The unique topologies of N^6 -Adenosine methylation (m^6A) in land-plant mitochondria and their putative effects on organellar gene-expression

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Running title: “ N^6 -adenosinemethylation (m^6A) modifications in plant mitochondria”

Summary

Mitochondria are the main source of ATP production and also contribute to many other processes central to cellular function. Mitochondrial activities have been linked with growth, differentiation and aging. As relicts of bacterial endosymbionts, these organelles contain their own genetic system (i.e., mitogenome or mtDNA). The expression of the mtDNA in plants is complex, particularly at the posttranscriptional level. Following transcription, the polycistronic pre-RNAs in plant mitochondria are processed into individual RNAs, which then undergo extensive modifications, as trimming, splicing and C→U editing, before being translated by organellar ribosomes. Our study focuses on *N*⁶-methylation of Adenosine ribonucleotides (m⁶A-RNA) in plant mitochondria. m⁶A is the most common modification in eukaryotic mRNAs. The biological significance of this highly dynamic modification is under investigation, but it's widely accepted that m⁶A mediates structural switches that affect RNA stability and activity. By performing m⁶A-pulldown/RNA-seq (m⁶A-RIP-seq) analyses of Arabidopsis and cauliflower mitochondrial transcripts (mtRNAs), we provide with detail information on the m⁶A landscapes in angiosperms mitochondria. The results indicate that m⁶A targets different types of mtRNAs, including coding sequences, UTRs, introns and non-coding RNA species. While introns and noncoding-RNAs undergo multiple m⁶A modifications along the transcript, in mRNAs m⁶A-modifications are preferably positioned near start-codons, and may modulate the translatability of the m⁶A-modified transcripts.

Keywords: *N*⁶-methyladenosine (m⁶A); mitochondria; gene-expression; translation; Arabidopsis; Cauliflower; Brassicales

Introduction

Mitochondria house the oxidative phosphorylation (OXPHOS) system and many metabolic pathways (*e.g.* amino acids, nucleotides and lipids biosynthesis), which are critical to the plant cell (Millar *et al.* 2011, Schertl and Braun 2014). As descendants from an ancestral bacterial symbiont, mitochondria contain their own genome (mtDNA), which encodes a limited set of genes. An exception is the dinoflagellate *Amoebophrya ceratii*, where the entire mitogenome was lost and essential component of the respiratory apparatus were all translocated into the nuclear genome (John *et al.* 2019). These proteins, as well as various organellar cofactors that have been recruited from the host to function in mitochondrial metabolism and organellar gene-expression, are imported into the mitochondria from the cytosol post-translationally (reviewed by *e.g.*, (Ferne *et al.* 2004, Millar, *et al.* 2011, Moller 2001).

The mtDNAs in plants are large and highly variable in size (reviewed by *e.g.*, (Grewe *et al.* 2014, Gualberto *et al.* 2014, Knoop 2012, Skippington *et al.* 2015, Sloan *et al.* 2012, Small 2013). Yet, the number of mitochondrial genes seems more conserved in the land-plant kingdom, with 60~70 known genes found in different terrestrial plant species (*i.e.*, angiosperms, gymnosperms, ferns, lycophytes, hornworts, mosses and liverworts) (Bonen 2018, Grewe, *et al.* 2014, Gualberto and Newton 2017, Guo *et al.* 2016, Mower *et al.* 2012, Park *et al.* 2015, Sloan, *et al.* 2012). These genes typically include various tRNAs, rRNAs, ribosomal proteins, subunits of the respiratory complexes I (NADH dehydrogenase), III (cytochrome *c* reductase or bc1), and IV (cytochrome *c* oxidase), subunits of the ATP-synthase (also denoted as CV), cytochrome *c* biogenesis (CCM) factors, and at least one component of the twin-arginine protein translocation complex (for a recent review see (Bonen 2018). In mistletoe, the mitochondrial genome undergone substantial gene losses including all CI subunits (Maclean *et al.* 2018, Petersen *et al.* 2015, Senkler *et al.* 2018).

The coordination of growth and development in plants is achieved by cellular signaling, which allow them to regulate and coordinate the energy demands during particular growth and developmental stages. These involve both anterograde (nucleus to organelles) and retrograde (organelles to nucleus) signaling cascades (Woodson and Chory 2008), but the identity of the messenger molecules involved in these pathways remains elusive. The expression of mtDNAs in plants is complex, particularly at the posttranscriptional level (reviewed by *e.g.* (Hammani and Giege 2014, Zmudjak and Osterseztzer-Biran 2017). While in Animalia, the mtDNAs are symmetrically transcribed in opposite directions, yielding two large polycistronic RNAs, the mitochondrial genes in plants are arranged in numerous shorted transcription units. Following

transcription, the mtRNAs in plants undergo extensive processing steps, which include the removal of many group II-type intron sequences (i.e., splicing) and the conversion of numerous cytidines (C) to uridines (U) deaminations (i.e., C→U RNA editing) (reviewed by e.g., (Binder *et al.* 2016, Gualberto and Newton 2017, Hammani and Giege 2014, Liere *et al.* 2011, Small 2013, Zmudjak and Ostersetzer-Biran 2017). These processing steps are critical for the mtRNAs to carry out their functions in protein synthesis, but may also serve as key control points in plant mitochondrial gene-expression, i.e., linking organellar functions with environmental and/or developmental signals.

The manuscript focuses on *N*⁶-adenosine methylation (m⁶A) patterns in the mitochondria of two key Brassicales species, *Arabidopsis thaliana* and *Brassica oleracea* (cauliflower). Various data suggest that m⁶A is the most prevalent RNA modification within mRNA transcripts in eukaryotes (Burgess *et al.* 2016, Liu *et al.* 2015, Niu *et al.* 2013, Patil *et al.* 2018, Visvanathan and Somasundaram 2018), which its biological significance is currently under intense investigation. In animals, m⁶A is a dynamic RNA-base modification which plays important roles for RNA, e.g. splicing, stability, and translation, akin to those of DNA methylation and histone modification (Liu, *et al.* 2015, Meyer and Jaffrey 2017, Patil, *et al.* 2018, Visvanathan and Somasundaram 2018). Co-immunoprecipitation of m⁶A-modified transcripts followed by RNA-seq analyses (i.e., m⁶A-RIP-seq) provided with detailed information regarding to the m⁶A landscapes in mammalian cells (see e.g., (Dominissini *et al.* 2012). Specific methyltransferases (MTAs, designated as ‘Writers’) and demethylases (termed as ‘Erasers’) regulate the distribution of m⁶A on the transcriptome, whereas proteins that interact and mediate m⁶A-dependent functions are termed as Readers (Meyer and Jaffrey 2017, Patil, *et al.* 2018, Visvanathan and Somasundaram 2018).

In plants, *N*⁶-methylation of Adenosine residues in nuclear-encoded mRNAs is positioned predominantly towards the 3'-end, about 100 bps upstream to the poly A tail (Bodi *et al.* 2012, Burgess, *et al.* 2016, Dominissini, *et al.* 2012, Ke *et al.* 2015, Li *et al.* 2014, Luo *et al.* 2014, Wan *et al.* 2015). This was further supported by a recent RNA-seq data of *Arabidopsis* plants, using nanopore direct RNA sequencing (Parker *et al.* 2019). Mutant plants with reduced levels of m⁶A, due to the knockout or knockdown of genes homologs of writers and readers show embryogenesis defects and altered growth and developmental phenotypes (Bodi, *et al.* 2012, Shen *et al.* 2016, Zhong *et al.* 2008). Interestingly, global m⁶A-RIP-seq analyses of plant transcriptomes suggested the presence of m⁶A methylations also in plant organelles (i.e., plastid and mitochondrial) as well (Luo, *et al.* 2014, Wan, *et al.* 2015, Wang *et al.* 2017). However, the occurrence and significance of this modification within organellar RNAs are still elusive.

In order to gain detailed insights into the biological significance of this modification in plant organelles, we analyzed the m⁶A patterns of mtRNAs extracted from purified mitochondria obtained from *Arabidopsis thaliana* and *Brassica oleracea* var. botrytis (cauliflower), two key Brassicales species (Grewe, *et al.* 2014). Here, we present several lines of evidence indicating that mtRNAs of *Arabidopsis* and cauliflower undergo extensive N⁶-methyladenosine modifications, and that the m⁶A-RNA methylation affects plant mitochondria gene-expression. Mass-spectroscopy (*i.e.* LC-UV-MS/MS) and m⁶A-RIP-seq analyses indicate that in mitochondria of *Arabidopsis* and cauliflower plants, the m⁶A is found within different RNA types (*i.e.*, mRNAs, structural RNAs, introns, UTRs and intergenic-expressed regions), but seems particularly prevalent in the noncoding-gene regions. Moreover, while noncoding RNAs contain multiple m⁶A sites found along the transcripts, in mRNAs the m⁶A is predominantly positioned within the AUG translation initiation codon. Biochemical analyses indicate that the extent of m⁶A modifications within the transcript is detrimental for translation. These data strongly support the notion that m⁶A modifications play important roles in regulating the expression of organellar genes in plants.

Results

m⁶A is presented within various mitochondrial transcripts in *Arabidopsis thaliana* and *Brassica oleracea* plants

Global RNA-seq analyses revealed the presence of numerous m⁶A modifications within various nuclear-encoded mRNAs in the model plant *Arabidopsis thaliana*, but also suggested that the organellar (*i.e.* chloroplasts and mitochondrial) transcripts undergo extensive N⁶-methylations (Bodi, *et al.* 2012, Li, *et al.* 2014, Luo, *et al.* 2014, Shen, *et al.* 2016, Wang, *et al.* 2017). However, the topology of m⁶A in plant mitochondria and significance to organellar biogenesis need to be further investigated. For this purpose, pull-downs of m⁶A-RNAs from highly-enriched mitochondria preparations, obtained from two key Brassicales species (*i.e.*, *Arabidopsis thaliana* seedlings and *Brassica oleracea* (cauliflower) inflorescences), to analyzed by chromatography-mass spectrometry (LC-MS) and RNA-seq analyses. *Arabidopsis* serves a prime model system for molecular genetics, while cauliflower is employed for the biochemical analysis of plant mitochondria RNA metabolism (Grewe, *et al.* 2014, Keren *et al.* 2009, Neuwirt *et al.* 2005, Sultan *et al.* 2016).

The presence of N⁶-methylated Adenosine residues in mitochondrial transcripts (mtRNAs) of *Arabidopsis* (Luo, *et al.* 2014, Wang, *et al.* 2017) and cauliflower was supported

by northern blot analyses (Keren *et al.* 2011) (Supporting information Fig. S1 and Table S1). Measurements of the bulk levels of m⁶A in total RNA preparations from isolated mitochondria, using a calorimetric m⁶A-RNA quantification kit, indicated to m⁶A/A ratios of 0.44% and 0.38% (*i.e.* 4~5 m⁶A modifications per 1,000 adenosine nucleotides) (Table S2). These data are in agreement with the reports by Luo, *et al.* (2014) and Wang, *et al.* (2017), which proposed that in global m⁶A-RIP-seq analyses of Arabidopsis the extent of m⁶A modifications within organelles (*i.e.*, chloroplasts and mitochondria) are greater than that of nuclear-encoded mRNAs. Analyses of the relative m⁶A levels, using calorimetric measurements, were previously shown to be well correlated with those of LC-MS analyses (Engel *et al.* 2017, Slobodin *et al.* 2017). Although the use of m⁶A antibodies provide with a sensitive approach to identifying m⁶A methylations, these methods can also lead to erroneous data due to non-specific association of various proteins with the antibodies. LC-MS analyses were used to confirm the presence of m⁶A organellar RNAs isolated from cauliflower mitochondria (Figure 1) (Keren, *et al.* 2009, Neuwirt, *et al.* 2005, Sultan, *et al.* 2016). RNA isolated from purified mitochondria was digested to single nucleosides and the presence of modified Adenosine residues (m⁶A) was assayed by LC-MS, based on specific retention times from standard and modified nucleosides separated by the same chromatographic procedure (see Materials and Methods). As indicated in Figure 2 and supplementary Figure S2, the LC-MS data indicated the presence of N⁶-methyladenosine modifications within the transcripts of cauliflower mitochondria.

