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Adenosine methylation in Arabidopsis mRNA is associated with the 3′ end and reduced levels cause developmental defects

Zsuzsanna Bodi, Silin Zhong†, Surbhi Mehra, Jie Song†, Neil Graham, Hongying Li, Sean May and Rupert George Fray *

School of Biosciences, University of Nottingham, Loughborough, UK

ADULT TEXT

INTRODUCTION

N6-methyladenosine (m6A) is a ubiquitous base modification found inherently in the mRNA of most Eukaryotes. Levels of methylation equivalent to at least 50% of transcripts carrying m6A are common (Zhong et al., 2008; Bodi et al., 2010), although its relative abundance may vary between species, or between tissue types within a species (Desrosiers et al., 1974; Adams and Cory, 1975; Perry et al., 1975; Wei et al., 1976; Beemon and Keith, 1977; Levis and Penman, 1978; Aloni et al., 1979; Kennedy and Lane, 1979; Nichols, 1979; Haugland and Cline, 1980). Ribonuclease fragmentation and labeling studies on mRNA show m6A to be present only at the central A within the sequence context GAC and AAC, with a 75% preference for GAC, and this consensus appears to be conserved amongst plants, yeast and mammals (Wei et al., 1976; Nichols and Welder, 1981; Harper et al., 1990; Shimba et al., 1995; Zhong et al., 2008; Bodi et al., 2010). Whilst other types of post-transcriptional base modification, such as C-to-U editing, or A-to-I conversions result in a change following reverse transcription, m6A is recognized as adenosine and so is not revealed by cDNA sequencing. For this reason it is technically demanding to map its position in specific messages, and this has only been achieved in two, relatively abundant, transcripts.

We previously showed that the N6-methyladenosine (m6A) mRNA methylase is essential during Arabidopsis thaliana embryonic development. We also demonstrated that this modification is present at varying levels in all mature tissues. However, the requirement for the m6A in the mature plant was not tested. Here we show that a 90% reduction in m6A levels during later growth stages gives rise to plants with altered growth patterns and reduced apical dominance. The flowers of these plants commonly show defects in their floral organ number, size, and identity. The global analysis of gene expression from reduced m6A plants show that a significant number of down-regulated genes are involved in transport, or targeted transport, and most of the up-regulated genes are involved in stress and stimulus response processes. An analysis of m6A distribution in fragmented mRNA suggests that the m6A is predominantly positioned toward the 3′ end of transcripts in a region 100–150 bp before the poly(A) tail. In addition to the analysis of the phenotypic changes in the low methylation Arabidopsis plants we will review the latest advances in the field of mRNA internal methylation.

