 members (ALKBH1-8 and FTO) in humans (Xu et al. 2014a). Enzymes of the ALKB family excise the methyl group through a two-step oxidative alkylation process and can act on DNA or RNA. Both FTO and ALKBH5 are found in nuclear speckles, suggesting that erasing of mRNA m^6A is mostly nuclear (Jia et al. 2011; Zheng et al. 2013). In mice, loss of FTO leads to increased m^6A levels and is associated with several metabolic disorders and cell differentiation (Zhoa et al. 2014), while loss of ALKBH5 also affects m^6A levels and is characterized by impaired fertility resulting from spermatocyte apoptosis (Zheng et al. 2013). These findings indicate that these two demethylases function in different physiological processes and strongly suggest that they are crucial for the development and reproduction.

The Arabidopsis genome codes for thirteen proteins of the ALKB family, among which, based on sequence alignment, five (ALKBH9A, 9B, 9C, 10A, and 10B) are potential homologs of the mammalian ALKBH5 m^6A-RNA demethylase

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(Table 11.1; Mielecki et al. 2012; Duan et al. 2017). The Arabidopsis genome codes for a sixth putative homolog of human ALKBH5 (AtALKBH10C), but it is most likely not an active demethylase as it has a degenerate catalytic site (our unpublished data). Besides Arabidopsis, these enzymes can be found in agronomically important plants, for instance, the presence of *ALKB* demethylase orthologues was detected in *Nicotiana sylvestris* (Li et al. 2018), *Zea mays*, *Oryza sativa*, *Marchantia polymorpha*, and *Solanum lycopersicum*. No homolog of the FTO demethylase was found to exist in plant genomes (our unpublished data). Based on transcript level measurements, *ALKBH9B, 9C,* and *10B* are the most expressed of all five Arabidopsis *ALKBH5* genes. Across development, it is always one (or few) of these three genes, whose transcript levels show the highest expression. In seedlings and leaves (juvenile, adult, and cauline), *ALKBH9B*, *9C,* and *10B* mRNAs show similar levels and are by far the most highly expressed genes. In buds and young siliques, *9B* and *10B* are almost the sole demethylases to be expressed and they show similar levels. Finally, *9B* is nearly the only demethylase expressed in the apical meristem and *10B* is by far the major eraser gene to be expressed in flowers and matured siliques. Recently, *in vitro* assays showed that ALKBH9B and 10B have m^{6}A-demethylase activities on RNA (Duan et al. 2017; Martínez-Pérez et al. 2017) and 10B was shown to have a demethylase activity *in planta* on polyadenylated transcripts (Duan et al. 2017). ALKBH10B-mediated mRNA demethylation is required

Table 11.1 The Arabidopsis m^{6}A modification regulatory system

Function	Name	Arabidopsis locus	Mammalian homolog	Biological role	References
mRNA m^{6}A writer	MTA	AT4G10760	METTL3	Embryo development	Zhong et al. (2008) Bodi et al. (2012)
	MTB	AT4G09980	METTL14	Embryo development	
	FIP37	AT3G54170	WTAP	Meristem maintenance	Shen et al. (2016)
	VIRILIZER	AT3G05680	KIAA1429	Embryo development	Ruzicka et al. (2017)
	HAKAI	AT5G01160	HAKAI	Embryo development	
mRNA m^{6}A eraser	[a]ALKBH9A	AT1G48980	ALKBH5	–	Duan et al. (2017)
	ALKBH9B	AT2G17970	ALKBH5	Viral infection response	Martínez-Pérez et al. (2017)
	[a]ALKBH9C	AT4G36090	ALKBH5	–	Duan et al. (2017)
	[a]ALKBH10A	AT2G48080	ALKBH5	–	
	ALKBH10B	AT4G02940	ALKBH5	Flowering	
	Name	*Arabidopsis locus*	*YT512-B domain YTH-type*	*Biological role*	*References*

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Table 11.1 (continued)

