mRNA adenosine methylase (MTA) deposits m\(^6\)A on pri-miRNAs to modulate miRNA biogenesis in Arabidopsis thaliana

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In Arabidopsis thaliana, the METTL3 homolog, mRNA adenosine methylase (MTA) introduces N\(^6\)-methyladenosine (m\(^6\)A) into various coding and noncoding RNAs of the plant transcriptome. Here, we show that an MTA-deficient mutant (mta) has decreased levels of microRNAs (miRNAs) but accumulates primary miRNA transcripts (pri-miRNAs). Moreover, pri-miRNAs are methylated by MTA, and RNA structure probing analysis reveals a decrease in secondary structure within stem-loop regions of these transcripts in mta mutant plants. We demonstrate interaction between MTA and both RNA Polymerase II and TOUGH (TGH), a plant protein needed for early steps of miRNA biogenesis. Both MTA and TGH are necessary for efficient colocalization of the Microprocessor components Dicer-like 1 (DCL1) and Hyponastic Leaves 1 (HYL1) with RNA Polymerase II. We propose that secondary structure of miRNA precursors induced by their MTA-dependent m\(^6\)A methylation status, together with direct interactions between MTA and TGH, influence the recruitment of Microprocessor to plant pri-miRNAs. Therefore, the lack of MTA in mta mutant plants disturbs pri-miRNA processing and leads to the decrease in miRNA accumulation. Furthermore, our findings reveal that reduced miR393b levels likely contribute to the impaired auxin response phenotypes of mta mutant plants.

Significance

Recently N\(^6\)-methyladenosine (m\(^6\)A) methylation has emerged as a biological process with significant impact on cellular functions. However, almost all the research regarding m\(^6\)A methylation has been based on mRNAs. In our research, we focus on how m\(^6\)A methylation affects microRNA (miRNA) biogenesis in Arabidopsis. In brief, we show that m\(^6\)A methylation is necessary to maintain proper levels of mature miRNAs as well as their precursors. m\(^6\)A mark affects pri-miRNA secondary structures and affects the recruitment of the Microprocessor to pri-miRNAs. We also demonstrate the interactions of MTA (m\(^6\)A writer) with other proteins involved in miRNA biogenesis, namely RNA Polymerase II and TOUGH. Our study provides evidence of the role played by m\(^6\)A in plant miRNA biogenesis.

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ALKB9B represent the characterized Arabidopsis m^6A erasers (24, 25).

Null mutants of MTA or any other members of the core writer complex (i.e., MTB, FIP37, and VIR) are embryo-lethal (18–20), indicating an essential function for this modification. However, hypomorphic knockdown mutants of these writers have been obtained and typically show an 80 to 90% reduction in m^6A levels (19, 20, 26). In contrast, reader and eraser knockouts are viable (23–25). Studies utilizing these various mutant plant lines indicate a role of this modification in embryogenesis, proper plant development (trichome morphology, meristem maintenance, vascular development), flowering time and flower morphology, and pathogen response (18–20, 24–26).

Many aspects of plant development and metabolism are controlled by microRNAs (miRNAs), and the complex phenotypes of low methylation plants have aspects reminiscent of miRNA biogenesis pathway mutants (see ref. 27 for review) and might be, at least partially, explained by the influence of m^6A on miRNA biogenesis. miRNAs are small endogenous noncoding RNAs that are ~21 nt in length and are important players in regulating cellular metabolism. miRNAs are derived from hairpins present in primary miRNAs (pri-miRNAs) that are synthesized by RNA Polymerase II (RNA Pol II), and further processed by RNAase III-type enzymes associated with and assisted by other proteins. In animals, m^6A is important for biogenesis of mature miRNAs, m^6A plays the role of a mark, identified by a reader protein (heterogeneous nuclear ribonucleoprotein A2/B1; HNRNPA2B1) that facilitates the recruitment of downstream enzymes and associated proteins to pri-miRNAs, thus facilitating their processing to miRNAs. Accordingly, it was shown that depletion of METTL3 leads to decreased accumulation of miRNAs and to an accumulation of pri-miRNAs due to their impaired processing (27, 28). While most of the findings related to m^6A in plants concern miRNAs, its role in miRNA biogenesis in plants remains unknown.