Keywords: post-transcriptional, mRNA methylation, MT-A70, IME4, METTL3

In Rous sarcoma virus a 1865 nt region of the genomic RNA contains seven m6A sites in a GAC context (Kane and Beemon, 1985; Csepany et al., 1990), and in bovine prolactin mRNA, methylation occurs at a single AGACU site within the 3′ untranslated region (UTR; Horowitz et al., 1984). In both cases, this conformed to an extended consensus sequence RRACH (where R represents purine, A is the methylation site, and H is any base other than G), previously proposed by Schibler et al. (1977) Consistent with this, the partially purified mammalian mRNA methylase shows strongest activity for GGACU and, to a lesser extent, AGACU sequences (Harper et al., 1990). Whilst m6A is clearly abundant within the mRNA pool, very few methylated transcripts have been identified to date. However, using an anti-m6A immunoprecipitation approach, four Saccharomyces cerevisiae messages, expressed during early meiosis were recently shown to be methylated (Bodi et al., 2010). The position of m6A in the meiotic kinase IME2, was mapped to the 3′ half of the message, a region where the translational suppressor Khd1 has previously been predicted to bind (Hasegawa et al., 2009; Bodi et al., 2010). Bokar et al. (1997) identified MT-A70 (assigned as METTL3 by HUGO Gene Nomenclature Committee) as the human mRNA m6A methyltransferase. A phylogenetic analysis of proteins...
homologous to METTL3 has identified conserved orthologs among most sequenced eukaryote genomes, including mammals, fish, amphibians, insects, plants, and some fungi (S. cerevisiae; Bujnicki et al., 2002). In S. cerevisiae, the METTL3 homolog (IME4) is only expressed in starved, diploid cells. Under these conditions, sporulation would normally occur, but disruption of IME4 abolishes mRNA methylation and prevents the initiation of this developmental pathway (Clancy et al., 2002; Bodi et al., 2010). Arabidopsis thaliana homozygous for an insertional knockout of the METTL3 homolog (MTA) arrest at the globular stage of embryonic development and the poly(A) RNA purified from these embryos lacks m6A (Zhong et al., 2008). More recently, a deletion of the Drosophila METTL3 homolog (Dm ime4) was shown to inhibit oogenesis, though the methylation status of the wild type or knockout flies was not reported (Hongay and Orr-Weaver, 2011). The consequence of knockout or silencing of the mammalian METTL3 is currently not known, and may well be lethal. However, in a recent paper (Jia et al., 2011) it is shown that the primary function of the human fat mass and obesity associated protein (FTO) is to remove the methyl groups from N6 methylated adenosines in mRNA. Overexpression of FTO (implying reduced methylation) is associated with obesity, diabetes, and risk of Alzheimer’s. Thus there is an intriguing link between nutrition and mRNA methylation in both yeasts and mammals. This link may also exist in plants, as AtFIP37 (a protein partner of the Arabidopsis MTA) is the only known target of Arabidopsis FKB12, a component of the target of rapamycin (TOR) pathway – a conserved pathway that regulates cellular responses to growth factors and nutrient availability in Eukaryotes.

Whilst an association between mRNA methylation and stress, particularly nutrient, signaling may exist, the function that m6A performs at a molecular level remains a mystery. Tuck et al. (1999) proposed that the presence of m6A in a message increases translatability. These authors observed an in vitro methylated dihydrofolate reductase message translated more efficiently than the non-methylated transcript in an in vitro translation assay. Roles in mRNA nuclear export or splicing have also been suggested. A role in splicing was also supported by the interaction between A. thaliana FIP37 (FKBP12 interacting protein) and MTA (Zhong et al., 2008), as AtFIP37 is a homolog of Drosophila female-lethal(2)idal, required for sex-specific splicing. However, in yeast none of the methylated messages identified so far are spliced (Bodi et al., 2010) and no involvement of AtFIP37 in splicing has so far been demonstrated. Other functions for mRNA methylation could also be envisaged, such as regulating siRNA or miRNA susceptibility, sub-cellular localization, nonsense mediated decay (NMD) or message turnover.

In this manuscript we demonstrate that Arabidopsis plants with decreased m6A levels show reduced apical dominance, abnormal organ definition, and increased number of trichome branches. In addition, a global transcriptome analysis revealed that groups of messages involved in localization and establishment of localization are significantly down regulated, whilst genes associated with response to external and internal stimuli are up-regulated in these plants. We also demonstrate that globally, the m6A is predominantly positioned toward the 3’ end of transcripts in a region 100–150 bp before the poly(A) tail.

MATERIALS AND METHODS

CONSTRUCTION OF MTA PARTIAL COMPLEMENTATION PLANTS

The MTA cDNA was cloned into the plant binary vector pGHAB3GWG (GenBank accession number FM177581) via an LR clonase (Invitrogen) reaction from a PENTR/D-TOPO (Invitrogen) vector containing the full MTA coding sequence (Zhong et al., 2008). After transfer to Agrobacterium tumefaciens C58, this vector was used for floral dip transformation of A. thaliana ecotype Columbia (Col) plants heterozygous for the SALK_074069 insertion. Transgenic seedlings were selected on hygromycin, and lines heterozygous for the SALK_074069 were identified by the production of a 1050-bp fragment following amplification with oligonucleotides Lba1 (TCGTTACGTTAGGCCATCG) and A63UTRR (GACATTGGCTTTGGGTATGGAA). After self-pollination, T2 plants homozygous for SALK_074069 were identified by the absence of the wild type 1080 bp fragment obtained following PCR amplification with oligonucleotides A63UTRR and A6E4F (GACTTACGACGTGGCATCG) and the presence of the 1050 bp fragment obtained with oligonucleotides Lba1 and A63UTRR (Figure 1). In each case the presence of the transgene was confirmed by amplification of a fragment of 799 bp after PCR amplification with oligonucleotides A6rev (CTAAAGCTGATTAGCATTAG) and A6E4F.