Function	Name	Arabidopsis locus	Mammalian homolog	Biological role	References
YTH m^6A readers	[a]ECT1	AT3G03950	YTHDF	Calcium-mediated signaling	Ok et al. (2005)
	ECT2	AT3G13460	YTHDF	Leaf and trichome morphogenesis	Scutenaire et al. (2018) Wei et al. (2018) Arribas-Hernández et al. 2018
	ECT3	AT5G61020	YTHDF	Leaf and trichome morphogenesis	
	ECT4	AT1G55500	YTHDF	Leaf morphogenesis	Arribas-Hernández et al. 2018
	[a]ECT5	AT3G13060	YTHDF	–	Ok et al. (2005) Scutenaire et al. (2018)
	[a]ECT6	AT3G17330	YTHDF	–	
	[a]ECT7	AT1G48110	YTHDF	–	
	[a]ECT8	AT1G79270	YTHDF	–	
	[a]ECT9	AT1G27960	YTHDF	–	
	[a]ECT10	AT5G58190	YTHDF	–	
	[a]ECT11	AT1G09810	YTHDF	–	
	[a]ECT12	AT4G11970	YTHDC	–	Scutenaire et al. (2018)
	[a]CPSF30-L	AT1G30460	YTHDC	[b]Nutrient uptake/ oxidative stress response	Scutenaire et al. (2018) Li et al. (2017a, b)

[a]These uncharacterized genes are potential players of m^6A regulation
[b]CPSF30-L isoform contains most of the short form polypeptide fused at its C-terminus with a canonical YTH domain of the DC-type; however, it is unknown if the biological role involves the m^6A and the CPSF30-L reader function

for the proper transition from vegetative to reproductive stage. This is at least in part linked to the role of ALKBH10B in demethylating, in a timely manner, transcripts required for the floral transition and as a result stabilizing them (Duan et al. 2017). Arabidopsis ALKBH9B, was so far not found to affect m^6A levels *in vivo*, but one cannot exclude the possibility that it works redundantly with other ALKBH5 orthologues, such as ALKBH9C. ALKBH9B was found to influence m^6A abundance on the viral genome of Alfalfa mosaic virus (AMV) and regulate its infectivity (Martínez-Pérez et al. 2017).

It is important to note that the dynamic nature of m^6A on mRNAs (e.g., the erasing of the m^6A marks on mature cytoplasmic transcripts) is still highly debated in the scientific community (Rosa-Mercado et al. 2017). Nonetheless, mRNA demethylases were found to exist and to be evolutionarily conserved, their downregulation and overexpression shown to significantly alter the pattern of m^6As on the polyadenylated transcriptome, and their loss to have drastic physiological impacts. Hence,

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they have roles to play in m^{6}A-based post-transcriptional regulation, however, where and how do they intervene remains to be understood.

11.2.3 m^{6}A Physiological, Cellular, and Molecular Roles

The biological consequences of m^{6}A methylation are multiple, but a common feature of most organisms is that it has pleiotropic physiological functions and is necessary for reproduction, differentiation, growth, development, biotic and abiotic stress responses. Arabidopsis is no exception to this. Except for HAKAI, loss-of-function and hypomorphic mutants of any of the constituents of the plant writer complex show total to drastic decrease of the levels of m^{6}A on the polyadenylated transcriptome and display identical phenotypes (Ruzicka et al. 2017). Complete loss of the m^{6}A mark results in embryogenesis defects leading to lethality of the embryos, whose development is arrested at the globular stage (Vespa et al. 2004; Zhong et al. 2008; Bodi et al. 2012; Shen et al. 2016; Ruzicka et al. 2017). Downregulation of N^6-methyladenosines at post-embryonic stages has drastic pleiotropic consequences. Plants show delayed growth and development with reduced apical dominance (Bodi et al. 2012; Shen et al. 2016; Ruzicka et al. 2017). Seedlings with reduced levels of m^{6}A show an over proliferation of the vegetative shoot apical meristem (SAM), accompanied by a dramatic delay in leaf emergence and aberrant leaf morphology (Shen et al. 2016; Arribas-Hernández et al. 2018). Plantlets, with very low levels of m^{6}A, fail to develop a reproductive SAM and eventually die (Shen et al. 2016). Hypomethylated plants also show trichome morphogenesis defects, with leaves accumulating overbranched trichomes, due to abnormally high ploidy levels (Vespa et al. 2004; Bodi et al. 2012; Scutenaire et al. 2018). Root growth and development also require normal m^{6}A levels. Indeed, hypomethylated mutants show reduced root growth, aberrant gravitropic responses, abnormal root cap formation, and deficient vascular development (linked to defective protoxylem development).