In this study, we provide evidence that biogenesis of at least 25% of Arabidopsis miRNAs is affected by the absence of the m^6A mark. We show that plant pri-miRNAs are m^6A-methylated by MTA, and deficiency of MTA (and thus m^6A) leads to accumulation of pri-miRNAs accompanied by lower miRNA levels. We also show that MTA interacts with RNA Pol II and TOUGH (TGH), suggesting that MTA acts at early stages of miRNA biogenesis. Lack of m^6A leads to the stem-loop region of pri-miRNAs becoming less structured, which negatively impacts the binding of Hiyomastase 1 (HYL1) to these precursors. Together, these results suggest that MTA affects Microprocessor assembly via its influence on secondary structure of methylated miRNA precursors, as well as direct interactions between MTA and a miRNA biogenesis protein TGH. Thus, the lack of m^6A methylation in pri-miRNAs in the mta mutant plants results in the inefficient recruitment of Microprocessor components to plant pri-miRNAs, resulting in their less-efficient processing, and ultimately leading to the decrease in miRNA production. We also suggest that the impaired auxin response in plants with MTA deficiency is caused, at least partly, by decreased miR393b levels in Arabidopsis plants of both WT Col-0 and a mutant line with MTA cDNA under the ABI3 promoter in a homozygous MTA T-DNA insertion mutant background (mta ABI3:MTA). The ABI3 promoter drives a very low level of mTA expression postgermination, giving rise to plants with 80 to 90% less m^6A modification compared to their WT counterparts (26).

Our sequencing data showed a decrease in the overall abundance of mature miRNAs in mta mutant as compared to WT plants (SI Appendix, Fig. S1). Specifically, we identified 60 differentially expressed miRNAs that had high confidence scores (probability ≥ 0.9 or false-discovery rate ≤ 0.1), and 51 of these 60 miRNAs were found to be down-regulated, while only 9 miRNAs were up-regulated (Fig. I and SI Appendix, Table S1). This down-regulation of miRNAs in mta plants was confirmed by quantitative real-time PCR (RT-qPCR) for six arbitrarily selected miRNAs (miR159b, miR169a, miR319b, miR393b, miR399a, and miR850) (Fig. 1B).

Next, we went on to investigate the levels of primary miRNAs (pri-miRNAs) in the low methylation plants (mta mutants) by using the mirEX^2 platform (www.combio.pl/mirex2) developed by our group (30, 31). This platform utilizes a repository of 298 primer pairs, specifically for Arabidopsis pri-miRNA quantification by RT-qPCR. Of the 298 pri-miRNAs tested, 68 pri-miRNAs were at undetectable levels in our samples, leaving 230 for further analysis. The results of RT-qPCR revealed that 85 pri-miRNAs had statistically significant (P ≤ 0.05, n = 3) changes in their accumulation (WT vs. mta): 56 of these (~66%) were found to be up-regulated, while 29 were down-regulated (Fig. 1C and SI Appendix, Table S1). Upon comparison of the RT-qPCR results with sRNA sequencing data, we identified 20 cognate pri-miRNA/miRNA pairs that showed higher accumulation of pri-miRNAs and lower levels of mature miRNAs (Fig. 1D). These results point toward an impaired processing of these pri-miRNAs in the absence of MTA. The general trend, showing an accumulation of pri-miRNAs and decreased levels of miRNAs in the conditions of reduced m^6A methylation, raised the possibility that the presence of m^6A is necessary for proper processing of some pri-miRNAs. Therefore, we tested the methylation status of the pri-miRNAs using m^6A-RNA immunoprecipitation followed by sequencing (m^6A-IP Seq). This method is not an equivalent of the commonly used MeRIPSeq (32), as we avoided the fragmentation of polyA RNA. We validated this protocol using spiked-in methylated and nonmethylated controls (SI Appendix, Fig. S2). We further tested the robustness of our data by comparing them to previously published data by Shen et al. (19) and Anderson et al. (33). In our dataset, we were able to identify 14,870 genes whose transcripts (15,562) are depleted in the mta mutant, indicating that these gene transcripts are methylated. Upon comparison of our data to the published datasets, we found an overlap of 2,868 (80%) and 3,100 genes (65%) between our data and data from Shen et al. (19) and Anderson et al. (33), respectively; 930 genes were common among all three datasets. A Venn diagram showing these overlaps is presented in SI Appendix, Fig. S3.