MAPPING m6A DISTRIBUTION IN THE mRNA POOL

Total RNA was extracted from Arabidopsis tissue samples using the method of Chomczynski and Sacchi (1987). Poly(A) RNA
was purified using oligo(dT)-Cellulose columns (Fluka) following a
standard protocol (Sambrook and Russel, 2001). The oligo(dT)
chromatography was carried out at least three times on each
sample and the quality of the mRNA was checked on an RNA
6000 LabChip, with Agilent Bioanalyzer (Ambion). The alkaline
fragmentation of the mRNA samples was carried out using a 100-
mM carbonate/bicarbonate buffer system. The buffered mRNA
samples had 60 mM Na$_2$CO$_3$ and 40 mM NaHCO$_3$ as final con-
centrations, and were incuated at 60°C. The size of fragments
was determined by the time of incubation, which was calculated
according to the following formula:

$$t = \frac{(L_o - L_t)}{k (L_o L_t)}$$

where $L_o =$ initial length of transcript (in kb)
$L_t =$ desired RNA fragment length (in kb)
$k =$ constant $= 0.11$ kb/min
$t =$ time (min)

The initial length of the mRNA was approximated as 1.2 kb
thus a desired fragment length of 150 nt was achieved by a 53-min
incubation time. The fragmentation was stopped by adding 3 M
sodium acetate, one tenth of the original volume, and three vol-
umes of absolute Ethanol. After overnight precipitation (−20°C)
the RNA pellet was obtained by centrifugation, washed with
80% ethanol, and resuspended in water. The size of fragments
was checked on an RNA 6000 LabChip, with Agilent Bioanalyzer
(Ambion). To prevent unwanted labeling of free 5′ ends, the frag-
ments were phosphorylated using 20 units of T4 polynucleotide
kinase (PNK; Fermentas) in the presence of 10 mM cold ATP and
1X PNK buffer in a final volume of 40 μL at 37°C for 1 h. The fragments
containing poly(A) tails were purified away from the
non-polyadenylated species by using oligo(dT) magnetic beads,
PolyTtrack System 1000 (Promega). These were released by elution
with 20 μL water. The m$^6$A content of this sample was measured as
previously described (Zhong et al., 2008). The unbound fraction,
representing the 5′ end and middle region of the mRNA pool,
was separated from the excess ATP using a micro-spin column
(BioRad) with a 6 nt cut-off. This step was also used for buffer
exchange (Tris–HCl). An aliquot of these fragments was used for
m$^6$A quantification. The rest of the sample was further processed
for the analysis of fragments from capped 5′ ends. This sample was
digested with 1 μL Terminator$^\text{TM}$ nuclease (Epicerent Biotechnolo-
gies) in a final volume of 70 μL with 1X reaction buffer at 30°C for
1 h. The reaction mixture was put through a micro-spin column
and extracted with phenol–chloroform to stop the reaction. After
ethanol precipitation, the RNA was resuspended in 7 μL of water
and the m$^6$A content was determined.