The unique topologies of m⁶A methylomes in Brassicales mitochondria

Following the confirmation of N⁶- adenosine methylation events (Fig. 2, and supplementary Figures S1 and S2), we further characterized the landscapes of m⁶A modifications in Arabidopsis and cauliflower mitochondria by the m⁶A-RIP-seq method (Dominissini, *et al.* 2012, Luo, *et al.* 2014) (Fig. 1). rRNA-depleted RNA preparations, obtained from Arabidopsis and cauliflower mitochondria, were fragmented into 60~200 nucleotides (*i.e.* input mtRNA) and immunoprecipitated with monoclonal anti-m⁶A antibodies (see Materials and Methods). The precipitated m⁶A-mtRNAs were then used to construct libraries that were subjected to RNA-seq analyses. The corresponding nucleotide reads were aligned to the reference Arabidopsis and cauliflower mitochondrial transcriptomes (Fig. S3) (Grewe, *et al.* 2014, Sultan, *et al.* 2016, Unseld *et al.* 1997) to generate detailed landscapes of m⁶A-RNAs in Arabidopsis and cauliflower mitochondria. As control we analyzed the RNA-seq's of rRNA-depleted mtRNAs from Arabidopsis and cauliflower (SRA accession PRJNA472433, Table S3).

The mitogenome of *Arabidopsis thaliana* (Col-0) harbors 57 identified genes, including 38 protein coding frames, 22 tRNAs and 3 rRNAs (Sloan *et al.* 2018, Unseld, *et al.* 1997). The remaining four genes include an intronic ORF, annotated as maturase-related (*matR*) gene encoded in the fourth intron in *nad1* (Sultan, *et al.* 2016, Wahleithner *et al.* 1990), *atp4* (previously annotated as *orf25*) (Heazlewood *et al.* 2003), *atp8* (previously denoted as *orfB*) (Heazlewood, *et al.* 2003, Sabar *et al.* 2003) and *mttB* (or *tatC*, previously annotated as *orfX*) (Bogsch *et al.* 1998). In addition to known genes, about 460 open reading frames (i.e., mtORFs; larger than 60 codons) are found within the sequenced mtDNA of *Arabidopsis thaliana* (Col-0) (Unseld, *et al.* 1997). The m⁶A/RNA-seq data is presented Table 1 and supplementary Tables S3 and S4, which corresponds to two independent m⁶A-RIP-seq analyses. The m⁶A modifications were mapped to 37 ~ 41 annotated genes and 7 ~ 10 ORFs in the mitogenome of *Arabidopsis* (Table 1 and Tables S3 and S4).

In cauliflower mitochondria, 54 known genes (i.e., 33 annotated protein coding frames, 18 tRNAs and 3 rRNAs) and 35 ORFs are annotated (KJ820683.1) (Grewe, *et al.* 2014). Between 18 to 23 genes and 21 or 22 mtORFs were identified in the two replicates of the m⁶A-RIP-seq analyses of *B. oleracea* mitochondria (see Table 1, and supplementary Tables S3 and S4). The distribution of m⁶A signals in *Arabidopsis* and cauliflower mitochondria (i.e., average of the two independent m⁶A-RIP-seq analyses) is provided in Figure 3.

The identity of the genes and the numbers of modifications within each gene were different between *Arabidopsis* and cauliflower, but the m⁶A-related patterns in *Arabidopsis* and cauliflower mitochondria generally shared similar patterns. In animals, m⁶A sites are in particularly enriched within the 3' UTRs, near stop codons (Bodi, *et al.* 2012, Deng *et al.* 2015, Dominissini, *et al.* 2012, Hoernes *et al.* 2016). Yet, in both *Arabidopsis* and cauliflower, the majority of the m⁶A peaks (i.e., about half of the signals) are mainly found in noncoding RNAs (49.3 ± 7.8 % in *Arabidopsis* and 50.3 ± 4.8 % in cauliflower) (Fig. 3a, Table 1 and supplementary Table S4). Within known gene regions, about quarter to fifth of the m⁶A methylation sites are mapped to the coding sequences (i.e., 26.6 ± 1.1 % in *Arabidopsis* and 22.7 ± 0.5 % in cauliflower), about 20% are found within group II intron sequences (17.0 ± 3.0 % in *Arabidopsis* and 18.1 ± 2.9 % in cauliflower), and only 2 to 4 % (3.9 ± 3.0 % in *Arabidopsis* and 2.2 ± 0.5 % in cauliflower) of the sites occur within the untranslated regions (UTRs, Fig. 3a). Higher m⁶A signals in mRNAs, versus introns and other non-coding regions (Fig. 3 and Fig. S3), may be expected from their expression patterns and RNA metabolism in plants mitochondria (Grewe, *et al.* 2014, Zmudjak and Ostersetzer-Biran 2017). As these analyses were performed with rRNA-depleted mtRNAs, any conclusions regarding the presence of m⁶A

in tRNAs and rRNAs (Table 1) should be taken with care. The differences in the m⁶A landscapes between the two closely-related Brassicales species may correspond to variations in the experimental procedure, or indicate to altered m⁶A modifications due to growth conditions and the use of different plant tissues (i.e., vegetative plant tissue in Arabidopsis and floral tissues in cauliflower). Analyses of the m⁶A landscapes in different tissues and at various growth stages are underway.

To evaluate the m⁶A-enrichment within coding sequences systematically, we aligned the m⁶A peaks of Arabidopsis and cauliflower mitochondria to their mtDNA sequences. These included coding sequences, group II introns and noncoding regions. While in introns (Fig. 3b) and intergenic regions (Fig. S4) the m⁶A signals are distributed along the transcript, in protein coding sequences (i.e., exons and mRNAs) the m⁶A sites are predominantly located within the range of ± 100 nucleotides around the first codon (i.e., about 25% of the reads in Arabidopsis and 12% in cauliflower mitochondria) (Fig. 3a,b). These data may indicate that in mRNAs of both Arabidopsis and cauliflower mitochondria, many of the m⁶A modifications occur in proximity or within the translation initiation site. We also noted to differences in the quantities of m⁶A modifications per organellar transcript between mRNAs and noncoding transcripts. Many mRNAs contain a single or a few m⁶A modification site (Fig. 3b and Fig. 4a), while in noncoding sequences often contain more than 5 sites per transcript (see Fig. 3b and Fig. 4b). In summary, the m⁶A methylation patterns in plant mitochondria differ markedly from those of bacteria, where the m⁶A is found mostly inside ORFs, or nuclear-encoded mRNAs which are modified mainly in the 3' UTRs and regions adjacent to the stop codon (Bodi, *et al.* 2012, Deng, *et al.* 2015, Dominissini, *et al.* 2012, Hoernes, *et al.* 2016).

Analysis of common m⁶A motifs in Brassicales mitochondrial RNAs

To determine whether the m⁶A-RNA reads in plant mitochondria share a common sequence motif, we used the HOMER (Heinz *et al.* 2010) and MEME (Bailey *et al.* 2009) programs. Integration of results from the motif discovery tools, and clustering of the enriched mRNAs or noncoding sequences from Arabidopsis and cauliflower mitochondria, suggested the following m⁶A elements: DRm⁶AYHSYS (where 'D' is A, G or U, 'R' is A or G, 'Y' is C or U, 'S' is C or G, and 'H' is A, C or U) for mRNAs (Fig. 4c) and HRm⁶AYS for the noncoding RNAs (Fig. 4d). These putative mitochondrial m⁶A sequence elements share a similar core motif, i.e., Rm⁶AY (Fig. 4c,d), which was described for nuclear-encoded mRNAs both animals and plants (i.e., RRm⁶ACH) in (Liu and Pan 2016, Maity and Das 2016, Patil, *et al.* 2018), and to a lesser degree with the m⁶A motif (i.e., UGCCm⁶AG) of bacterial RNAs (Deng, *et al.* 2015).

The consensus DRm⁶AYHSYS and HRm⁶AYS sequences are somewhat different from the previously suggested m⁶A motif (i.e., RRm⁶ACH site) for organelles in Arabidopsis plants (Wang, *et al.* 2017). The variations between these analyses may relate to the use of m⁶A-RNA-seq with global RNA-seq analyses in Wang, *et al.* (2017), which might be only partially represented by mitochondrial transcripts.

The m⁶A modification has no obvious effects on the stability of recombinant transcripts, in vitro

The identity of the m⁶A-modifying enzymes in bacteria or organelles in eukaryotic organisms is currently unknown. To gain some insights regarding the physiological significance of m⁶A in angiosperms mitochondria, we analyzed the effects of N⁶-adenosine methylations on the transcription, stability and translatability of several mtRNAs, in vitro. For this purpose, cDNA fragments corresponding to ATP-synthase *atp1* gene, *nad4* subunit of respiratory complex I (i.e., NADH dehydrogenase enzyme), whose coding region is interrupted by several group II-type introns in both Arabidopsis and cauliflower mitochondria (Grewe, *et al.* 2014, Sloan, *et al.* 2018, Unseld, *et al.* 1997), and *matR* ORF, encoded within *nad1* intron 4 (Sultan, *et al.* 2016, Wahleithner, *et al.* 1990), were obtained by RT-PCR with total RNA isolated from Arabidopsis mitochondria and cloned into pGEM vector, such as each gene-fragment contains a T7 RNA polymerase (RNAP) promoter site at the 5' termini and a C-terminal 6x His tag in its 3' termini (Fig. 5, and Materials and Methods). The use of RT-PCR instead of PCR allowed us to retain potentially important C→U RNA editing sites, which are highly common in plant mitochondria and affect *atp1*, *nad4* and *matR* mRNAs (Hammani and Giege 2014, Ichinose and Sugita 2016b, Sloan, *et al.* 2018, Takenaka *et al.* 2008, Unseld, *et al.* 1997, Zmudjak and Ostersetzer-Biran 2017). The integrity of each construct was verified by DNA-sequencing, and then transcribed in vitro, in the absence or presence of different m⁶ATP/ATP nucleotides ratios, in order to obtain 'body-labeled' m⁶A-modified transcripts (Fig. 5).

The decay patterns of control (i.e., unmodified) versus m⁶A-modified *atp1*, *nad4* and *matR* RNAs were examined in vitro, by incubating the transcripts with purified cauliflower mitochondrial extracts. Samples were collected at different time points, as indicated in Figure 6 and supplementary Figure S5. As indicated by the 'in organello' assays, the presence of m⁶A had no or only minor effect on the stabilities of *atp1*, *nad4* and *matR* transcripts (Fig. 6 and Fig. S5). Both the control RNAs and the m⁶A-modified transcripts showed similar degradation patterns, where the signal of the recombinant RNAs was reduced by about 40~50%, following two hours of incubation with the plant organelle extracts (Figs. 6b and S5).

m⁶A affects the translation of mitochondrial transcripts, *in vitro*

Next, we considered a possible link between posttranscriptional m⁶A modification and organellar translation (Akichika *et al.* 2019, Choi *et al.* 2016, Hoernes, *et al.* 2016, Meyer and Jaffrey 2017, Meyer *et al.* 2015, Slobodin, *et al.* 2017, Sun *et al.* 2019). Attempts to induce the translation of *in vitro* transcribed RNAs in Arabidopsis or cauliflower mitochondrial extracts were generally unsuccessful. We therefore analyzed the translation efficiencies of m⁶A-modified *atp1*, *nad4* and *matR* transcripts (Fig. 5), using the TNT[®] wheat germ extract (WGE) system (see Materials and Methods). To enable the synthesis of recombinant proteins, specifically by organellar ribosomes (i.e., the crude wheat germ extracts should contain both cytosolic and organellar ribosomes), the translation of recombinant ATP1, MATR and NAD4 fragments was assayed in the presence of cycloheximide (CycH, 100 µg/ml), which blocks translation by affecting peptidyl transferases activity in eukaryotic ribosomes, and Kanamycin (Kan, 50 µg/ml) which specifically interrupt organellar (i.e., mitochondria and plastids) proteostasis (Wang *et al.* 2015a).

As indicated in Figure 7a, the translation of *atp1*, *matR* and *nad4* was reduced in the presence of CycH by about 50% when the transcripts contained a 10% m⁶A/A ratio, while no translation products corresponding to *atp1*, *matR* and *nad4* were observed when their transcripts were synthesized in the presence of m⁶A only (i.e., 100% m⁶A/A ratio). Excessive m⁶A modification was found to be detrimental for the translation of m⁶A modified *atp1*, *matR* and *nad4* *in vitro* (i.e., in the WGE system), as indicated by the reduced translation efficiencies of the modified mtRNAs containing a higher m⁶A/A ratio. As indicated in Figure 7a, the addition of Kanamycin abolished the translation by organellar ribosome in the plant extract.