miRNAs can be encoded within intergenic regions as independent transcriptional units or can be embedded within protein-coding genes sharing transcriptional units with their host protein genes (34). For our analysis from the m^6A-IP Seq data we selected transcripts of only those miRNA genes that are independent transcriptional units. Using this approach, we identified transcripts of 11 MIR genes that were enriched more than 1.5-fold in WT vs. mta, indicating that they are m^6A-methylated in WT plants (hence can be immunoprecipitated with m^6A antibody), and lack this methylation in the absence of MTA (Fig. 2A and SI Appendix, Fig. S4). All of the identified pri-miRNAs that passed the enrichment threshold were either absent or had significantly reduced abundance in the mta IP sample relative to WT IP. As an orthogonal approach to verify that these pri-miRNAs were bona fide targets for MTA directed methylation, we carried out RNA IP (RIP) using an anti-GFP antibody and the GFP-tagged MTA (35S:MTA-GFP) plant line. As a control, the plants expressing only GFP in the WT Col-0 background were used.
RIP was performed on the nuclear fraction and followed by RT-qPCR on oligo(dT) primed cDNA. Eighteen pri-miRNAs (10 from our m6A-IP Seq data and 8 randomly chosen) were selected for verification by RT-qPCR, and of these all but 4 showed a statistically significant enrichment \( (P < 0.05) \) in the MTA–GFP sample when compared to the GFP control (Fig. 2B and SI Appendix, Fig. S5). This indicates that pri-miRNAs are frequently bound by MTA. Taken together, these data suggest that MTA binds and methylates at least a set of pri-miRNAs, and that this binding promotes their processing to mature miRNAs.

**Lack of m6A Affects Stem–Loop Structure of pri-miRNAs and Their Ability to Bind HYL1.** The effect of m6A on secondary structure of RNA has been well documented. Such alterations in RNA structure induced by m6A are known as “m6A switches” (35–37). These m6A switches influence the ability of RNA-binding proteins to recognize the RNA molecules, which further determines the substrate RNA fate. We used protein interaction profile sequencing (PIP-seq) in order to determine whether such structural changes could be observed in miRNA precursors and hence influence miRNA biogenesis. Using this approach, double-stranded RNAs and single-stranded RNAs can be detected after single- and double-stranded specific RNase treatment, respectively (38–40), and thus we were able to identify and distinguish between the structured and unstructured regions of the miRNA precursors. We noticed that the nucleotide accessibility for double- or single-stranded RNase(s) is altered in mta mutants. Our data show a significant reduction in the level of pri-miRNA stem–loop structure in the mta mutant as compared to WT plants (Fig. 3A, tw o box plots on the left). We observed even stronger differences when we focused only on those pri-miRNAs that we identified as m6A-methylated (Fig. 3A, two box plots on the right). By constraining a folding algorithm with PIP-seq–derived structure scores, we obtained 2D structural models for a few examples among the m6A-methylated pri-miRNAs (models for pri-miR160a, pri-miR163a, pri-miR166a, and pri-miR830 are presented in SI Appendix, Fig. S6). Presented models represent the most frequently interrogated structural conformation among the potentially multiple existing folding patterns for each RNA. These data-based structural models suggest that the pri-miRNA regions containing the miRNA/miRNA* stem regions tend to be in an unpaired conformation in the absence of the m6A mark (mta mutants) as compared to the methylated pri-miRNAs (WT plants) (SI Appendix, Fig. S6).