**MICROARRAY ANALYSIS**

Trays of wild type and homoygous SALK_074069 ABI3MTA
plants were grown in parallel for 3 weeks, at which point the 3rd
to 6th rosette leaves were taken from each of four plants and these
were pooled for RNA extraction. Three biological repeats of the
wt and reduced m$^6$A samples were prepared. The Affymetrix Ara-
bidopsis ATH1 GeneChip$^\text{TM}$ Genome Array was used for microarray
analysis. Hybridizations were carried out at the NASC’s Affymetrix
service (Nottingham Arabidopsis Stock Centre, University of Not-
ttingham, UK). Total RNA samples were labeled, hybridized, and
scanned following the standard protocol from the manufacturer
(GeneChip Expression Analysis, Affymetrix$^\text{TM}$). The GeneChip
Command Console Software (AGCC; Affymetrix$^\text{TM}$) was used to gen-
erate “.cel” files for each of the hybridizations. These are available
from the NASCArrays database (accession ID: NASCARRAYS-
612) and from GEO (accession ID: GSE34924$^3$). The raw chip
data were normalized using the Robust Multichip Average (RMA)
pre-normalization algorithm (Irizarry et al., 2003) in the Gene-
Spring GX (version 11; Agilent Technologies) analysis software
package. Following RMA pre-normalization, the signals were fur-
ther normalized by standardizing the signal value of each probe-set
to the median of that probe-set across all hybridizations. Differ-
tentially expressed probe-sets were identified using a two-step
process (i) fold-change $\geq 1.5$ between mutant and wild type and
(ii) a t-test ($p \leq 0.05$) using the Benjamini and Hochberg multiple
testing correction (Benjamini and Hochberg, 1995). All further
analysis was carried out using different functions in GeneSpring
GX. Gene Ontology analysis was performed using the GO analy-
sis function in GeneSpring GX, with the p-value calculated using a
hypergeometric test with Benjamini–Yekutieli correction (Ben-
jamini and Yekutieli, 2001). The significance of gene list overlaps
was calculated using a hypergeometric distribution test$^4$.

**RESULTS**

**COMPLEMENTATION OF MTA HOMOZYGOUS LINES WITH THE MTA
cDNA UNDER THE CONTROL OF THE ABI3 PROMOTER**

We previously reported that the SALK_074069 T-DNA insertion in the
AT4G10760 (MTA) gene in homozygous form results in an
arrest at the globular stage during embryonic development (Zhong
et al., 2008). This embryo lethality can be successfully rescued by
a constitutively expressed MTA cDNA. All mature tissues tested
were also shown to contain m$^6$A in their mRNA and we wished to
test if this was also performing an important or essential function.
The role of mRNA methylation in normal mRNA metabolism is
not known, and it remains possible that methylation plays a posi-
tive or negative role in post-transcriptional gene silencing; for this
reason we wished to avoid an RNAi based approach for reducing
MTA levels. Instead, we used the MTA cDNA under the control
of the embryo-specific ABI3 promoter to rescue the homozygous
embryo-lethal phenotype of the SALK_074069. This promoter
construct directs the expression of MTA during embryonic devel-
opment but limits the production of MTA beyond this stage. The
expression of ABI3 has previously been extensively characterized
and the promoter has been used to rescue other embryo-lethal
mutations in order to study vegetative gene function (Rhode et al.,
1999; Despres et al., 2001).

The full length MTA cDNA expressed under the control of the
ABI3 promoter was used to transform plants hemizygous for the
SALK_074069 insertion. Three transgenic lines that contained the
SALK insertion were selected and allowed to self-pollinate. Among

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1. [www.affymetrix.com](http://www.affymetrix.com)
2. [http://affymetrix.arabidopsis.info](http://affymetrix.arabidopsis.info)
3. [http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)
4. [http://stattrek.com/Tables/Hypergeometric.aspx](http://stattrek.com/Tables/Hypergeometric.aspx)
the progeny of each of these plants were individuals homozygous for the SALK_074069 insertion (Figures 1A,B), demonstrating that this construct was able to complement the embryo-lethal phenotype. Reduced levels of m^6A methylation were assayed using thin layer chromatography (TLC) for separation and quantification of radio labeled nucleotides (Zhong et al., 2008). As expected, the m^6A to A ratio in the seeds was similar to that of the wild type plants. However, in leaves, and in flowers there was a 90–93% reduction when the homozygous SALK_074069 insertion plants were compared to wild type (Figure 2A). This was consistent with the lower level of expression of the MTA cDNA on the Affymetrix ATH1 GeneChip (Tables S1 and S4 in Supplementary Material) and verified by qRT-PCR (Figure A1 in Appendix).