The m^{6}A mark and its control is also most likely necessary not only for the response of the plant to viral infection (Martínez-Pérez et al. 2017; Li et al. 2018) but also for environmental growth conditions and stress exposure (Luo et al. 2014; Anderson et al. 2018). In Arabidopsis, the viral RNA of AMV was found to have m^{6}A residues upon infection and to be demethylated *in vivo* by ALKBH9B (Martínez-Pérez et al. 2017). Loss of ALKBH9B provokes a hypermethylation of the viral RNA and downregulates AMV replication and infectivity. The current model suggests that m^{6}A could control AMV viral infection by signaling the viral transcript to the nonsense mediated decay (NMD) pathway. This m^{6}A-based response to viral infection is likely not restricted to AMV (Martínez-Pérez et al. 2017), nor to Arabidopsis. Recently, a report by Li et al. (2018) correlated endogenous m^{6}A-levels to tobacco mosaic virus (TMV) infection in *N. tabacum*. Upon infection, global m^{6}A content decreased and the levels of transcripts coding for putative homologs of ALBKH5 and MTA70 were, respectively, up- and downregulated. These observations support a putative m^{6}A-mediated control of viral infection

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in tobacco as well. Methylome profiling of the transcriptomes of two Arabidopsis accessions [Can-0 (from Canary Islands) and Hen-16 (from Northern Sweden)] shows that most methylation peaks are shared by both ecotypes, supporting the crucial role of m^6A-mediated regulation in development. Nonetheless, a portion of the detected methylated sites are specific to each ecotypes, and the presence of m^6A correlates with highest expression levels of the marked genes. Considering the Can-0 and Hen-16 are originally from very distinct climates, one can postulate that m^6A could play a role in plant acclimation to the environment (Luo et al. 2014). Along the same idea, a recent work by the Gregory lab (Anderson et al. 2018), profiled m^6A on the transcriptome of salt treated Arabidopsis leaves and found that upon stress, transcripts coding for salt and osmotic stress response proteins gain m^6A and are stabilized. This supports a role of m^6A in promoting the plant response to stress, at least salinity.

Our understanding of the molecular and cellular bases of m^6A physiological functions in plants is so far quite modest and limited to their role in the control of cytoplasmic mRNA stability (Luo et al. 2014; Shen et al. 2016; Duan et al. 2017; Wei et al. 2018; Anderson et al. 2018). At the global transcriptome scale, m^6A acts to stabilize transcripts by preventing their endonucleolytic cleavage (4–5 nt upstream to the mark) and subsequent 5′-3′ digestion by XRN4, the plant homolog of XRN1 (Anderson et al. 2018). This is coherent with previous observations showing that the m^6A mark correlates with elevated transcript levels (Luo et al. 2014). However, this is opposite to the situation in animals where the m^6A mark is an mRNA-decay triggering signal at the global level (Ke et al. 2015, 2017). This transcriptome-wide observation does not stand for all Arabidopsis mRNAs, as there are cases where the presence of m^6A directs a signal to turnover. Shen et al. (2016) found that the lack of m^6A on two key SAM regulators (*WUSCHEL* and *SHOOTMERISTEMLESS*) prevents the timely degradation of their transcripts and proper regulation of SAM proliferation. Furthermore, ALKBH10B-mediated demethylation was found to stabilize transcripts of *FLOWERING LOCUS T* (*FT*), *SPL3,* and *SPL9*, which are key regulators of the floral transition (Duan et al. 2017).

In animals, m^6A also acts as a translation stimulatory signal, at transcriptome-wide level, and is known to control a handful of alternative splicing events (Lence et al. 2016; Haussmann et al. 2016), directs primary miRNA transcripts to processing (Alarcon et al. 2015), and acts directly on chromatin, where it contributes to DNA repair (Xiang et al. 2017) and to the XIST-dependent gene silencing (Patil et al. 2016). Whether m^6A also acts on these processes in plants remains to be explored.