In light of these data, we reasoned that these changes in structure could in principle influence the binding of HYL1 to these precursors. HYL1 is a double-stranded RNA binding protein. **Fig. 1.** miRNA biogenesis is impaired in mta mutant plants. (A) sRNA sequencing analysis of miRNAs in mta and WT plants. Each black dot represents one miRNA. The red horizontal bar represents the threshold (probability 0.9). (B) Relative abundance (as determined by TaqMan RT-qPCR) of miRNAs identified with altered abundance in WT vs. the mta mutant monitored by sRNA sequencing. *P < 0.05, **P < 0.005, and error bars represent SD (n = 3). (C) Levels of 230 pri-miRNAs as determined by RT-qPCR with separation of statistically significant pri-miRNAs (above the red horizontal bar). Each dot represents one pri-miRNA and the red horizontal bar represents P-value threshold (P value 0.05). (D) A set of cognate pairs of pri-miRNA/miRNAs (selected from A and B) where pri-miRNA levels are up-regulated, while the levels of their cognate miRNA are down-regulated.
protein and is an important player in miRNA biogenesis (41, 42). Hence, we studied the binding of HYL1 to pri-miRNAs in WT and mta mutant plants using RIP (HYL1-RIP) followed by RT-qPCR. We found that 7 of the 10 pri-miRNAs tested were significantly less abundant in HYL1-IP samples from the mta mutants as compared to WT (Fig. 3B). A decrease in the binding efficiency of HYL1 that is a double-stranded RNA binding protein, to pri-miRNAs in the mta mutant is an additional indication that a double-stranded conformation of miRNA/miRNA* containing stem-loop regions of pri-miRNAs are formed less frequently in low methylation (mta) than in WT plants.

**MTA Interacts with RNA Pol II In Situ.** The strong tendency toward accumulation of pri-miRNAs and low abundance of miRNAs in mta mutants is reminiscent of various Arabidopsis mutants of genes encoding the proteins that participate in miRNA biogenesis, like Dicer-like 1 (DCL1), HYL1 (42–44), Serrate (SE) (45), and TGH (46). These proteins are involved in early stages of miRNA biogenesis and are needed for efficient processing of pri-miRNAs to miRNAs. These observations led us to suggest that MTA might act at early stages of miRNA biogenesis, probably cotranscriptionally. The deposition of m^6^A is usually assumed to be cotranscriptional; this assumption is supported by the fact that m^6^A can influence other processing events, such as selection of polyadenylation sites or pre-mRNA splicing, which are themselves cotranscriptional (47–49). However, a direct association between METTL3 and RNA Pol II has only been shown in mammalian cells when the rate of transcription was artificially slowed down (50). We confirmed colocalization of MTA and RNA Pol II in Arabidopsis using immunolocalization (SI Appendix, Fig. S7). Furthermore, we used the proximity ligation assay (PLA) to confirm in vivo interactions of MTA with RNA Pol II in plants under physiological conditions. Our results show direct interactions between MTA–GFP and RNA Pol II in cell nuclei with MTA–GFP expression but not in the GFP control (Fig. 4). These results were obtained for RNA Pol II phosphorylated at both Serine 5 and Serine 2. Thus, we show that MTA is associated with the RNA Pol II from an early stage of transcription and likely methylates pri-miRNAs (among other RNA Pol II transcripts) cotranscriptionally.

**MTA Acts at Early Stages of miRNA Biogenesis.** While MTA interacting with RNA Pol II is an indication of it methylating RNA Pol II transcripts cotranscriptionally in general, we investigated whether specific proteins involved in miRNA biogenesis could interact with MTA. To address this possibility, we performed a screen on possible MTA interactors involved in early steps of miRNA biogenesis, like HYL1, Cap binding protein 20 (CBP20), SE, TGH, and Dawdle (DDL1), using a microscopic approach. We performed FRET analyzed by fluorescence lifetime imaging microscopy (FLIM). As FRET can occur only when two proteins are within nanometers of each other, FRET–FLIM helps to quantify direct protein–protein interactions. This FRET–FLIM analysis confirmed the close association and interactions of MTA and TGH (Fig. 5). No such interactions could be seen in the case of any other tested proteins (SI Appendix, Fig. S8). Thus, we identified the miRNA biogenesis factor TGH as an MTA partner.

**Lack of m^6^A/MTA Impairs Microprocessor Complex Assembly.** Having identified TGH as an interactor of MTA, we focused on the m^6^A methylation status of pri-miRNAs in the tgh mutant. m^6^A–IP followed by RT–qPCR revealed that except for two pri-miRNAs, there is no overall significant reduction of m^6^A methylation in pri-miRNAs in the tgh mutant (SI Appendix, Fig. S9). This result indicates that TGH is not required for m^6^A methylation, and MTA acts upstream of TGH in miRNA biogenesis.