Less than one quarter of the T2 generation was homozygous for the SALK_074069 insertion, and all of these had various developmental defects. Most of the observed plants had reduced inflorescence internode lengths, and a small, but bushy rosette compared to wild type plants. A general increase in the number of rosette leaves was also observed (Figure 2B) and these were more crinkled. However, the severity of the phenotypes varied between and amongst the three lines. This is probably due to variations in the copy number of the complementing ABI3MTA construct, since one third of homozygous plants are expected to have two copies of the construct. The architecture of flowers is also affected (Figure 2C), particularly in the first flowers of the inflorescence, where stamens often showed partial conversion to petals. Reduced seed set was also observed, but viable seed were recovered.

It was previously reported that overexpression of AtFIP37, the interacting partner of MTA (Zhong et al., 2008) increases trichome branching in Arabidopsis (Vespa et al., 2004). Trichomes have been used as a model for cell shape and polarity control in plants, and the
degree of branching may also be an indication of endoreduplication levels, as appeared to be the case with AtFIP37 overexpression (Vespa et al., 2004). Interestingly, the reduced m6A plants showed a trichome phenotype similar to that of the AtFIP37 over-expressing lines. The fraction of trichomes with four or more branches is increased from 24 to 60% in the low methylation plants (Figure 3).

GLOBAL GENE EXPRESSION ANALYSIS IN THE LOW LEVEL METHYLATION PLANTS

Transcriptional changes in the reduced m6A plants compared to wild type were determined by hybridizing RNA from 3-week-old rosette leaves to the Affymetrix Arabidopsis ATH1 GeneChip. We identified 1537 differentially expressed genes (fold-change ≥1.5, corrected p ≤ 0.05). The differentially expressed gene set had 883 up-regulated and 654 down-regulated genes (Table S1 in Supplementary Material). Gene Ontology analysis was performed on the up and down-regulated genes separately to identify over represented terms. The most significantly enriched GO categories were “response to stimulus,” “response to stress,” “response to other organism,” and “response to biotic stimuli” 36, 28, 14, and 14% respectively of the up-regulated genes falling in these groups, this was followed by “response to external stimulus” 11%. This may suggest a function for mRNA methylation in the proper execution of responses to external, environmental and internal stimuli, or may be the consequence of a perceived stress due to reduced methylase activity. We also observed that there are a significant number of up-regulated genes involved in metabolism (58%), response to carbohydrate stimulus (4%), or carbohydrate binding (4%; Table S2 in Supplementary Material). The most significant GO terms enriched in the down-regulated set were associated with “establishment of localization,” “localization,” and “transport” (22% each). As well as “phospholipid biosynthetic process,” “phospholipid metabolic process,” “cellular lipid metabolic process” (4% each) “nitrate metabolic process,” and “nitrate assimilation” (2.5% each) were also represented (Table S3 in Supplementary Material). It is possible that loss of methylation in the vegetative state may interfere with localization of proteins, organelles, or mRNA. However, gene expression changes in the low methylation plants are likely to be a complex combination of superimposed secondary and primary outcomes of the lack of mRNA methylation in the mature plant.

We were particularly interested in genes which belong to GO categories such as RNA methyl transferase activity, RNA metabolic processes, vegetative to reproductive transmission, and meristem maintenance (Table S4 in Supplementary Material). Not surprisingly, the MTA gene was the most significantly down-regulated member of the RNA metabolic processes and methyl transferase activity groups.

The flowering time regulator FLOWERING LOCUS T (FT) gene was also found as a significant down-regulated gene in the vegetative to reproductive transmission group. We also found that ATFIP37, the only documented interacting partner of MTA (Zhong et al., 2008), is significantly up-regulated. This is consistent with our observation of increased trichome branch number.

We compared gene lists generated from the methylation deficient plants data and a published data set of trichome expressed genes (Jakoby et al., 2008). In total, 28 (p < 0.01) overlapping genes (Figure 4) were identified and are listed in Table S5 in Supplementary Material. The most frequently represented categories in the trichome gene list are genes involved in responses to external or internal stimuli.