11.2.4 The Plant m^6A Readers: YTH-domain-Containing Proteins

So far, only one type of m^6A readers has been recognized in plants: those containing YTH domains. The YT521-B Homology domain (YTH) is a highly structured conserved RNA binding domain among eukaryotes. After being first identified as a

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human splicing factor, YT521-B proteins carrying a YTH domain (now called YTHDC1) were further identified and classified as DC type (YTH-domain-containing protein) and DF type (YTH-domain family proteins), depending on the subcellular localization (Imai et al. 1998; Hartmann et al. 1999; Stoilov et al. 2002; Zhang et al. 2010). A recent analysis of YTH domains from yeast, metazoan and Viridiplantae, found that they are of two evolutionary types: the DC-type group comprising YTH domains of human YTHDC1 and 2 and the DF-type group containing human YTHDF1-3 (Scutenaire et al. 2018).

The structural resolution of YTH domains from yeast and animal proteins showed that both DC- and DF-type motifs adopt a conserved canonical fold of three α-helices and six β-strands that creates an aromatic pocket (formed with three highly conserved tryptophan residues) that tightly accommodates m^{6}A (Li et al. 2014a, b; Luo and Tong 2014; Theler et al. 2014; Xu et al. 2014b; Zhu et al. 2014; Xu et al. 2015). Sequence comparisons support that the m^{6}A-binding mode of the YTH domains is largely conserved across eukaryotes (Scutenaire et al. 2018). AU6

In plant genomes, genes coding for YTH-domain proteins experienced a large expansion with thirteen genes in Arabidopsis (Table 11.1). Viridiplantae YTH-proteins also carry DC- and DF-type domains that are further subdivided into two (DCA and DCB) and three (DFA, DFB, and DFC) subgroups, respectively. This observation suggests that plant YTH domain likely underwent neo-functionalization and that they are not fully redundant (Scutenaire et al. 2018).

In plants, all the functional work done on YTH-domain m^{6}A readers is from Arabidopsis. Arabidopsis YTH domain was initially identified in two proteins found to directly bind the CIPK1 (Calcineurin B-like-interacting protein kinase 1) calcium-dependent kinase. Eleven proteins were found to share the YTH domain at their C-terminus and called ECT1 to 11 (for evolutionarily conserved C-terminal region) (Ok et al. 2005). Subsequent searches identified two additional proteins, which are of the DC-type (while ECT1-11 is of DF-type): ECT12 of unknown function and CPSF30-L, which is encoded by the long isoform of the gene encoding CPSF30, the cleavage and polyadenylation subunit factor 30 (Addepalli and Hunt 2007).

The physiological and molecular roles of ECT proteins have been just recently explored with the first functional analysis of a plant m^{6}A reader, the Arabidopsis ECT2 protein. *In vitro* and *in planta* assays showed that ECT2 binds to m^{6}A-containing RNAs and requires an intact aromatic pocket (Scutenaire et al. 2018; Wei et al. 2018). *ECT2* transcript is the most abundant and ubiquitously expressed of all ECTs, nonetheless, the pattern of expression of its protein is distinct (Scutenaire et al. 2018; Wei et al. 2018; Arribas-Hernández et al. 2018). Consistently with its expected role as m^{6}A reader, *ect2* loss-of-function mutants, although not displaying dramatic phenotypes, recapitulate some of the defects observed in hypomethylated plants. First, ECT2 and its m^{6}A-reading activity were found to be required for proper trichome morphogenesis (Scutenaire et al. 2018; Wei et al. 2018; Arribas-Hernández et al. 2018). In the absence of ECT2, or the sole presence of a mutant allele coding for a protein with a mutated aromatic pocket, trichomes are over-branched—a phenotype that arises from increased ploidy levels. ECT3 was also found to be required for normal trichome morphogenesis, acting together (but not

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redundantly) with ECT2. ECT2 and ECT3 were also found to act redundantly to ensure the timely emergence and proper leaf formation. This role also requires their m^6A reading activities (Arribas-Hernández et al. 2018). Leaf morphogenesis also requires ECT4 but solely in backgrounds where both ECT2 and ECT3 are absent.