We further analyzed the effects of m⁶A on the translation of *atp1*, *matR* and *nad4* transcripts specifically modified at their AUG sites (i.e., m⁶AUG). For this purpose, synthetic RNA fragments, containing the consensus T7 polymerase promoter upstream to a modified Adenosine residue of the initiation codon (i.e., TTTAAGAAGGAGATATACC[m⁶A]TG-xxxx; xxx indicates the *atp1*, *nad4* or *matR* fragments), were translated in the absence or the presence of cycloheximide (CycH, 100 µg/ml). We noticed that the efficiency of translation of *atp1* and *nad4* transcripts, modified only at the Adenosine residue of the initiation codon, was increased to some extent in the WGE (Fig. 8a). Yet, when the translation efficiencies of the m⁶AUG-RNAs were assayed in the presence of Kanamycin, similar intensities of the His-tagged polypeptides, corresponding to ATP1, MATR and NAD4 fragments, were seen in the non-

modified and m⁶A-AUG modified transcripts. These analyses indicate that excessive m⁶A modification is detrimental for the translation of m⁶A-modified transcripts, while a single modification at the start codon may enhance to some extent the trainability by plant organellar ribosomes.

Arabidopsis mutants affected in mitochondria gene expression show altered m⁶A patterns

The biogenesis of mitochondria in plants is tightly controlled by nuclear loci, which regulate organellar gene expression and metabolic functions. The plant organellar pre-RNAs undergo extensive posttranscriptional processing steps, including RNA editing and intron splicing. These essential processing steps are regulated by various cofactors (reviewed by *e.g.* (Zmudjak and Ostersetzer-Biran 2017), such as the maturase-related nMAT1 (Keren *et al.* 2012, Nakagawa and Sakurai 2006) and nMAT2 (Keren, *et al.* 2009, Zmudjak *et al.* 2017) proteins, which affect the splicing efficiencies of different subsets of mitochondrial introns in Arabidopsis. Both *nmat1* and *nmat2* mutants show altered mitochondria gene expression profiles, with notable accumulations of various group II intron-containing pre-RNAs and decreased steady-state levels of the corresponding mRNAs (Keren, *et al.* 2009, Keren, *et al.* 2012, Nakagawa and Sakurai 2006, Zmudjak, *et al.* 2017).

m⁶A-RIP analyses followed by RT-qPCRs of *nmat1*, *nmat2* and wild-type plants suggested that pre-RNAs that their maturation (*i.e.*, splicing) was affected are also more extensively modified by m⁶A (Fig. 9 and Table S5). These including the first exons in *nad1* (*i.e.*, *nad1-ab*), *nad2-ab* and *nad4-bc* RNAs in *nmat1* mutant, and *cox2*, *nad1-de*, *nad7-bc*, and to a lesser degree the different exons in *nad2* and *nad5* genes, and the intronless transcripts of *ccmB*, *ccmC* and *nad9* mRNAs in *nmat2* mutant (Fig. 9). Higher accumulation was observed in the levels of *nad1-i1*, *nad2-i2* and *nad4-i2* in *nmat1* (Keren, *et al.* 2012). The steady-state levels of several other intron-containing pre-mRNAs, in particularly *cox2*, *nad1*, *nad5* and *nad7*, were also higher in *nmat2* mutants than in wild-type plants (Keren *et al.*, 2009; Zmudjak *et al.*, 2017). Yet, the m⁶A profiles of various intronless transcripts in *nmat1* or *nmat2* mitochondria (Fig. 9), did not any show significant differences compared with the wild-type (*col-0*) plants. These results suggest that organellar transcripts affected in their metabolism may be more prone to N⁶-adenosine methylations.

m⁶A mRNAs are enriched to some extent in the mitoribosomes

Published data (Akichika, *et al.* 2019, Choi, *et al.* 2016, Hoernes, *et al.* 2016, Meyer and Jaffrey 2017, Meyer, *et al.* 2015, Slobodin, *et al.* 2017, Sun, *et al.* 2019) and own results (Figs. 7 and

8a) indicate that the posttranscriptional modification of m⁶A can affect the translation of both nuclear and organellar-encoded polypeptides. The reduction in translation efficiency in the WGE system is correlated with magnitude of N⁶-adenosine modifications within a given transcript, while a single (or a few) m⁶A modification near initiation codons may enhance in some cases the translatability of the organellar transcripts. Polysomal-profiling experiments of purified cauliflower mitochondria were used to identify m⁶A-mRNA that are stably bound to the mitoribosomes (Waltz *et al.* 2019) (see Materials and Methods).

Figure 9a shows a typical polysome profile of cauliflower mitochondria. The fractions were pooled (bottom to top) into the heavier polysomal fractions (tubes 5 – 50) and the lighter monosomal fractions (tubes 65 – 95). In addition to the polysomal fractions, we also analyzed the m⁶A profiles in small-RNPs or subribosomal fractions (tubes 105 – 150), in comparison with those of the input RNA (rRNA-depleted). As shown in Figure 9b and Table S2, an enrichment in the m⁶A signals was apparent in both the ‘monosomal’ and ‘polysomal’ fractions. The relative levels of various mitochondrial transcripts in the polysomes (i.e., monosomal and polysomal fractions) were assayed by m⁶A-RIP followed by RT-qPCR analyses, using the input RNA (Δ rRNAs) as calibrator (Fig. 9c). The data indicated to small, yet statistically significant, increases (between 2x and 4x) in the levels of ribosome-associated m⁶A-mRNAs versus their ratios in the input RNA (Fig. 9c).

Discussion

The N⁶-methyladenosine (m⁶A) was reported in the early 1970s (Desrosiers *et al.* 1974), and is currently considered as the most prevalent modification in nuclear-encoded mRNAs. co-IPs coupled to high-throughput RNA sequencing methods (Dominissini, *et al.* 2012), provided with comprehensive data regarding the m⁶A landscapes across the transcriptomes of various organisms (Burgess, *et al.* 2016, Liu and Pan 2016, Parker, *et al.* 2019, Wang, *et al.* 2017, Yue *et al.* 2015). Unlike pseudouridylation (U $\rightarrow$ Ψ), the most abundant posttranscriptional modification in RNAs (Ge and Yu 2013), or C $\rightarrow$ U exchanges, which are highly common in plant organelles (Barkan and Small 2014, Chateigner-Boutin and Small 2010, Colas des Francs-Small and Small 2014, Hammani and Giege 2014, Ichinose and Sugita 2016a, Schallenberg-Rüdinger and Knoop 2016, Shikanai 2015, Zmudjak and Ostersetzer-Biran 2017), m⁶A is not expected to rewire the genetic-code for translation (Dai *et al.* 2007). Instead, the m⁶A-modification is anticipated to serve important regulatory roles in gene expression in eukaryotic and prokaryotic organisms (see *e.g.*, (Bodi, *et al.* 2012, Deng, *et al.* 2015, Dominissini, *et al.* 2012, Hoernes, *et al.* 2016).

In bacteria, many of the m⁶A sites are mapped to genes of the respiratory apparatus (Deng, *et al.* 2015). These data may suggest an important regulatory role for m⁶A in controlling the OXPHOS capacity (Deng, *et al.* 2015), an activity which may have retained in the energy producing organelles (i.e., mitochondria and chloroplasts) in eukaryotic systems. Global transcriptomic analyses indicated the presence of m⁶A-modifications in mitochondrial (and plastidial) transcripts in the model plant, *Arabidopsis thaliana* (Bodi, *et al.* 2012, Chen and Liu 2014, Luo, *et al.* 2014, Shen, *et al.* 2016, Wang, *et al.* 2017). However, as the RNA-seq analyses involved total plant RNA preparations, the occurrence and significance of N⁶-methyladenosine modifications within the organelles in plants (and animals) need to be further investigated. In this study, we provide with detailed analyses of the m⁶A-RNA landscapes in the mitochondria of two *Brassicaceae* species, i.e., *Arabidopsis* and cauliflower.

Northern blots (Fig. S1), calorimetric assays (Table S2) and LC-MS analyses (Figs. 2 and S2), indicated the presence of m⁶A within organellar transcripts in *Arabidopsis* and cauliflower mitochondria, with a calculated m⁶A occurrence of about 0.4 to 0.5% of the total organellar A residues, on average. Following the confirmation of m⁶A modification in plants organellar transcripts, the topologies of m⁶A-RNA methylomes in the mitochondria of *Arabidopsis* and cauliflower were analyzed by the m⁶A-RIP-seq method (Fig. 1, and (Dominissini, *et al.* 2012, Luo, *et al.* 2014).

The m⁶A-RIP-seq analyses indicated to different m⁶A modification patterns in *Arabidopsis* and cauliflower mitochondria (Fig. 3, Tables 1, S3 and S4). Such differences could relate to the analyses of different plant species, altered growth conditions, or may indicate differences in m⁶A-RNA modifications patterns between different tissues (i.e., whole plant versus inflorescences). Despite the differences, the m⁶A-RIP-seq of *Arabidopsis* and cauliflower mitochondria generally showed a similar distribution of the m⁶A sites, where about half of the reads are mapped to noncoding RNAs and about quarter (i.e., 23% in *Arabidopsis* seedlings and 27% in cauliflower inflorescences) of the m⁶A signals are found in known protein-coding genes (Fig. 3a and Table 1). While in nuclear-encoded transcripts in animals and plants, the majority of the m⁶A sites are mapped to regions adjacent to stop codons within the 3' UTRs (Bodi, *et al.* 2012, Deng, *et al.* 2015, Dominissini, *et al.* 2012, Hoernes, *et al.* 2016), in *Arabidopsis* and cauliflower mitochondria, the majority of the m⁶A sites within known genes are mapped to the coding regions (i.e., 60.8% in *Arabidopsis* and 52.3% in cauliflower). In fact, less than 5% of the m⁶A reads are mapped to UTRs in both *Arabidopsis* and cauliflower mitochondria (Fig. 3a and Table 1). These data also correlate with the distribution of m⁶A in the mtRNAs of *Arabidopsis* by the global RNA-seq analyses s (Luo, *et al.* 2014). Notably, a

significant portion of m⁶A reads were found to reside within group II intron sequences (i.e., 17% in Arabidopsis and 18% in cauliflower) (Figs. 3, S3 and Table 1).

Beside to their unique topology, plant mitochondria also exhibit a distinctive distribution of m⁶A modifications within their transcripts. While noncoding RNAs contain multiple m⁶A sites distributed along the transcript (Figs. 3b and S4), the coding sequences contain a fewer number of m⁶A-modifications per transcript, with a notable preference to regions close (± 100 nts) to the AUG sites (i.e., 27.3% in Arabidopsis and 12.0% in cauliflower) (see Fig. 3b). These data may indicate that the m⁶A-modification plays a regulatory role in the expression of organellar genes in angiosperms mitochondria. The m⁶A-RIP-seqs data also revealed the presence of various RNA reads which mapped to the plastid genome (see supplementary Table S6 and Fig. S6). However, these data represent preliminary data and the topology of m⁶A modifications in the plastids need to be further investigated.

Through its impact on splicing (Xiao *et al.* 2016), mRNA stability (Wang *et al.* 2014) and rate of translation in animals (Meyer, *et al.* 2015, Wang *et al.* 2015b), m⁶A regulate essential features of a cell by affecting the expression of nuclear-encoded genes. In Arabidopsis mutants, altered m⁶A methylation patterns result with embryogenesis and developmental defect phenotypes (Bodi, *et al.* 2012, Luo, *et al.* 2014, Růžicka *et al.* 2017, Shen, *et al.* 2016, Zhong, *et al.* 2008). It is therefore anticipated that m⁶A would also affect the expression of organellar genes.

Following the analysis of the topologies of m⁶A in Brassicales mitochondria, we next sought to infer the roles of m⁶A-modifications in organellar biogenesis and gene-expression. As we were unable to determine the significance of m⁶A to mitochondria biology by forward or reverse genetis, we used an in vitro approach to study the effects of N⁶-adenosine methylations on organellar gene expression, using the wheat germ extract (WGE) system. No significant effects on the stability of recombinant *atp1*, *nad4* or *matR* transcripts was observed between unmodified and m⁶A-modified transcripts in vitro (Figs 6 and S5). However, excessive m⁶A modifications were found to be detrimental for the translation of the m⁶A-modified transcripts in vitro (Fig. 7). This effect are related to organellar translation, as the translatability of ATP1, NAD4 and MATR were reduced in the presence of cycloheximide, which affects the translation of mRNAs bound to cytosolic ribosomes (Fig. 7a,b).