![FIGURE 3 | Altered levels of trichome branching in 3-week-old seedlings.](image)

The third and fourth leaves from 3-weeks-old seedlings of wild type and homozygous SALK_074069 ABI3/MTA plants were examined for changes in their trichome phenotypes. Light micrographs of trichomes from plants with low m6A levels and from wild type plants (A). The low m6A plants are characterized by a higher proportion of trichomes with four or more branches. Trichomes from the abaxial side were analyzed for the third and fourth leaves from four wild type and four low m6A plants, each from different transgenic lines (554 and 521 trichomes respectively). Sixty-three percentage of trichomes from low methylation plants had four or more branches compared to just 24% in wild type (B). Error bars represent SD of the replicates.

![FIGURE 4 | Venn diagram showing overlapping expression changes of genes between low methylation plants and a trichome specific set.](image)

For the overlap search all genes with significant change were used from the low methylation versus wild type dataset (1537 genes). The central region corresponds to genes with changed expression in both trichomes and low methylation plants. The gene list is in Table S6 in Supplementary Material.
m^6A IS ENRICHED IN THE 3′ END OF THE TRANSCRIPT

The 5′ cap and the 3′ poly(A) tail of Eukaryotic mRNA have a well recognized function in translation and RNA processing. The adjacent 5′ and 3′ UTRs may also contain binding sites for regulatory proteins or (particularly in animals) microRNAs. Nucleotide modification in these regions might alter RNA–protein interactions and so could impact upon gene expression. In order to determine if m^6A is randomly distributed in the body of a transcript or shows a positional bias, we fragmented mRNA and separated the fragments into those derived from the 3′, 5′, or 5′ and middle of the transcripts. The amount of m^6A in the different fractions was then determined using the previously described TLC quantification method (Zhong et al., 2008).

Poly(A) RNA was prepared from Arabidopsis wild type seedlings and was subjected to alkali hydrolysis using a sodium carbonate/hydrocarbonate buffer system. This method allowed a controlled fragmentation of the mRNA, where the fragment size was determined by the length of incubation time. An average 180 or 120 nt fragment size was achieved under our conditions, which was confirmed by RNA6000 LabChip (Agilent; Figure A2 in Appendix).

Following the hydrolysis, the exposed 5′ ends were polished using PNK and an excess of cold ATP to prevent subsequent labeling of the free ends. The fragments containing a poly(A) tail were obtained by hybridization to an oligo(dT) column, and labeling of the free ends. The fragments containing a poly(A) end were further treated with terminator nuclease which eliminated all fragments with a 5′ monophosphate end and left only the 5′ caped fragments behind. This fraction was regarded as representing the 5′ end of the transcriptome. The enrichment of these three fractions was confirmed by hybridization to labeled probes from the 3′, 5′, and mid region of an ATP synthase cDNA (ATI G15700; Figure A2 in Appendix). All three fractions were then assayed for their m^6A content in a GA context using the established TLC method (Zhong et al., 2008). When the mRNA was fractionated to 120 or 180 nt, the majority of the m^6A was observed in the most 3′ fragments (m6A:A ratios of 3.5 and 4.7% respectively; Figures 5A,B). When mRNA was fragmented to 90 nt or less, this 3′ bias was no longer apparent, possibly due to the inefficient enrichment of the respective fractions. Thus, globally at least 85% of the m^6A appears to be in the 3′ end of the transcripts. Using a PATMATCH (TAIR5) search for the GGACU expanded methylation consensus established in mammals, we identified a list of 1159 genes with at least one GGACU in the last 200 nt of their 3′ UTR. This list was compared to the list of differentially expressed genes from low methylation plants for overlaps.

In total, 99 genes in the differentially expressed genes from low methylation plants for overlaps.

$\text{m}^6\text{A}$ IS ENRICHED IN THE 3′ END OF THE TRANSCRIPT

FIGURE 5 | m6A-methyl adenosine is enriched in the 3′ end of transcripts. Fragmented high purity mRNA was fractionated into 3′, middle + 5′ and 5′ regions and the m6A content was measured for each fraction using the published TLC based method. The positions of m6A, A, C and A are indicated in the left hand panel. The spot corresponding to m6A is enriched on the TLC carried out on the 3′ end, compared to the 5′ and 5′ + middle samples (A). (The example is from mRNA fragmented to 180 nt). The graph shows the distribution of m6A for three different fragmentation experiments (B). When the fragment size is 90 nt or less, m6A enrichment associated with polyadenylated fragments is no longer seen.