It is known that during miRNA biogenesis, TGH facilitates Microprocessor assembly either by attracting the additional m^6^A reader protein (unknown so far). MTA Regulates the Level of mir393b which Is Involved in Auxin Response. Auxin response defects have been reported for hypomorphic mutants of the known plant m^6^A methyltransferase complex (20). We found this to be the case for the auxin-responsive DR5pro:GUS reporter construct when introduced into both WT and the mta mutant background. Upon induction of 14-d-old seedlings with 2,4-dichlorophenoxyacetic acid (2,4-D), the mta plants showed much less GUS expression (Fig. 7A).
miR393b has been described to be involved in auxin response regulation, and in mutants lacking miR393b, auxin signaling is reduced (51). In our sRNA sequencing data, we found that miR393b is down-regulated in mta mutants, although we could not detect its precursor in our m6A-IP Seq. However, because of its recognized importance in regulating auxin responses, we further investigated the requirement of m6A for miR393b accumulation. We confirmed that pri-miR393b is m6A-methylated using m6A-IP followed by RT-qPCR. IP of RNA bound to MTA also confirmed MTA binding to pri-miR393b (Fig. 7B). Interestingly, our PIP-seq analysis revealed that pri-miR393b is less structured in mta than in WT plants. Moreover, the 2D model of this precursor revealed that its stem–loop region that contains mature miR393b sequence is formed more efficiently when pri-miR393b contains m6A (WT) in comparison to unmethylated precursors (mta mutant), similar to the other miRNAs showing underaccumulation in mta mutant plants in comparison to WT (SI Appendix, Fig. S10).

To further demonstrate that the low expression level of miR393b is in fact caused by the lack of MTA, we designed a transient expression assay in Nicotiana benthamiana. We used constructs designed to express pri-miR393b, MTA, or a catalytically inactive version of MTA, ΔMTA (D482A). ΔMTA was prepared by primer-induced point mutation resulting in the following change: Aspartic acid at position 482 to alanine (D482A) at the catalytic DPPW motif (52), making the protein catalytically inactive. These were introduced individually or in combination into Nicotiana leaves by agroinfiltration. We then assessed mature miR393b levels by Northern blot and found that the pri-miRNA393b transgene produces ∼2.3 times more miR393b when it is coexpressed with MTA, while this effect was abolished to a large extent when MTA was replaced by its catalytically inactive version (ΔMTA) (Fig. 7C). Thus, we show that the reduced auxin response in mta mutant plants is partially caused by the regulatory defects of miR393b biogenesis due to the lack of MTA activity.

**Discussion**

Here, we provide evidence that, in addition to influencing mRNA metabolism, m6A methylation is present in pri-miRNAs and affects miRNA biogenesis in A. thaliana. We show that the lower level of MTA (and hence m6A) leads to a reduction of miRNA levels in the case of at least 25% of miRNAs, whereas pri-miRNAs tend to overaccumulate in the mutant plants. This anticorrelation of pri-miRNAs with miRNA levels is reminiscent of observations from the Arabidopsis lines carrying mutations in genes encoding proteins involved in early stages of miRNA biogenesis: For example, hyl1 (42–44) and se (45). In contrast, mutants of HEN1 [a protein that is involved in methylation of miRNA/miRNA* duplexes at the 3′ ends (53), thus acting at the later step of miRNA biogenesis] do not show accumulation of pri-miRNAs, while down-regulation of miRNAs is observed in hen1 mutant plants (54). This suggests that MTA regulates miRNA production at early stages of their biogenesis.

Since MTA is a known mRNA methyltransferase and m6A is abundant in mRNA, we considered the possibility that the
observed effects were indirect and can be a result of altered metabolism of other miRNA biogenesis proteins. We used two different and independent approaches to exclude this possibility. MTA–GFP-mediated RNA IP and m6A-IP experiments revealed that for at least a group of miRNAs, this effect is direct and is a result of MTA binding to pri-miRNAs and introducing m6A marks. Additionally, structural changes in the pri-miRNAs characterized by the loss of secondary structure and increase in single-stranded regions in the mta mutant background, specifically in the stem–loop regions of these miRNA precursors, also indicate the importance of m6A in miRNA biogenesis in plants. These structural analyses go in line with less efficient binding of HYL1, a double-stranded RNA binding protein, to unmethylated pri-miRNAs in the mta mutant.