We further analyzed the effect of a single modification within the start (AUG) codon on the translation efficiencies of ATP1, MATR and NAD4 fragments, using synthetically modified RNAs. While reduced translation was observed in the case of ‘body-labeled’ *atp1*, *nad4* and *matR* RNA (i.e., modified at multiple sites along their transcripts) (Figs. 5 and 7), the translation

efficiencies of m⁶AUG-modified transcripts increase, at least to some extent, in the case of *atp1* and *nad4* (Fig. 8a). Also, no significant effects on the translatability of *atp1*, *matR* and *nad4*, modified at the initiation codon, was seen in the presence of Kanamycin, which affects the translation of organellar proteins (Wang, *et al.* 2015a). Together, these results suggest that multiple m⁶A modifications are detrimental for translation, while a single modification in the start codon (and maybe also of other A residues found in proximity to the translation initiation site), can enhance the translation of some organellar transcripts. The molecular basis for these effects are currently unknown. It was previously shown that posttranscriptional m⁶A-modifications affect the translation of various nuclear-encoded transcripts (Akichika, *et al.* 2019, Choi, *et al.* 2016, Hoernes, *et al.* 2016, Meyer and Jaffrey 2017, Meyer, *et al.* 2015, Slobodin, *et al.* 2017, Sun, *et al.* 2019). In a recent study, (Akichika, *et al.* 2019) indicated that the translation efficiency of some mRNAs in yeast, are significantly decreased upon KO of *CAPAM* gene a cap-specific adenosine-N⁶-methyltransferase. It is possible that an organellar writer protein for N⁶-methylation may possess similar functions. Following the modifications in sites adjacent to the translation start sites, specific readers may associate with the modified transcripts and enhance their translatability.

Under the *in vitro* conditions, the transcription efficiency of synthetic RNA-fragments corresponding to *atp1*, *nad4* and *matR* was not significantly affected by m⁶A, even at m⁶A/A ratio's as high as 50% (Fig. 5). A reduction in the transcription efficiencies of cDNAs corresponding to *atp1*, *nad4* and *matR* was observed only when the reaction mix contains a 100% m⁶A/A ratio (Fig. 5). It is expected that m⁶A modifications occur posttranscriptionally. Accordingly, it was recently shown that both plant and animal cells require an N⁶-mAMP deaminase (MAPDA) to catabolize N⁶-mAMP to inosine monophosphate, most likely to prevent misincorporation of m⁶A into the RNAs during transcription (Chen *et al.* 2018). This is further supported by *in vitro* assays that show that eukaryotic RNA polymerases (RNAPs) can also use N⁶-mATP as a substrate (Chen, *et al.* 2018). As, the single-subunit RNA polymerase of plant mitochondria (mtRNAP) is related to the T7/T3 RNAPs (Cermakian *et al.* 1996, Masters *et al.* 1987, Zmudjak and Ostersetzer-Biran 2017), we speculate that the incorporation of m⁶A may precedes efficiently in the presence of m⁶ATP ribonucleotides in the mitochondria *in vivo*, as well (unless N⁶-mAMP is catabolized in these organelles).

Examining common m⁶A motifs in Arabidopsis and cauliflower mitochondria, using the HOMER (Heinz, *et al.* 2010) and MEME (Bailey, *et al.* 2009) programs, we observe a statistical enrichment for the 5'-Rm⁶AY-3' sequence (P<0.005; Fig. 4). The predicted mitochondrial m⁶A sites shares homology with the m⁶A core motif suggested for nuclear-

encoded mRNAs (i.e., RRm⁶ACH) in animals and plants (Liu and Pan 2016, Maity and Das 2016, Patil, *et al.* 2018), and to a lesser degree with the m⁶A motif of bacterial RNAs (i.e., UGCCm⁶AG). However, the identities of the enzymes which regulate m⁶A patterns in plant mitochondria remain unknown.

The nuclear genome of *Arabidopsis thaliana* contains several genes which are closely related to m⁶A methyltransferases (i.e., Writers) in mammals (see *e.g.* (Růžicka, *et al.* 2017) and Table S7). These include an MTA-related protein (At4g10760) and an ortholog of the mammalian WTAP (AT3G54170), known as FIP37. The two factors serve as core components of the m⁶A methyltransferase complex, and play indispensable roles in determining the m⁶A mRNA modification patterns in Arabidopsis plants (Bodi, *et al.* 2012, Luo, *et al.* 2014, Růžicka, *et al.* 2017, Shen, *et al.* 2016, Zhong, *et al.* 2008). Here, we performed sequence similarity searches with PSI-BLAST, in an effort to identify putative components of the organellar m⁶A methyltransferase complex from Arabidopsis and rice genomes. These analyses revealed the existence of several genes which share homology with known m⁶A enzymes, are predicted to reside with the mitochondria or chloroplasts, and are conserved between dicot and monocot species (Table S7). Among the factors which match such criteria are At-MTA and FIP37 proteins, which were also identified in proteomic analyses of plant organellar preparations (see Table S7). These observations may also relate to the similarities in the m⁶A sequence motifs found between the nuclear- and organellar-encoded transcripts (Fig. 4b). The location and functions of candidate factors (*e.g.*, At-MTA and FIP37) in m⁶A-RNA modifications in plant organelles are currently under investigation.

Conclusions

The expression of the mitochondrial genomes in plants is complex, particularly at the posttranscriptional level. RNA processing events that contribute to mitochondrial gene expression include the processing of polycistronic transcripts, trimming, splicing and deamination of many Cytidine residues to Uracil (reviewed in *e.g.*, (Zmudjak and Ostersetzer-Biran 2017). More than 100 types of RNA-base modifications are currently known (Nachtergaele and He 2017). These RNA modifications can affect cellular activities by altering the genetic code (*e.g.*, pseudouridylation and deamination), affecting the RNA structure, or by recruiting specific cofactors (readers) that influence a wide range of biological functions, *e.g.*, transcription, stability, processing and translatability. The m⁶A modifications show dynamic patterns, changing in their distribution and levels in response to various environmental or developmental signals. Here, we show that plant mitochondrial RNAs undergo many N⁶-

adenosine methylation (m⁶A) modifications in different RNA types (*i.e.*, intergenic-expressed transcripts, tRNAs, mRNAs, intron and UTR sequences), with an occurrence of about 5 m⁶A sites per 1,000 Adenosine residues. Does m⁶A affects organellar gene expression in plants? Currently, we cannot provide a definitive answer. The in vitro translation assays may indicate that m⁶A have a selective program: it is detrimental for the translation of RNAs modified at multiple sites (e.g., as introns and noncoding RNAs), while methylations within, or near, the start codon may rather enhance the translation. Based on the data, we speculate that m⁶A may adds an additional layer of posttranscriptional regulation in the expression program of plant mitochondria, which play critical roles in cellular metabolism. Accordingly, m⁶A seems enriched in ribosome-associated mRNAs, while mutants affected in organellar gene expression show specific changes in their m⁶A methylomes. However, such assumptions need to be supported experimentally. Analysis of Arabidopsis plants affected in genes that function in m⁶A modification in plant mitochondria would provide with more insights into the roles of m⁶A modifications to mitochondria biogenesis and gene expression. We further intend to define whether the m⁶A landscapes in plant mitochondria change in response to physiological and environmental stimuli.

Experimental procedures

Plant material and growth conditions

Plants growth and analyses generally followed the procedures described in (Sultan, *et al.* 2016). *Arabidopsis thaliana* ecotype Columbia (Col-0) seeds were obtained from the ABRC center, at Ohio State University (Columbus, OH). The seeds were sown on MS-agar plates, incubated in the dark for 2 days at 4°C, and then transferred to controlled temperature and light conditions in the growth chambers [*i.e.* 22°C under short day conditions (8 hrs. light, 150 µE·m⁻²·s⁻¹ and 16 hour dark)]. Cauliflower (*Brassica oleracea* var.) inflorescences were purchased fresh at local markets and used immediately.

Mitochondria extraction and analysis

Isolation of mitochondria from 3-week-old Arabidopsis seedlings grown on MS-plates was performed according to the method described in (Keren, *et al.* 2012). Preparation of mitochondria from cauliflower inflorescence followed the protocol described in detail in (Neuwirt, *et al.* 2005). In brief, about 1.0 kg cauliflower inflorescences were cut off from the stems, and ground with ice-cold extraction buffer [0.9 M mannitol, 90 mM Na-pyrophosphate,

6 mM EDTA, 2.4% PVP25 (w/v), 0.9% BSA (w/v), 9 mM cysteine, 15 mM glycine, and 6 mM β -mercaptoethanol; pH 7.5]. Mitochondria were recovered from the extract by differential centrifugations and purification on Percoll gradients, aliquoted and stored frozen at -80°C.

RNA extraction and analysis

RNA extraction from highly-purified mitochondria was performed essentially as described previously (Cohen *et al.* 2014, Keren, *et al.* 2011, Zmudjak, *et al.* 2017). In brief, RNA was prepared following standard TRI Reagent[®] protocols (Molecular Research Center Inc., TR-118). The RNA was treated with RNase-free DNase (Ambion, Cat no. AM2222) prior to its use in the assays. Reverse transcription was carried out with Superscript III reverse transcriptase (Thermo Fisher Scientific, Cat no. 18080093), using 1 μ g of total Arabidopsis or cauliflowerer mtRNA and 250 ng of a random hexanucleotide mixture (Promega, Cat no. C1181) and incubated for 50 min at 50°C. Reactions were stopped by 15 min incubation at 70°C. Quantitative reverse transcription PCR (RT-qPCR) was performed essentially as described previously (Sultan, *et al.* 2016, Zmudjak, *et al.* 2017).

Detection of N^6 -Adenosine methylations (m^6A) in mitochondrial RNAs by LC-UV-MS/MS

Detection of m^6A methylations in mitochondrial RNA (mtRNA) was carried out by LC-UV-MS/MS, essentially as described previously (Thuring *et al.* 2016). In brief, 75 μ g total mtRNA was digested to single nucleosides, using 10 U S1 nuclease (Thermo Fisher Scientific, Cat no. EN0321), 1 U snake venom phosphodiesterase (Exo-PI; Sigma-Aldrich, Cat no. P4506), and 10 U alkaline phosphatase (Thermo Fisher Scientific, EF0654). The nucleosides products were then analyzed by liquid chromatography-mass spectrometry, using Luna[®] polar (RP-C18) column (Phenomenex, Aschaffenburg, Germany). Data were acquired and processed using the MassHunter software (version B.06.00, Agilent Technologies, Wilmington, DE, USA). Table S1 summarizes the chromatographic data, including the retention times (RT), masses, abundances and molecular formula of the analyzed products.

Calorimetric m^6A measurements

Global m^6A modifications in total organellar RNAs was assessed by the EpiQuik m^6A RNA Methylation Quantification Kit (Epigentek Group Inc., Farmingdale, NY) according to the manufacturers' specifications, with 200 ng total RNA, in duplicates.

m⁶A-RNA co-immunoprecipitation (m⁶A-RIP)

Monoclonal anti-m⁶A antibody (Synaptic systems, Cat no. 202011) was incubated with Protein A/G Magnetic Beads (Pierce, 88802) for 30 min at room temperature in IPP buffer (0.2 M NaCl, 0.1% NP-40, 10 mM Tris-HCl, pH 7.5, 1 U/μl RNasin). The antibody-bound beads were washed twice with IPP buffer and resuspended in 200 μl of IPP buffer. Prior to m⁶A-RIP, the ribosomal RNA was removed from the total mtRNA using the RiboMinus™ Plant Kit for RNA-seq (Thermo Fisher Scientific, Cat no. A1083808). The purified organellar RNA was then fragmented using an RNA Fragmentation kit (Ambion, Cat no. AM8740), yielding fragments in the range of 60 to 200 nts. The fragmented RNA was denatured at 70°C for 2 min and then incubated for 2~4 hrs. at 4°C with 5 μg anti-m⁶A antibody coupled to magnetic protein A/G beads, in the presence of RNase inhibitor (Ambion, Cat no. AM2682). The beads were then washed twice with IPP buffer, twice with low salt buffer (50 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl pH 7.5), twice with high salt buffer (0.5 M NaCl, 0.1% NP-40, 10 mM Tris-HCl pH 7.5) and once with IPP buffer. The m⁶A-RNA was released from the beads with RLT buffer (Qiagen, 79216) and purified using the Dynabeads™ MyOne™ Silane RNA isolation Kit (Thermo Fisher Scientific, Cat no. 37002D). For the analysis of m⁶A in mtRNAs (i.e., cauliflower) we further used a non-radioactive northern blotting (Keren, *et al.* 2011), with anti-m⁶A antibodies (Fig. S1).