The loss of ECT2 induces the rapid downregulation, through degradation, of three trichome-morphogenesis transcripts (*TTG1*, *ITB1,* and *DIS2*) that carry m^6A. This observation is consistent with the role of ECT2 as m^6A reader, as in its absence, the m^6A-signal is likely improperly decoded and transcripts targeted for degradation. Furthermore, it also suggests that aberrant trichome morphogenesis could be, at least in part, the consequence of the improper expression of these three transcripts (Wei et al. 2018).

In planta, ECT2 accumulates mostly in the cytoplasm, but is also found in the nucleus. Upon stress-induced downregulation of translation initiation (heat and osmotic stress), ECT2 relocalizes to stress granules, which are messenger ribonucleoprotein particles (mRNPs) triage and storage centers, also containing factors of the translation machinery. The formation of cytoplasmic foci upon stress is also a feature of ECT4, but not ECT3, which is coherent with the presence in ECT2 and ECT4 (but not ECT3) of YPQ-rich regions, reminiscent of that found in human YTHDF proteins and aggregation-prone factors. The dynamic and complex subcellular distribution of these readers suggests that they might decode the m^6A signal in several post-transcriptional processes, such as splicing/maturation and/or nucleocytoplasmic export step.

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11.3 The m^5C Epitranscriptomic Mark in Plants

Compared to m^6A modification, m^5C is less abundant and much less research has been conducted so far. Transcriptome-wide m^5C represents 0.4% of the total number of cytosines on human polyadenylated transcripts (Squires et al. 2012), whereas m^6A represents 1–1.5% of the adenosines on mRNA (Ke et al. 2015). This cytosine methylation mark is widespread and mainly detected in tRNAs and rRNAs, affecting RNA conformational structure and translational process (Chow et al. 2007; Motorin and Helm 2010; Squires and Preiss 2010), but it was also identified in mRNAs and noncoding RNAs (Squires et al. 2012). Consensus sequence for m^5C sites has been distinguished in Archaea, and until recently, none were found in animal and plant species (Edelheit et al. 2013). However, two enriched sequence motifs around m^5C sites were recently detected in Arabidopsis, with the most significantly enriched motif at the consensus HACCR (where H = U > A > C and R = A/G) (Cui et al. 2017). Additionally to the consensus motif, David et al. (2017) suggested that RNA secondary structure may also be important to confer methylation at m^5C sites, by demonstrating that a 50-nucleotide sequence flanking at m^5C site is essential for methylation in a transient expression system in *N. benthamiana* (David et al. 2017).

The Arabidopsis transcriptome-wide profiling of m^5C-containing RNAs has been recently mapped by two distinct approaches. First, David et al. (2017) identified

more than a thousand m^5C sites in mRNAs, lnRNAs, and sRNAs by RNA bisulfite sequencing, using several tissues and RNA methyltransferase mutants. Quantitative differences in methylated sites between roots, shoots, and siliques revealed a dynamic pattern to suggest a tissue-specific function of m^5C modification (David et al. 2017). The second approach, using RNA immunoprecipitation followed by deep-sequencing, also revealed a tissue-specific regulation of m^5C in various tissues and at different developmental stages (Cui et al. 2017). Thousands of m^5C sites were found to be enriched around start and stop codons of thousands of expressed genes in young seedlings (Cui et al. 2017).

Two classes m^5C writer proteins were identified in eukaryotes, the transfer RNA aspartic acid methyltransferase 1 (TRDMT1) [also known as DNA methyltransferase 2 (DNMT2)] found in yeast, plants, and animals (Goll et al. 2006; Burgess et al. 2015), and the yeast tRNA specific methyltransferase 4 (TRM4) [also known as the human NOP2/Sun domain protein 2 (NSUN2)] (Motorin and Grosjean 1999; Auxilien et al. 2012). The Arabidopsis genome encodes eight potential m^5C methyltransferases, two are the TRM4-like proteins, TRM4A and TRM4B (Chen et al. 2010; Cui et al. 2017), from which the latter has been already characterized in plants (David et al. 2017; Cui et al. 2017). Further analysis was undertaken, using loss-of-function mutants for the tRNA-specific m^5C methyltransferase (TRM4B), revealing that m^5C modification is required for proper root development and oxidative stress responses. David et al. (2017) observed defects in primary root elongation due to impaired cell division at the meristematic tissue, and showed that loss of TRM4B increases sensitivity to oxidative stress and decreases tRNA stability. Accordingly, Cui et al. (2017) showed that TRM4B loss-of-function mutants exhibit downregulation of key genes of root development, namely *SHORT HYPOCOTYL 2* (*SHY2*) and *INDOLE ACETIC ACID-INDUCED PROTEIN 16* (*IAA16*), which is positively correlated with the stability and m^5C modification in their transcripts (Cui et al. 2017).