Our studies also revealed that MTA can directly interact with TGH, a protein involved in early stages of miRNA biogenesis. As many other miRNA biogenesis mutants, tgh shows a decrease in accumulation of miRNAs and overaccumulation of many pri-miRNAs. Moreover, TGH has been described as a factor important for the recruitment of HYL1 to pri-miRNAs (46). Thus, MTA can influence miRNA biogenesis in two different ways: 1) by m6A methylation of miRNA precursors, and/or 2) by direct interactions with TGH. We show that the m6A marks introduced by MTA stabilize the secondary structure of the stem loop regions of pri-miRNAs, likely stimulating their recognition by HYL1, and afterward by the endonuclease DCL1 as well as the rest of the components of the Microprocessor complex. However, MTA may also stimulate Microprocessor assembly by its direct interactions with TGH, which in turn interacts with HYL1 and stimulates/stabilizes HYL1 binding to double-stranded regions of pri-miRNAs. Upon comparison of our results to the previously published data regarding miRNA levels in the tgh mutant, we found a 45% overlap within down-regulated miRNAs in both tgh and mta mutants, despite these two datasets originating from two different tissues (46) (SI Appendix, Fig. S11). These 23 miRNAs that are down-regulated in both mta and tgh mutants could be regulated by the direct MTA–TGH interactions.

However, the mechanism of this MTA–TGH interaction-related alteration of miRNA biogenesis requires further studies.

Here, we also provide evidence that MTA interacts with RNA Pol II phosphorylated at both Serine 5 and Serine 2. This shows...
that MTA is associated with the RNA Pol II from the initiation of transcription and is present during the elongation step, modifying growing transcripts (see ref. 55 for review). In both mta and tgh mutants, colocalization of HYL1 and DCL1 with the elongation form of RNA Pol II (phosphorylated at Serine 2) is impaired, suggesting that both MTA/m6A as well as TGH contribute to cotranscriptional assembly of the Microprocessor.

In addition to the miRNAs that show decreased levels in the absence of m6A methylation, we also noticed nine miRNA species whose levels were up-regulated in mta hypomorphic plants. Of these 29 pri-miRNAs, only 1 (pri-miR472) has been shown to be recognized and bound by MTA, and is m6A-modified. The additional 28 pri-miRNAs are most likely not m6A-methylated, so their lower accumulation cannot be directly explained by the presence of m6A marks, but rather by indirect effects. We show that HYL1 binds to pri-miR472 independently whether it is methylated (WT) or not (mta plants), and the level of mature miR472 in the mta mutant is similar to that observed in WT plants. This may suggest that the lack of MTA does not influence Microprocessor assembly on miR472 precursors. However, loss of MTA, hence m6A methylation of pri-miR472, affects the level of pri-miR472, which is decreased in low m6A methylation plants. It is worth noting that

Fig. 6. Microprocessor assembly is impaired in both mta and tgh mutants. (A) Localization of RNA Pol II (green), DCL1 (red), and DNA (blue) in the nuclei of WT plants as well as mta and tgh mutants. (B) Colocalization scores of DCL1 and RNA Pol II are significantly lower in mta and tgh mutants as calculated using three different approaches (Pearson, Spearman, and LiQ). (C) Localization of RNA Pol II (green), HYL1 (red), and DNA (blue) in the nuclei of WT plants as well as mta and tgh mutants. The merge column shows all three channels. (D) Co-localization scores of HYL1 with RNA Pol II are significantly lower in mta and tgh mutants as calculated by three different approaches (Pearson, Spearman, and LiQ). ***P < 0.001, n = 50.
the Arabidopsis MIR gene encoding miR472 is very long (∼5 kbp) and contains four exons (31). Its transcription ends at several alternative polyadenylation sites and the transcripts undergo alternative splicing. Thus, it is also possible that m6A methylation of pri-miR472 changes maturation of MIR472 primary transcripts toward variants that are efficiently processed into mature miR472. This would explain the lower level of pri-miR472 and the constant level of miR472 in mta plants in comparison to WT. However, additional studies are needed to verify this interesting scenario.

As already mentioned above, while analyzing effects of m6A methylation on miRNA biogenesis, one should be aware that miRNA biogenesis is affected by a variety of processes like splicing, alternative polyadenylation, degradation, and so forth (56–60). The changes in structure caused by m6A can influence all of these processes by altering binding of proteins to MIR primary transcripts. The possible role of an m6A reader protein in the processing of pri-miRNAs should also not be overlooked.