Transcriptome mapping by high-throughput (RNA-seq) analysis

Total mtRNA was extracted from mitochondria preparations, obtained from Arabidopsis and cauliflower inflorescence. Sequencing was carried out on an Illumina Genome Analyzer (The Center for Genomic Technologies, The Hebrew University of Jerusalem, Israel), generally as described previously (Grewe, *et al.* 2014). Reads were first filtered for low quality and primers or adapters contaminations using trimomatic (version 0.36) (Bolger *et al.* 2014). Filtered reads were mapped to the *A. thaliana* and *B. oleracea* mitochondrial genomes (NC_001284 and KJ820683.1, respectively) using Bowtie2 (version 2.2.9) (Langmead and Salzberg 2012). BEDTools (Quinlan and Hall 2010) was used to determine the coverage of mapped reads of the m⁶A-RIP-seq and input RNA RNA-seq libraries on the genomes in a 1 bp intervals, and m⁶A peaks were called using an R-script modified from (Luo, *et al.* 2014) (Supplementary script 1). Only significant peaks (p-value < 0.05) were selected for further analysis (Supplementary datasets 1&2). Resulting m⁶A peaks were mapped to annotated genes using BEDTools intersect. Consensus of methylation sites was predicted using HOMER (Heinz, *et al.* 2010). Sequences are available at the Sequence Read Archive (SRA, accession PRJNA472433).

Synthesis of recombinant His-tagged mitochondrial proteins

In vitro transcription assays were performed as described previously (Ostersetzer and Adam 1997, Ostersetzer and Adam 1999). Gene-fragments corresponding to ATP1, MatR and NAD4 were generated by RT-PCRs with specific oligonucleotides designed to each gene-fragment (i.e., containing the *Nco* I and *Xho* I restriction sites) (supplementary Table S8). The fragments were digested with the restriction endonucleases *Nco* I and *Xho* I and cloned into same sites in pET28b (see Table S8). The integrity of each construct was confirmed by DNA-sequencing (Center for Genomic Technologies, HUJI, Israel). For *in vitro* transcriptions, 1 U of T7 RNA polymerase (Promega, Cat no. P2075) was added to 500 ng of the cDNA-fragments in T7 transcription reaction buffer (Promega, Cat no. P2075), and incubated for 2 hours at 37°C in the absence or presence of *N*⁶-methyladenosine (Jena Biosciences, Cat no. NU-1101L) to generate ‘body-labeled’ m⁶A-RNAs. mRNAs labeled in the start codon (i.e. m⁶AUG) were generated by custom synthesis (i.e., Tamar Laboratories, Israel, Integrated DNA Technologies, Inc., IDT), with the following sequence: TTTAAGAAGGAGATATACC/iN6Me-rA/TG[xxx], where the ribosome binding (rbs, or Shine-Dalgarno) sequence is underlined, iN6Me-rA corresponds to the m⁶A modification (according to IDT), and the ‘xxx’ region corresponds to specific ATP1, MatR or NAD4 gene-fragments containing a 6x His-tag ligated to the synthetic m⁶AUG-RNA fragments (see Table S8). Recombinant His-tagged ATP1, MatR and NAD4 proteins were obtained by *in vitro* translation assays, using a coupled transcription-translation system containing wheat germ extract (TNT kit; Promega, Cat no. L5030), according to the manufacturer protocol.

RNA stability assay

Transcript fragments corresponding to *matr*, *nad4* and *atp1* were transcribed *in vitro* with Biotin-14-CTP (ThermoFischer Scientific, 19519016) and in the absence or presence of m⁶ATP (see Table S1). The *in vitro* transcribed RNAs were incubated with mitochondrial extracts for 2h at 30°C. For the analysis of RNA stability, organellar samples were taken at specific time points, as indicated in the blots, and RNA stability was analyzed by Filter binding assays (Barkan *et al.* 2007, Keren *et al.* 2008, Ostersetzer *et al.* 2005). Detection of RNAs was carried out by non-radioactive northern blots, using anti-Biotin antibodies and chemiluminescent detection.

Isolation of ribosome-bound mRNAs from cauliflower mitochondria

Organellar polysomes were obtained generally as described previously (Barkan 1998, Kahlau and Bock 2008, Uyttewaal *et al.* 2008, Zoschke *et al.* 2013), with some modifications. Mitochondria isolated from cauliflower inflorescences were suspended in polysomal extraction buffer (*i.e.*, 40 mM Tris-acetate pH 8.0, 10 mM MgCl₂, 0.2 M Sucrose, 10 mM β-mercaptoethanol, 1% Triton X-100, 2% polyoxyethylen-10-tridecylether, 0.5 mg/ml heparin, chloramphenicol 100 µg/ml, cycloheximide 25 µg/ml). The mitochondrial suspension was centrifuged (15,000 x g, 10 min at 4°C) to remove insoluble material, and the clear lysate was then loaded onto a step gradient containing 15%, 30%, 40% and 55% sucrose in polysome gradient buffer (0.4 M Tris-HCl pH 8.0, 0.2 M KCl and 0.1 M MgCl₂) and centrifuged at 45,000 x g at 4 °C for 65 min at 4°C. Each gradient was then separated into several fractions (*i.e.*, bottom-to-top) of equal volumes (about 100 µl) using a fraction collector (BioRad, Hercules, USA). RNA from all fractions was prepared following standard TRI Reagent® protocols (Molecular Research Center Inc., TR-118).

Accession numbers

SRA accessions PRJNA472433

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Short legends for Supporting Information

Table S1. Chromatographic data and MS parameters for m⁶A nucleoside analysis.

Table S2. Quantification of the bulk levels of m⁶A by calorimetric assays.

Table S3. RNA-seq’s of rRNA-depleted total RNAs from Arabidopsis and cauliflower mitochondria (PRJNA472433, replicate 1).

Table S4. List of mitochondrial genes in Arabidopsis and cauliflower identified by the m⁶A-RIP-seq analyses (PRJNA472433, replicate 1).

Table S5. Lists of oligonucleotides used for the analysis of the splicing profiles of wild-type and mutant plants by RT-qPCR experiments.

Table S6. List of chloroplast genes in Arabidopsis identified by the m⁶A-RIP-seq analyses.

Table S7. List of putative m⁶A modification enzymes in Arabidopsis mitochondria.

Table S8. Oligonucleotides used to the construction of gene-fragments corresponding to MatR, NAD4 and ATP1.

Figure S1. Analysis of m⁶A modifications in cauliflower mtRNA.

Figure S2. Identification of m⁶A by LC-MS/MS in mitochondrial RNAs isolated from wild-type cauliflower inflorescences.

Figure S3. Analysis of m⁶A methylomes in Arabidopsis mtRNAs.

Figure S4. Distribution of m⁶A modifications in noncoding mtRNAs of Arabidopsis and cauliflower.

Figure S5. The effect of m⁶A modifications on mtRNA stability.

Figure S6. Analysis of m⁶A methylomes in plastid RNAs of Arabidopsis plants.

Supplementary script 1. The R script used to in this study for m⁶A peaks discovery from RIP-RNA-seq data.

Supplementary Data Set 1. Arabidopsis RNA-seq data set.

Supplementary Data Set 2. Cauliflower RNA-seq data set.

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Tables

Table 1. m⁶A-RIP-seq data

(a) m⁶A-RIP-seq data corresponding to SRA accessions PRJNA472433, replicate 1.

m ⁶ A-RNA		<i>Arabidopsis thaliana</i> (No. of peaks)	<i>Brassica oleracea</i> (No. of peaks)
Known genes		189 peaks / 41 genes	310 peaks / 18 genes
	UTRs	8	17
	Exons/coding regions	115	162
	Introns	66	131
mtORFs		11 peaks / 10 genes	51 peaks / 21 genes
Intergenic regions		195	349
rRNAs* ¹		(12)	(9)
tRNAs* ¹		(1)	(8)

(b) m⁶A-RIP-seq data corresponding to SRA accessions PRJNA472433, replicate 2.

m ⁶ A-RNA		<i>Arabidopsis thaliana</i> (No. of peaks)	<i>Brassica oleracea</i> (No. of peaks)
Known genes		235 peaks / 37 genes	266 peaks / 23 genes
	UTRs	27	12
	Exons/coding regions	122	150
	Introns	85	104
mtORFs		16 peaks / 7 genes	35 peaks / 22 genes
Intergenic regions		244	305
rRNAs* ¹		(18)	(2)
tRNAs* ¹		(1)	(6)

*¹ - The m⁶A-RIP-seq data corresponding to rRNAs and tRNAs should be taken with care due to rRNA and tRNA depletion prior to the RNA-seq analyses (see Material and Methods).

Figure legends

Figure 1. m⁶A-RIP-seq analyses of plant mitochondrial RNAs.

Schematic diagram of the m⁶A-seq protocol. Total RNA was isolated from mitochondria obtained from *Arabidopsis thaliana* or *Brassica oleracea* (var. botrytis; Cauliflower) inflorescences. (1) The presence of N⁶-adenosinemethylated (m⁶A) RNAs in the mitochondrial transcripts was confirmed by Liquid chromatography/mass spectrometry (LC-MS) analysis. (2) rRNA-depleted mtRNA was fragmented, and the organellar m⁶A-RNAs were immunoprecipitated, using monoclonal m⁶A antibodies and analyzed by RNA-seq.

Figure 2. Identification of m⁶A by LC-MS/MS in mitochondrial RNAs isolated from wild-type cauliflower inflorescences.

Total mtRNA, isolated from cauliflower mitochondria, was digested to single nucleosides with S1 nuclease and phosphodiesterase, and the presence of modified Adenosine residues (m⁶A) was assayed by LC-MS, based on specific retention times from standard and modified nucleosides separated by the same chromatographic procedure. The UV absorption spectra of (a) different nucleosides (A, G, C, and U) and m⁶A standards, (b) nucleosides obtained by total RNA digestion and (c) the specific MS spectrum of m⁶A in mitochondrial RNA preps are given in each panel. The LC-MS total ion chromatogram data of mtRNA nucleosides is presented in Figure S2. The chromatographic data, including the retention times (RT), masses, abundances and molecular formula of the analyzed products are summarized within Table S1

Figure 3. The m⁶A landscapes of plant mtRNAs reveal a unique topology.

(a) pie-charts presenting the fraction of m⁶A peaks in coding-regions (exons), introns, UTRs and intergenic regions in *Arabidopsis* (left panel) or *Cauliflower* (right panel) mitochondria, represented in the m⁶A-RNA-seq data (*i.e.*, SRA accessions PRJNA472433, replicate 1). (b) Distribution of m⁶A peaks in *Arabidopsis* (left panel) or *Cauliflower* (right panel) sequences corresponding to exons (upper panels) and intron regions (lower panels), according to normalized exons and introns lengths (PRJNA472433, replicate 1). A list of mitochondrial genes in *Arabidopsis* and *cauliflower* identified by the m⁶A-RIP-seq analyses is presented in Table S4.

Figure 4. Common m⁶A patterns in *Arabidopsis* and *cauliflower* mitochondria.

Relative abundances of N^6 -adenosinemethylated nucleotide sites in organellar transcripts in both Arabidopsis and cauliflower, corresponding to (a) coding-regions (or exons) and (b) non-coding RNAs. The identity of putative consensus sequence motifs in m^6A -modified mtRNA, was analyzed by the HOMER (Heinz, *et al.* 2010) and MEME (Bailey, *et al.* 2009) programs. Integration of results from the motif discovery tools, indicated to the common m^6A elements, DRm⁶AYHSYS (where ‘D’ is A, G or U, ‘R’ is A or G, ‘Y’ is C or U, ‘S’ is C or G, and ‘H’ is A, C or U) for mRNAs (c) and HRm⁶AYS for noncoding mtRNAs (d).

Figure 5. In vitro transcription of body-labeled m^6A -RNAs.