Together, these studies identified the m^5C modification as another important methylation mark on RNA that has an impact on plant development and adaptive responses. Further research is needed to elucidate the mechanisms and functional roles of m^5C-mediated regulation of protein-coding genes, and to perhaps identify potential m^5C readers and erasers. A recent study showing that an Arabidopsis RRM motif-containing ALY protein preferentially binds to an m^5C-modified RNA (Pfaff et al. 2018) has encouraged future research efforts on this potential m^5C reader. Arabidopsis ALY protein family functions on mRNA export, and *aly* mutant plants exhibited various defects in vegetative and reproductive development, including shorter primary roots, altered flower morphology and reduced seed production (Pfaff et al. 2018). Altogether, it seems that the m^5C modification may influence protein-coding genes with widespread consequences for the development and stress responses.

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11.4 Concluding Remarks

The advances of new technologies, such as sequencing-based transcriptome-wide mapping, revolutionized the field of RNA chemical modifications and permitted to unveil a novel layer in the control of gene expression that is now known as epitranscriptomics or RNA epigenetics. Advances on animal epitranscriptomic regulation have been dazzling in the past years and several epitranscriptomic marks (including m^1A, m^5C, m^6A, m^6Am, ac^4C, or h^5mC) have been mapped transcriptome-wide in different cell types and environmental conditions. We learned from animal studies the crucial importance of these regulatory marks that control constitutive cellular processes and allow their reprogramming to permit organism development and acclimation. In plants, our current understanding of epitranscriptomics is limited to the m^6A and m^5C-based regulations in a single model plant. Nonetheless, Arabidopsis studies revealed that in plants also these modifications are crucial to growth and acclimation. It is hence now a necessity to foster more knowledge on this novel field of biology in model, but also in cultivated plants.

A first step is to get a global vision of the nature and patterning of chemical modifications on the polyadenylated transcriptome of plants. With the advent of global approaches such as LC-MS/MS or next-generation sequencing, one is now capable of not only knowing the nature and relative abundance of mRNA modifications but also to decipher their distribution on each expressed genes. Such repertoires might easily be obtained from diverse species, organs, environmental conditions, and even populations. We anticipate these data to give insights on the role and agronomical importance of RNA epigenetics, as did, for example, the 1001 Arabidopsis epigenomes. Analyses and comparisons of these repertoires will give us clues regarding the interplay that exists between the various marks or their respective importance in acclimation and growth.

Of course, several fundamental questions remain to be addressed in model plants that will contribute to our understanding of the importance of RNA epigenetics in crop development and resistance to stressful conditions, encountered in cultivated fields. What are the actors (writers, readers, and erasers) of the different epitranscriptomic mark-based regulations? Understanding the molecular, cellular, and physiological roles of these actors will help comprehend the role of the mark and the interplay between marks. As an example, data already obtained from Arabidopsis studies on the features and role of the m^6A mark can be exploited to understand the importance of this mark in cultivated species. With the advent of genome editing technologies, reverse genetic approaches on proteins of the writer complex, m^6A-readers, and erasers can easily be conducted.

RNA epigenetics in animals is no longer an emerging field but a fast growing new topic of biology that appeals to more and more scientists. Of course, several deficiencies in the epitranscriptomic control of gene expression were linked to cancers and diseases. In plants, the m^6A mark controls development at the embryonic and post-embryonic stages, and very likely required for defense against viral infections and stress responses. The community of plant scientists interested in RNA

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epigenetics is so far quite small and must grow to foster sufficient knowledge to understand this novel extremely complex field of biology.

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