Interestingly, the reduced auxin responsiveness of m6A writer mutant plants can be partially explained by the altered miR393b level. We show that pri-miR393b is m6A-methylated by MTA, and the level of miR393b is lower in the mta mutant in comparison to WT plants. miR393b is involved in homeostasis of AUX/IAA genes the expression of which is regulated by a complex feedback loop that involves regulation of these genes by the proteins they encode (51, 61, 62). miR393b has been shown to accumulate in leaves and is induced in response to auxin (52). We have found that expression of the auxin responsive DR5pro:GUS reporter construct in the mta mutant background is reduced upon treatment with auxin (2,4-D). Strong inhibition of auxin response after induction is observed in the mta mutant. (Scale bars, 2 mm.) (B) The miR393b level is down-regulated in the mta mutant (sRNA sequencing data) as compared to WT plants (Left, n = 3), pri-miR393b carries m6A mark (m6A-IP followed by RT-qPCR, Center, n = 3), and pri-miR393b is bound by MTA (MTA-GFP RIP, Right, n = 3). Graphs represent fold-change values between WT and mta mutant plants (Left and Center) and GFP and MTA-GFP plants (Right). (C) N. benthamiana leaves were agroinfiltrated with one of following constructs: MTA, catalytically inactive MTA (ΔMTA), and pri-miR393b (lanes 2, 3, and 4, respectively) or with a combination of two constructs (encoding pri-miR393b + the construct encoding MTA (lane 5) or pri-miR393b + ΔMTA (lane 6)). The levels of miR393b were monitored for each experimental variant using Northern blotting. Mock represents transfection with no plasmid control, and U6 serves as a loading control. Numbers above the last three lanes represent relative amounts of miR393b in the presence of MTA (2.3, penultimate lane) or ΔMTA (1.4, last lane) as compared to the expression of pri-miR393b alone (1, lane 4).
reporter is reduced in miR393b mutant plants (51) and a similar reduction is seen with the same reporter in the mta background. Thus, the altered miR393b biogenesis in plants with the low level of m^6^A methylation of pri-miRNAs likely contributes to the reduced auxin response in the m^6^A writer mutant, linking this modification with miRNA biogenesis and, as a physiological effect, with one of the most crucial hormone response pathways.

Based on our results, we propose a model showing the involvement of MTA/m^6^A in miRNA biogenesis in plants. m^6^A methylation of pri-miRNAs influences miRNA precursor secondary structure that in consequence stimulates the recognition of pri-miRNAs by HYL1. The direct interactions between MTA and the TGH protein and recognition of m^6^A marks by an unknown m^6^A reader that may also interact with TGH may also be involved in the HYL1 recruitment to the methylated miRNA precursor. This is strongly supported by the previously described involvement of TGH in the HYL1 recruitment to pri-miRNAs (46). Thus, the recognition of pri-miRNAs by HYL1, and in consequence assembly of the whole active Microprocessor, is controlled by pri-mRNA m^6^A methylation, which induces the proper secondary structure of the precursor as well as a specific MTA–TGH interaction that both support efficient assembly of the plant miRNA biogenesis machinery (Fig. 8).

Materials and Methods

**Plant Material.** *Arabidopsis thaliana* WT Col-0, mta mutants (26), MTA-GFP (p3SS:MTA-GFP), and GFP plants were sown on Jiffy pots and stratified for 2 d in dark at 4 °C. Thereafter, the plants were grown in plant growth chambers at 22 °C with 16-h light and 8-h dark cycles (70% humidity, 150- to 200-μmol m^−2^ s^−1^ photon flux density). Rosette leaves from 4-wk-old plants were harvested, immediately flash-frozen in liquid nitrogen, and used or stored at −80 °C until further use.

**sRNA Sequencing and Analysis.** sRNA fraction from 4-wk-old *A. thaliana* WT Col-0 and mta mutant plants was used to prepare libraries that were sequenced at Fasteris on the HiSeq 4000 platform. A detailed description of the library preparation and data analysis can be found in *Supplementary Materials and Methods*.