(a) Schematic representation of constructs used for in vitro transcription assays. cDNAs corresponding to *atp1*, *matR* and *nad4* were obtained by RT-PCR, with total RNA isolated from Arabidopsis mitochondria, such as each gene-fragment contained a T7 RNA polymerase (RNAP) promoter site at the 5’ termini and a C-terminal 6x His tag (see Materials and Methods). mtRNAs modified only in the initiation codon (m^6A Ug-mRNA) were generated by custom RNA synthesis (see Materials and Methods). (b) *In vitro* transcription of cDNAs (*i.e.*, *atp1*) with T7 RNA polymerase, in the absence (0) or presence of 10, 20, 50 or 100% m^6A ATP/ATP ratios, were used in order to obtain ‘body-labeled’ m^6A transcripts. Different amounts (*i.e.*, 0.5, 1.0, 1.5 or 2.0 μ l) of *atp1* transcripts (diluted to a final RNA conc. of 100 ng/ μ l) were loaded onto agarose gel (1% w/v), and separated by gel-electrophoresis. Quantification data of the relative levels of *atp1* mRNA transcripts are indicated below the blots in panel ‘b’.

Figure 6. The effect of m^6A modifications on mtRNA stability.

(a) The decay patterns of control (*i.e.*, non-modified) versus m^6A body labeled mRNAs, were examined by incubating the in vitro transcribed *atp1* mRNAs with purified cauliflower mitochondrial extracts. Samples were collected at different time points, as indicated in the figure, and the relative levels of *atp1* mRNAs were examined by Filter binding assays (Keren, *et al.* 2008, Keren, *et al.* 2011, Ostersetzer, *et al.* 2005). (b) Quantification data of the relative levels of *atp1* mRNA transcripts following incubation with the organellar extracts. The RNA-degradation patterns of m^6A modified versus control (non-modified) *matR* and *nad4* mRNAs are presented in Figure S5.

Figure 7. m^6A is detrimental for the translation of mtRNAs modified at multiple sites.

(a) The translation efficiencies of transcripts corresponding to *atp1*, *nad4* and *matR*, containing increased levels of m⁶A modifications along the transcripts (*i.e.*, body-labeled RNAs), were examined by western-blot analyses of in vitro translated products, using a wheat germ extract system. To enable the synthesis of recombinant proteins by organellar ribosomes, the translation of the corresponding *atp1*, *matR* and *nad4* transcripts was assayed in the presence of cycloheximide (CycH, 100 µg/ml), which blocks peptidyl transferase activity in eukaryotic ribosomes. In addition, the translation efficiencies were also analyzed in the presence of Kanamycin (50 µg/ml), which interrupt the synthesis of organellar (*i.e.*, mitochondria and plastids) proteins (Wang, *et al.* 2015a). (b) The translation efficiencies of *atp1*, *matR* and *nad4* transcripts, modified in the presence of different, *i.e.*, 0, 2.5, 5, 10, 25, 50 and 100%) m⁶A/A ratios.

Figure 8. The translation efficiencies of transcripts corresponding to *atp1*, *nad4* and *matR*, modified specifically at the AUG site (*i.e.*, m⁶AUG).

(a) The translation efficiencies of transcripts corresponding to *atp1*, *nad4* and *matR*, modified by m⁶A specifically at the AUG sites, were examined by western-blot analyses of in vitro translated products, using a wheat germ extract system. (b) To enable the synthesis of recombinant proteins by organellar ribosomes, the translation of the corresponding *atp1*, *matR* and *nad4* transcripts was assayed in the presence of in the presence of Kanamycin (50 µg/ml), which interfere with the synthesis of mitochondrial (or plastidial) polypeptides (Wang, *et al.* 2015a). The authors wish to note that the image in the MATR panel in Fig. 8b was cropped in order to bring the m⁶A-MatR lane close to the control (unmodified) lanes (marked by a dashed-line). No other changes have been made to the original figure.

Figure 9. m⁶A methylomes of Arabidopsis mutants affected in mtRNA metabolism.

The m⁶A methylation landscapes of *nmat1* (a) and *nmat2* (b) were analyzed by RT-qPCR following m⁶A pulldown assays, using anti-m⁶A antibodies. Total RNA extracted from wild-type (Col-0) and *nmat1* (Keren, *et al.* 2012, Nakagawa and Sakurai 2006) and *nmat2* (Keren, *et al.* 2009, Zmudjak, *et al.* 2017) mutants was used in m⁶A-RIP assays, with monoclonal m⁶A antibodies. The relative accumulation of different m⁶A-modified RNAs was assayed by RT-qPCR analyses with oligonucleotides corresponding to different genes in *Arabidopsis thaliana* mitochondria (Table S5). Panels ‘a’ and ‘b’ represent the relative m⁶A-RNA abundances in *nmat1* and *nmat2* mutants versus wild-type plants, respectively. Data in panels ‘a’ and ‘b’

represent the mean ($\pm$ standard deviation, SD) of 15 replicates in 5 independent experiments (i.e., each performed in triplicate, and are presented relative to control).

Figure 10. m⁶A-mRNA methylation associated with different polysomal fractions.

Mitochondria isolated from cauliflower inflorescences were fractionated by sucrose gradient centrifugation. (a) Polysome profiles of cauliflower mitochondria (collected from bottom to top). (b) m⁶A mRNA extracted from different fractions quantified by calorimetric assay. (c) The relative levels of m⁶A-RNAs versus the input mRNA, evaluated by RT-qPCR of various mitochondrial mRNAs following m⁶A pulldown assays, using anti-m⁶A antibodies (i.e., log₂ ratios). Data represent the mean ($\pm$ standard deviation, SD) of 12 replicates in 4 independent experiments (i.e., each performed in triplicate, and are presented relative to control). Input correspond to ribosomal-depleted mtRNA before fractionation procedures, while fractions 105-150 indicate the sub-ribosomal fractions, fractions 65-95 correspond to mitochondrial monosomes, and fractions 5-50 relate to the organellar polysomes. Error bar shows standard deviation (STDEV) values. Each measurement was the average of three biological replicates. Small italic letters in panel 'c' indicate a significant difference from input RNA values (Student's T-test, P 0.05).

Figure 1

Figure 2

a

Standard Mix

b

total mtRNA

c

mtRNA-m⁶A

Figure 3

a

Arabidopsis thaliana

Brassica oleracea

b

Figure 4

Figure 5

a

b

Figure 6

Figure 7

a

b

Figure 8

a. In vitro translation using WGE

b. In vitro translation using WGE, + Kan (50 µg/ml)

Figure 9

a

b

Figure 10

a

b

m⁶A methylation associated with different polysomal fractions

c

Figure S1. Analysis of m⁶A modifications in cauliflower mtRNA. The presence of m⁶A modifications was analyzed by slot blots and immunoassays with anti-m⁶A antibodies.

a**Standard nucleosides Mix** **b****mtRNA nucleosides** **c****m⁶A**

Figure S2. Identification of m⁶A by LC-MS/MS in mitochondrial RNAs isolated from wild-type cauliflower inflorescences.

Total mtRNA, isolated from cauliflower mitochondria, was digested to single nucleosides with S1 nuclease and phosphodiesterase, and the presence of modified Adenosine residues (m⁶A) was assayed by LC-MS, based on specific retention times from standard and modified nucleosides separated by the same chromatographic procedure. The UV absorption spectra of (a) different nucleosides (A, G, C, and U) and m⁶A standards, (b) nucleosides obtained by in total RNA digestion and (c) the specific MS spectrum of m⁶A nucleosids in mitochondrial RNA preps are given in each panel.

a ATMG00560 (*rpl2* gene)

b ATMG00580 (*nad4* gene)

Figure S3. Representative analyses of *N*⁶-adenosine methylomes in Brassicales mtRNAs. The topology of m⁶A modifications was analyzed by m⁶A-RIP-seq analyses with anti-m⁶A antibodies. The reads were aligned to Arabidopsis mitochondrial genome (NC_001284.2; Unseld et al., 1997). Coverage of m⁶A (blue line) and control (input mtRNA, in red line) of two organellar genes, *rpl2* (a) and *nad4* (b) is indicated in the figure. To normalize for expression levels, coverage for each position is divided by the mean coverage in the control experiment.

Figure S4. Distribution of m⁶A modifications in noncoding mtRNAs of Arabidopsis and cauliflower. Distribution of m⁶A peaks in Arabidopsis (a) or Cauliflower (b) sequences corresponding to noncoding RNAs, according to normalized gene lengths.

Figure S5. The effect of m6A modifications on mtRNA stability.

The decay patterns of control (i.e., non-modified) versus m6A body labeled mRNAs corresponding to *matR* (a) or *nad4* (b), were examined by incubating the in vitro transcribed RNAs with purified cauliflower mitochondrial extracts. Samples were collected at different time points, as indicated in the figure, and the relative levels of *matR* and *nad4* mRNAs were examined by Filter binding assays (Ostersetzer et al., 2005; Keren et al., 2008; Keren et al., 2011).

a

b

Distribution of m⁶A sites within plastid RNAs

c

Distribution of m⁶A sites within plastid RNAs

Figure S6. Analysis of m⁶A methylomes in plastid RNAs of Arabidopsis plants. (a) The topology of m⁶A modifications was analyzed by m⁶A-RIP-seq analyses with anti-m⁶A antibodies. The reads were aligned to the chloroplast genome of Arabidopsis (NC_000932.1). (b) pie-charts presenting the fraction of m⁶A peaks in genes (62 reads) and expressed-intergenic regions (9 reads) in Arabidopsis chloroplasts. (c) Distribution of m⁶A peaks in Arabidopsis sequences corresponding to coding regions, according to normalized gene lengths. A list of mitochondrial genes in Arabidopsis and cauliflower identified by the m⁶A-RIP-seq analyses is presented in Table S6.

Table S1.

A. Chromatographic data.

Nucleoside label	Retention time (min)	Formula	Abundance (relative counts)	Target Mass (gr • mol ⁻¹)
Cytidine (C)	3.69	C ₉ H ₁₃ N ₃ O ₅	12300	243.08698
Uridine (U)	5.20	C ₉ H ₁₂ N ₂ O ₆	1931	244.07114
Guanosine (G)	9.18	C ₁₀ H ₁₃ N ₅ O ₅	3160	283.09308
Adenosine (A)	12.47	C ₁₀ H ₁₃ N ₅ O ₄	3113	267.09795
<i>N</i> ⁶ -methyladenosine (m ⁶ A)	15.79	C ₁₁ H ₁₅ N ₅ O ₄	2459	281.11359

B. ESI-MS and ESI-MS/MS parameters for m⁶A nucleoside analysis

Compounds	Precursor ions (m/z)	Product ions (m/z)	Collision energy(V)	ΔT (min)
M ⁶ A	282. 1211[M+H] ⁺	123.06384 [M-159+H] ⁺	15	14-17
		130.99616 [M-151+H] ⁺	15	
		133.04954 [M-149+H] ⁺	15	
		150.07742 [M-132+H] ⁺	15	

Table S2.

(a) Quantification of the bulk levels of m⁶A in organellar RNA preparations of Arabidopsis and cauliflower plants, using a commercial colorimetric m⁶A quantification assay.

	OD ₄₅₀ -NC* ¹	Calculated m ⁶ A (ng)	m ⁶ A/RNA ratio (ng/ng)	m ⁶ A/A ratio
Arabidopsis mitochondrial RNA	0.475 ± 0.085	0.247 ± 0.044	0.123 ± 0.022	0.436 ± 0.088
Arabidopsis plastid RNA	0.1575 ± 0.015	0.082 ± 0.008	0.041 ± 0.004	0.144 ± 0.016
cauliflower mitochondrial RNA	0.417 ± 0.016	0.217 ± 0.008	0.108 ± 0.004	0.380 ± 0.016

*¹ – OD₄₅₀-NC, The optical densities of organellar m⁶A-RNAs at 450 nm as indicated by the calorimetric kit, minus the negative control (no RNA).

(b) Quantification of m⁶A ratios in mitochondrial RNA preparations of Arabidopsis and cauliflower polysomic fractions, using a commercial colorimetric m⁶A quantification assay.

	m ⁶ A/A ratio	relative m ⁶ A/A ratios
Input RNA (ΔrRNA)	0.428 ± 0.025	1.0 ± 0.058
Subribosomal fractions	0.167 ± 0.087	0.39 ± 0.203
Monosomal fractions	0.702 ± 0.031	1.64 ± 0.073
Polysomal fractions	0.686 ± 0.047	1.60 ± 0.113

Table S3. RNA-seq's of rRNA-depleted total RNAs from Arabidopsis and cauliflower mitochondria.