**m^6^A-RNA IP of pri-miRNAs and Sequencing.** PolyA enriched RNA from 4-wk-old *A. thaliana* WT Col-0 and mta mutant plants was used to perform m6A IP. Libraries were prepared from RNA before IP (input) and after IP. A detailed description of the library preparation and data analysis can be found in *Supplementary Materials and Methods*.

**Data Submission and Availability.** The data obtained in this study (sRNA and m^6^A-IP Seq) has been deposited under the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) accession no. GSE122528. The Pip-seq data used for the secondary structure analysis of miRNA transcripts can be found under NCBI GEO accession no. GSE108852.

**RIP.** Transgenic *Arabidopsis* line p3SS:MTA-GFP and mta mutants were grown along with the GFP and WT Arabidopsis lines. RIP was performed as described by Raczyńska et al. (63). A detailed protocol for RIP and data analysis can be found in *Supplementary Materials and Methods*.

**Pip-seq Analysis to Access RNA Structure Scores of miRNA Processors.** A total of two biological replicates of Pip-seq, including single-stranded RNA-seq and double-stranded RNA-seq libraries, for leaves five to nine from 4-wk-old WT and mta mutant plants were constructed as previously described (37, 39). The raw data has been deposited under the GEO accession no. GSE108852. Refer to *Supplementary Materials and Methods* for the detailed protocol and data analysis pipeline.

**Immunolocalization.** The immunolabeling experiments using the Duolink PLA fluorescence protocol were performed on isolated nuclei of 4-wk-old *A. thaliana* leaves (WT, plants with MTA-GFP expression or GFP expression, control) lines. In situ PLA detection was carried out using the appropriate Duolink in situ Orange Kit Goat/Rabbit (Sigma-Aldrich) according to the protocol of the manufacturer. A detailed protocol for PLA can be found in *Supplementary Materials and Methods*.

**FRET-FLIM.** FRET-FLIM was performed using protoplasts were isolated from 3- to 4-wk-old WT Arabidopsis leaves using the method described in Knop et al. (65). For the detailed protocol, please refer to *Supplementary Materials and Methods*.

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**Fig. 8.** A schematic representation of the role of m^6^A/MTA in miRNA biogenesis in plants. (A) In WT plants (Left), MTA methylates at least a set of pri-miRNAs which in turn leads to folding the proper secondary structure of a miRNA precursor. MTA and/or a putative reader of the m^6^A marks introduced by this enzyme (a gray square marked with ‘+’1) facilitate the binding of TGH to the pre-miRNA. The properly folded miRNA precursor is recognized by HYL1. This eventually leads to efficient Microprocessor assembly and miRNA biogenesis at the appropriate level. (B) In the mta mutant, the lack of MTA, and thus m^6^A, leads to the loss of MTA–TGH interactions and inefficient formation of a stem–loop structure in the miRNA precursor, leading to reduced binding of HYL1 that in consequence disrupts Microprocessor assembly, and finally the down-regulation of the mature miRNA level is observed. Whether TGH can bind to the precursor in the mta mutant is unknown (marked in the scheme by a red question mark).

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Northern Blotting. Northern blotting was performed as described in Kruzka et al. (66). Briefly: 30 µg of RNA (per sample) isolated from transfected tobacco leaves was loaded on 8 M denaturing urea polyacrylamide gel (15%) in TBE buffer (0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA, pH 8.0). RNA was then transferred onto the Amersham Hybon-N nitrocellulose membrane (GE Healthcare) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) and fixed using CL-1000 UV Crosslinker (UVP). Prehybridization and hybridization were performed in hybridization buffer (3.5% SDS, 0.375 M sodium phosphate dibasic, 0.125 M sodium phosphate monobasic) at 42 °C with DNA oligo probes (Sigma) labeled at their 5’ ends with γ-32P ATP (Hartmann Analytic). U6 was used as a loading control. After washing, the blots were exposed for up to 3 d to a phosphorimaging screen (FujiFilm) and the results were visualized with the FujiFilm FLA5100 reader (FujiFilm) and quantified using Multi Gauge V2.2 (FujiFilm).

Data Availability. The data reported in this paper have been deposited in the NCBI GEO database, https://www.ncbi.nlm.nih.gov/geo (accession nos. GSE122528 and GSE108852).

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