Library	Plant	Total reads	QC filtered	Mapped to mtDNA
Input RNA	<i>A. thaliana</i>	62126071	61812498 (99.50%)	25125548 (40.65%)
m6A-RIP	<i>A. thaliana</i>	7528773	7425907 (98.63%)	1327196 (17.87%)
Input RNA	<i>B. oleracea</i>	51288692	51048195 (99.53%)	37467278 (73.40%)
m6A-RIP	<i>B. oleracea</i>	29371176	29201322 (99.42%)	20505049 (70.22%)

Table S4. List of mitochondrial genes in Arabidopsis and cauliflower identified by the m⁶A-RIP-seq analyses (SRA accession PRJNA472433).

Gene name	<i>Arabidopsis thaliana</i> (Col-0)	<i>Brassica oleracea</i> (var. botrytis)	m ⁶ A-RNA-seq	
	Accession No.	Accession No.	peaks	
A. Known genes				
ATP synthase subunit 1 (<i>ATP1</i>)	ATMG01190	BroleMp020	2	6
ATP synthase subunit 4 (<i>ATP4</i> ; <i>ORF25</i>)	ATMG00640	BroleMp040	2	4
ATPase subunit 6-1 (<i>ATP6-1</i>)	ATMG00410	BroleMp039	4	4
ATP synthase subunit 6-2 (<i>ATP6-2</i>)	ATMG01170	-	4	-
ATP synthase subunit 8 (<i>ATP8</i> or <i>ORFB</i>)	ATMG00480	BroleMp061	2	1
ATP synthase subunit 9 (<i>ATP9</i>)	ATMG01080	BroleMp076	1	1
cytochrome C biogenesis 203 (<i>CCB203</i>)	ATMG00960	-	2	-
cytochrome C biogenesis 206 (<i>CCMB</i> or <i>CCB206</i>)	ATMG00110	BroleMp027	-	2
cytochrome C biogenesis 256 (<i>CCMC</i> or <i>CCB256</i>)	ATMG00900	BroleMp055	1	2
cytochrome C biogenesis 452 (CCMFC, <i>CCB452</i>)	ATMG00180	BroleMp066	5	2
cytochrome C biogenesis 382 (CCMFN1; <i>CCB382</i>)	ATMG00830	BroleMp075	3	2
cytochrome oxidase (<i>COX1</i>)	ATMG01360	BroleMp045	2	7
cytochrome c oxidase subunit 2 (<i>COX2</i>)	ATMG00160	BroleMp053	4	6
cytochrome c oxidase subunit 3 (<i>COX3</i>)	ATMG00730	BroleMp068	2	3
cytochrome c oxidase 2-like	ATMG01280	-	2	-
cytochrome b/b6 protein	ATMG00590	-	2	-
cytochrome c oxidoreductase apocytoch. b (<i>COB</i>)	ATMG00220	BroleMp052	3	7
NADH dehydrogenase subunit 1a (<i>NAD1A</i>)	ATMG01275	BroleMp011	1	8
NADH dehydrogenase subunit 1b (<i>NAD1B</i>)	ATMG01120	-	3	-
NADH dehydrogenase subunit 1c (<i>NAD1C</i>)	ATMG00516	-	8	-
NADH dehydrogenase subunit 2a (<i>NAD2A</i>)	ATMG00285	BroleMp056	3	8
NADH dehydrogenase subunit 2b (<i>NAD2B</i>)	ATMG01320	-	9	-
NADH dehydrogenase subunit 3 (<i>NAD3</i>)	ATMG00990	-	2	-
NADH dehydrogenase subunit 4 (<i>NAD4</i>)	ATMG00580	BroleMp015	13	10
NADH dehydrogenase subunit 5a (<i>NAD5A</i>)	ATMG00513	BroleMp002	3	15
NADH dehydrogenase subunit 5c (<i>NAD5C</i>)	ATMG00060	-	4	-
NADH dehydrogenase subunit 6 (<i>NAD6</i>)	ATMG00270	BroleMp013	1	-
NADH dehydrogenase subunit 7 (<i>NAD7</i>)	ATMG00510	BroleMp058	11	5
NADH dehydrogenase subunit 9 (<i>NAD9</i>)	ATMG00070	BroleMp037	1	-
Maturase R (<i>MatR</i> , encoded within <i>NAD1</i> intron 4)	ATMG00520	BroleMp003	6	9
<i>mttB</i> (or <i>TatC</i> , previously annotated as ORF-X)	ATMG00570	-	2	-
ribosomal protein L5 (<i>RPL5</i>)	ATMG00210	BroleMp050	2	-
ribosomal protein L16 (<i>RPL16</i>)	ATMG00080	BroleMp049	1	3
ribosomal protein L2-1 (<i>RPL2</i>)	ATMG00560	BroleMp018	3	6
ribosomal protein L2-2 (<i>RPSL2</i> , <i>RPS12</i>)	ATMG00980		2	4

ribosomal protein S3 (<i>RPS3</i>)	ATMG00090	BroleMp048	2	10
ribosomal protein s4 (<i>RPS4</i>)	ATMG00290	BroleMp057	3	2
ribosomal protein s7 (<i>RPS7</i>)	ATMG01270	BroleMp008	1	4

B. Pseudogenes

ribosomal protein S14 (<i>ΨRps14</i>)	(At-pseudo)	BroleMp051	-	3
<i>ΨMttB/TatC</i>	(At-pseudo)	BroleMp017	-	7

B. Annotated mtORFs

ORF101A	ATMG00260	-	1	-
<i>ORF101C</i>	-	BroleMp026	-	2
<i>ORF101E</i>	-	BroleMp047	-	2
<i>ORF101F</i>	-	BroleMp054	-	4
<i>ORF105</i>	-	BroleMp042	-	2
<i>ORF107A</i>	ATMG00030	-	1	-
<i>ORF108A</i>	-	BroleMp065	-	1
<i>ORF109</i>	ATMG00530	BroleMp021	1	8
<i>ORF110</i>	-	BroleMp032	-	1
<i>ORF113A</i>	-	BroleMp063	-	1
<i>ORF114</i>	ATMG01000	-	2	-
<i>ORF115A</i>	-	BroleMp060	-	1
<i>ORF115B</i>	-	BroleMp001	-	2
<i>ORF116</i>	-	BroleMp016	-	1
<i>ORF117</i>	ATMG00970	-	1	-
<i>ORF119</i>	-	BroleMp007	-	1
<i>ORF120</i>	-	BroleMp059	-	1
<i>ORF120A</i>	-	BroleMp062	-	1
<i>ORF122</i>	-	BroleMp010	-	2
<i>ORF122A</i>	ATMG00470	-	1	-
<i>ORF128</i>	-	BroleMp023	-	2
<i>ORF141</i>	ATMG00500	-	1	-
<i>ORF146</i>	-	BroleMp067	-	1
<i>ORF159</i>	ATMG01050	BroleMp004	-	2
<i>ORF215A</i>	ATMG00910	-	1	-
<i>ORF257</i>	-	BroleMp046	-	6
<i>ORF266</i>	-	BroleMp044	-	3
<i>ORF311</i>	-	BroleMp030	-	7

Table S5. Lists of oligonucleotides used for the analysis of the splicing profiles of wild-type and mutant plants by RT-qPCR experiments.

gene target	oligo name	sequence (5'-to-3')
<i>atp1</i>	<i>atp1F</i>	TCACTTCGACACGTCTTTGC
	<i>atp1R</i>	GGAATGGCCTTGAATCTTGA
<i>atp4</i>	<i>atp4F</i>	GGATCAGCTTGCGAATTTGT
	<i>atp4R</i>	GCAAATTGCTTCCCCACTAA
<i>atp6-1</i>	<i>atp6-1F</i>	TCTTTTGCAGTCAATGCAC
	<i>atp6-1R</i>	TCTCGCGTATCTCACATTGC
<i>atp8</i>	<i>atp8F</i>	CCGTCGACTTATTGGGAAAA
	<i>atp8R</i>	TTCCTTGGCCATGTACAACA
<i>atp9</i>	<i>atp9F</i>	CATTCCCTCTGACGTCGAAT
	<i>atp9R</i>	TCGTCGATCTTACCCTCGT
<i>ccmb</i>	<i>ccmBF</i>	TCTTGGAATCACATCCAGCA
	<i>ccmBR</i>	CGAGACCGAAATTGGAAAAA
<i>ccmc</i>	<i>ccmCF</i>	AGCTACGCGCAAATTCTCAT
	<i>ccmCR</i>	GCCGTGGCGATATAAACAAAT
<i>ccmfc</i>	<i>ccmFcF</i>	CACATGGAGGAGTGTGCATC
	<i>ccmFcR</i>	GTGGGTCCATGTAAATGATCG
<i>ccmfn-1</i>	<i>ccmFN1F</i>	AGCTCTTGGCATTGCTTTGT
	<i>ccmFN1R</i>	AGTGCCACAATCCCATTTCAT
<i>cob</i>	<i>cobF</i>	TGCCGGAATGGTATTTCTTA
	<i>cobR</i>	GCCAAAAGCAACCAAAACAT
<i>cox1</i>	<i>cox1F</i>	GTAGCTGCGGTGAAGTAGGC
	<i>cox1R</i>	CTGCCTGGATTTCGGTATCAT
<i>cox2</i>	<i>cox2F</i>	TGATGCTGTACCTGGTCGTT
	<i>cox2R</i>	TGGGGGATTAATTGATTGGA
<i>cox3</i>	<i>cox3F</i>	CCGTAACCTGGGCTCATCAT
	<i>cox3R</i>	AAACCATGAAAGCCTGTTGC
<i>matR</i>	<i>matRF</i>	AATTTTTCGAGAGCTGGAA
	<i>matRR</i>	TTGAACCCCGTCCTGTAGAC
<i>mttb</i>	<i>mttBF</i>	GGGGTCTTTCTTTGAAACC
	<i>mttBR</i>	TCTCCCTCATTCCACTCGTC
<i>nad1 exons a-b</i>	<i>nad1 1-2F</i>	GACCAATAGATACTTCATAAGAGACCA
	<i>nad1 1-2R</i>	TTGCCATATCTTCGCTAGGTG
<i>nad1 exons b-c</i>	<i>nad1 2-3F</i>	ATTCAGCTTCCGCTTCTGG
	<i>nad1 2-3R</i>	TCTGCAGCTCAAATGGTCTC
<i>nad1 exons c-d</i>	<i>nad1 3-4F</i>	AAAAGAGCAGACCCCATTTGA
	<i>nad1 3-4R</i>	TCCGTTTGATCTCCAGAAG
<i>nad1 exons d-e</i>	<i>nad1 4-5F</i>	AGCCCGGGATCTTCTTGA
	<i>nad1 4-5R</i>	TCTTCAATGGGGTCTGCTC
<i>nad2 exons a-b</i>	<i>nad2 exons a-bF</i>	GCGAGCAGAAGCAAGGTTAT
	<i>nad2 exons a-bR</i>	GGATCCTCCCACACATGTTC
<i>nad2 exons b-c</i>	<i>nad2 exons b-cF</i>	AAAGGAACTGCAGTGATCTTGA
	<i>nad2 exons b-cR</i>	AATATTTGATCTTAGGTGCATTTTC
<i>nad2 exons c-d</i>	<i>nad2 exons c-dF</i>	GCGCAATAGAAAGGAATGCT
	<i>nad2 exons c-dR</i>	CTATGGGTCTACTGGAGCTACCC
<i>nad2 exons d-e</i>	<i>nad2 exons d-eF</i>	CAAAGGAGAGGGGTATAGCAA
	<i>nad2 exons d-eR</i>	TATTTGTCTTCGCCGCTTT

<i>nad3</i>	<i>nad3F</i>	CGAATGTGGTTTCGATCCTT
	<i>nad3R</i>	GCACCCCTTTTCATTCATA
<i>nad4 exons a-b</i>	<i>nad4 exons a-bF</i>	ATTCTATGTTTTTCCCGAAAGC
	<i>nad4 exons a-bR</i>	GAAAACTGATATGCTGCCTTG
<i>nad4 exons b-c</i>	<i>nad4 exons b-cF</i>	AATACCCATGTTTCCCGAAG
	<i>nad4 exons b-cR</i>	TGCTACCTCCAATTCCTGT
<i>nad4 exons c-d</i>	<i>nad4 exons c-dF</i>	TTCCTCCATAAATTCTCCGATT
	<i>nad4 exons c-dR</i>	TGAAATTTGCCATGTTGCAC
<i>nad5 exons a-b</i>	<i>nad5 exons a-bF</i>	TGGACCAAGCTACTTATGGATG
	<i>nad5 exons a-bR</i>	CCATGGATCTCATCGGAAAT
<i>nad5 exons b-c</i>	<i>nad5 exons b-cF</i>	TACCTAAACCAATCATCATATC
	<i>nad5 exons b-cR</i>	CTGGCTCTCGGGAGTCTCTT
<i>nad5 exons c-d</i>	<i>nad5 exons c-dF</i>	AACTCGGATTCGGCAAGAA
	<i>nad5 exons c-dR</i>	GATATGATGATTGGTTTAGGTA
<i>nad5 exons d-e</i>	<i>nad5 exons d-eF</i>	AACATTGCAAAGGCATAATGA
	<i>nad5 exons d-eR</i>	GTTCTGCGTTTCGGATATG
<i>nad6</i>	<i>nad6F</i>	TATGCCGGAAGGTACGAAG
	<i>nad6R</i>	GTGAGTGGGTCAGTCGTCCT
<i>nad7 exons a-b</i>	<i>nad7 exons a-bF</i>	ACCTCAACATCCTGCTGCTC
	<i>nad7 exons a-bR</i>	AAGGTAAAGCTTGAAGATAAGTTTTGT
<i>nad7 exons b-c</i>	<i>nad7 exons b-cF</i>	GAGGGACTGAGAAATTAATAGAGTACA
	<i>nad7 exons b-cR</i>	TGGTACCTCGCAATTCAAAA
<i>nad7 exons c-d</i>	<i>nad7 exons c-dF</i>	ACTGTCACTGCACAGCAAGC
	<i>nad7 exons c-dR</i>	CATTGCACAATGATCCGAAG
<i>nad7 exons d-e</i>	<i>nad7 exons d-eF</i>	GATCAAAGCCGATGATCGTAA
	<i>nad7 exons d-eR</i>	AGGTGCTTCAACTGCGGTAT
<i>nad9</i>	<i>nad9F</i>	GGATGACCCTCGAAACCATA
	<i>nad9R</i>	CACGCATTCGTGTACAAACC
<i>rpl2</i>	<i>rpl2F</i>	CCGAAGACGGATCAAGGTAA
	<i>rpl2R</i>	CGCAATTCATCACCATTTTG
<i>rpl5</i>	<i>rpl5F</i>	AAGGGGTTCGACAGGAAAGT
	<i>rpl5R</i>	CGTATTTGACCGGAAAATC
<i>rpl16</i>	<i>rpl16F</i>	GAGCATTTGCCAAACTCACA
	<i>rpl16R</i>	CGGACACTTTCATCGTGCTA
<i>rps3</i>	<i>rps3F</i>	CCGATTTGCGTAAGACTTGG
	<i>rps3R</i>	AGCCGAAGGTGAGTCTCGTA
<i>rps4</i>	<i>rps4F</i>	ACCCATCACAGAGATGCACA
	<i>rps4R</i>	TCACACAAACCCTTCGATGA
<i>rps7</i>	<i>rps7F</i>	CTCGAACTGAACGCGATGTA
	<i>rps7R</i>	AAGCTGCTTCAAGGATCCAA
<i>rps12</i>	<i>rps12F</i>	AGCCAAAGTACGGTTGAGCA
	<i>rps12R</i>	TTTGGGTTTTTCTGCACCAT
<i>26S rRNA</i>	<i>rm26F</i>	GACGAGACTTTCGCCTTTTG
	<i>rm26R</i>	CTTGAGCGAATTGGATGAT
<i>18S rRNA (At3g41768)</i>	<i>18S nucl-F</i>	AAACGGCTACCACATCCAAG
	<i>18S nucl-R</i>	ACTCGAAAGAGCCCGGTATT
<i>actin2 (At3g18780)</i>	<i>actin2-F</i>	GGTAACATTGTGCTCAGTGGTGG
	<i>actin2-R</i>	AACGACCTTAATCTTCATGCTGC

Table S6. List of Arabidopsis chloroplast genes identified by the m⁶A-RIP-seq analyses

GeneID:844757	<i>atpB</i>	protein_coding	ArthCp029
GeneID:844804	<i>ndhA</i>	protein_coding	gene_synonym=ndh1
GeneID:844756	<i>ndhD</i>	protein_coding	gene_synonym=ndh4
GeneID:844763	<i>ndhJ</i>	protein_coding	ArthCp025
GeneID:844729	<i>petB</i>	protein_coding	ArthCp053
GeneID:844732	<i>psbN</i>	protein_coding	ArthCp051
GeneID:844754	<i>rbcL</i>	protein_coding	ArthCp030
GeneID:844722	<i>rpl16</i>	protein_coding	ArthCp060
GeneID:844718	<i>rpl2</i>	protein_coding	ArthCp064
GeneID:1466246	<i>rpl2</i>	protein_coding	ArthCp085
GeneID:844717	<i>rpl23</i>	protein_coding	ArthCp065
GeneID:1466247	<i>rpl23</i>	protein_coding	ArthCp084
GeneID:844784	<i>rpoC1</i>	protein_coding	ArthCp013
GeneID:844801	<i>rps12</i>	gene=rps12	gene_biotype=protein_coding
GeneID:1466250	<i>rps12</i>	gene=rps12	gene_biotype=protein_coding
GeneID:844771	<i>rps14</i>	protein_coding	ArthCp020
GeneID:844786	<i>rps2</i>	protein_coding	ArthCp011
GeneID:844765	<i>rps4</i>	protein_coding	ArthCp024
GeneID:844712	<i>rps7</i>	protein_coding	ArthCp069
GeneID:4386140	<i>rps7</i>	protein_coding	ArthCp088
GeneID:1466254	<i>rrn16S</i>	rRNA	ArthCr086
GeneID:1466252	<i>rrn23S</i>	rRNA	ArthCr087
GeneID:1466258	<i>trnA</i>	tRNA	ArthCt108
GeneID:4042813	<i>trnA</i>	tRNA	ArthCt114
GeneID:1466260	<i>trnI</i>	tRNA	ArthCt107
GeneID:5563231	<i>trnI</i>	tRNA	ArthCt124

GeneID:1466259	<i>trnL</i>	tRNA	ArthCt101
GeneID:1466266	<i>trnY</i>	tRNA	ArthCt096
GeneID:4386139	<i>ycf1</i>	protein_coding	Arthcp087
GeneID:844715	<i>ycf2</i>	protein_coding	ArthCp066
GeneID:1466248	<i>ycf2</i>	protein_coding	ArthCp083
GeneID:844767	<i>ycf3</i>	protein_coding	ArthCp023

Table S6. List of putative m⁶A modification enzymes in angiosperms mitochondria.

Gene name	Gene annotation ¹	<i>Arabidopsis</i> gene i.d.	Ortholog in <i>Oriza Sativum</i> ²	Intracellular Location	REFs
Putative Writers					
MTA	An m ⁶ A writer protein required for N ⁶ -adenosine methylation in Arabidopsis	At4g10760	Os02g45110	M ³ , N ^{4,6} , P ⁵	(Zhong <i>et al.</i> , 2008; Růžicka <i>et al.</i> , 2017) ^{4,6} ; (Zybailov <i>et al.</i> , 2008) ⁵
MTB	Encodes a member of a core set of mRNA m ⁶ A writer proteins	At4g09980	Os01g16180	M ³ , N ³ , P ³ , C ^{3,5}	(Bujnicki <i>et al.</i> , 2002; Ito <i>et al.</i> , 2011) ^{4,5}
FIP37	A core component of the m ⁶ A methyltransferase complex in Arabidopsis	At3g54170	Os06g27970	M ³ , N ^{4,6} , P ⁵	(Zhong <i>et al.</i> , 2008; Růžicka <i>et al.</i> , 2017) ^{4,6} ; (Ferro <i>et al.</i> , 2010) ⁵
FPA	Encodes an homolog of RBM15 which induces 3' UTR extension in Arabidopsis	At2g43410	Os09g34070	?	(Batista, 2017; Sun <i>et al.</i> , 2017)
HAKAI	Contains a conserved E3 ubiquitin ligase domain	At5g01160	Os10g35190	N ^{4,6}	(Růžicka <i>et al.</i> , 2017) ^{4,6}
VIR	Encodes a splicing/methylation factor homolog of the mammalian VIRILIZER	At3g05680	Os03g35340	N ^{4,6}	(Růžicka <i>et al.</i> , 2017) ^{4,6}
Putative Erasers					
ALKBH5	Encodes and homolog of the RNA demethylase ALKBH5 in <i>H. sapiens</i>	At1g11780	Os11g29690	?	
TWN2	RNA binding; similar to Valyl-tRNA synthetase.	At1g14610	Os03g48850	M ^{4,6} , C ^{4,6}	(Souciet <i>et al.</i> , 1999) ^{4,6}
Putative Readers					
ECT3	Conserved C-terminal RNA binding domain; homolog of YTHDF2 in <i>H. sapiens</i>	At5g61020	Os07g07490	?	
ECT5	Conserved C-terminal RNA binding domain; homolog of YTHDF2 in <i>H. sapiens</i>	At3g13060	Os08g44200	C ⁵	(Ito <i>et al.</i> , 2011) ⁵
ECT8	Conserved C-terminal RNA binding domain; homolog of YTHDF2 in <i>H. sapiens</i>	At1g79270	Os01g48790	N ³	
EIF3A	Homolog of eukaryotic translation initiation factor 3 subunit A	At4g11420	Os01g03070	C ⁵ , M ³ , N ⁵	(Ito <i>et al.</i> , 2011; Sakamoto & Takagi, 2013) ⁵
RS40	Encodes an Arg/Ser-rich splicing factor; homolog of hnRNPC1/C2 in <i>H. sapiens</i>	At4g25500	Os02g03040	N ⁴	(Chen <i>et al.</i> , 2013) ⁴
RS41	Encodes an Arg/Ser-rich splicing factor with Conserved RRM domain	At5g52040	Os02g03040	C ⁵ , M ³ , N ⁵	(Ito <i>et al.</i> , 2011; Sakamoto & Takagi, 2013) ⁵

1. Gene annotation by 'The Arabidopsis Information Resource' (TAIR).
2. Orthologues obtained by the POG server (Walker *et al.*, 2007).
3. Localization prediction by the SUBA server (Heazlewood *et al.*, 2007). N, nucleus; M, mitochondria; P, plastid; C, cytosol.
4. Localization supported by experimental data.
5. Localization supported by LC-MS/MS analysis.
6. Localization supported by GFP localization assays.

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Table S8. Oligonucleotides used to the construction of gene-fragments corresponding to MatR, NAD4 and ATP1.

Gene fragment	Oligo name	Oligo sequence (5'→3')
ATP1	Atp1-NcoI-F	TTTTT <u>CCATGGA</u> ATTATCTCCTAGAGCTGCG
	Atp1-NcoI-R	TTTTT <u>CTCGAGA</u> ATTAATTGTCCACAGTC
MatR	MatR-NcoI-F	TTTTT <u>CCATGGA</u> AGAGGCGATCAGAAT
	MatR-NcoI-R	TTTTT <u>CTCGAG</u> CTGAACAATCGGAATTTTCG
Nad4	Nad4-NcoI-F	TTTTT <u>CCATGGT</u> AGAACATTTCTGTGAATGC
	Nad4-NcoI-R	TTTTT <u>CTCGAG</u> CATAGGGATTGGCACGCTTTTCG
ATP1-cRNA	Atp1-cR-F	TAATACGACTCACTATA (G)GTTTATCTCCTAGAGCTGCG
	Atp1-cR-R	TTTTT <u>CTCGAG</u> CATAGGGATTGGCACGCTTTTCG
MatR-cRNA	MatR-cR-F	TAATACGACTCACTATA (G)GTGAGGCGATCAGAAT
	MatR-cR-R	TTTTT <u>CTCGAG</u> CTGAACAATCGGAATTTTCG
Nad4-cRNA	Nad4-cR-F	TAATACGACTCACTATA (G)GTGAACATTTCTGTGAATGC
	Nad4-cR-R	TTTTT <u>CTCGAG</u> CATAGGGATTGGCACGCTTTTCG

- Restriction sites are indicated by underline
- The consensus T7 RNA polymerase site is indicated by bold letters, while the first transcribed 'G' residue is found in brackets.