FIGURE 6 | Venn diagrams representing overlapping genes between the list of cDNAs with GGACU in the last 200 nt of the 3′ UTR and the differentially expressed genes in the low methylation plants.

Ninety-nine Genes from the low methylation plants had at least one GGACU in the last 200 nt end of their 3′ UTR. Genes with no annotated 3′ UTR were not analyzed.

rather than as a direct outcome of methylation deficiency in the transcripts themselves. Thus, an over-representation of 3′ UTR GGACU containing transcripts in the messages that change following reduced methylation, may be even more significant than our overlap search suggests.
DISCUSSION

The *Arabidopsis* MTA gene encodes a close homolog of the mammalian mRNA methyltransferase and is required for the synthesis of m6A in * planta*. Disruption of MTA by T-DNA insertion results in embryo arrest at the globular stage (Zhong et al., 2008), however wild type plants have mRNA methylation in all tissues, and in order to study the role of this methylation during normal growth and development alternative approaches have to be employed. We used the ABI3 promoter to drive embryonic-expression of an MTA cDNA in order to bypass the embryo-leafy effects of the homozygous mutation (Rhode et al., 1999; Despres et al., 2001). Using this method allowed us to recover mature plants homozygous for the disrupted MTA gene and avoided RNAi based approaches which might themselves have been affected by altered methylation levels. Strong gene expression under the control of ABI3 promoter is restricted to embryonic development, however, low level expression can occur during different quiescent states, depending on light conditions (Rhode et al., 1999). We achieved a 90–93% decrease in m6A levels in the mature plant leaf and floral tissues. The remaining small amount of m6A most probably results from the low level basal expression of the MTA cDNA from the ABI3 promoter. Indeed full knockout of MTA in mature tissues may well be lethal as is suggested by certain inducible RNAi constructs (not shown). It is also possible that some of this remaining low level of m6A may be a result of mRNA methylation being carried out by the related methylase AT4G09980. AT4G09980 is a member of the MT-A70 family sub-lineage B (Bujnicki et al., 2002) that is also found in most Eukaryotes. Homozygous knockout of this methylase in *Arabidopsis* also results in embryo lethality, however, a role in mRNA adenosine methylation for this sub family has yet to be demonstrated. It also remains a possibility that, in addition to its role in mRNA methylation, MTA may also have an as yet unidentified function that is required for normal gene expression in these tissues.

The phenotypic changes in the leaf and flower structures of the reduced methylation plants indicate a reduced apical dominance and suggest problems with organ definition. We also observed significant changes in trichome branching. Trichomes have been used as a model for cell shape and polarity control in plants, and the degree of branching may also be an indicator of changed endoreduplication (Hulskamp et al., 1994). Endoreduplication is the first developmental step in trichome development after cell division ceases (Hulskamp et al., 1994). It was previously reported that overexpression of *AtFIP37*, the interacting partner of MTA (Zhong et al., 2008) increases trichome branching in *Arabidopsis* (Vespa et al., 2004) as a result of increased endoreduplication levels. The overlap of messages that change in the low methylation plants and with trichome-associated genes does not reveal any of the genes which are involved in the trichome branch determination processes, or in trichome cell differentiation. Though this is perhaps not surprising, as these cells would have contributed a very small proportion of the leaf RNA analyzed and these messages are likely expressed at a low level. Nevertheless, *AtFIP37* is up-regulated in the low methylation plants, and this is consistent with the previous observation that overexpression of *AtFIP37* causes increased branching (Vespa et al., 2004). Those trichome-associated messages which are also increased in the low methylation plants are mostly associated with stress related processes. Indeed, there was a general enrichment among the overexpressed genes for GO terms related to stress responses and responses to stimuli, both external and internal. It is not possible to determine if the overexpression of stress and stimuli response genes is a direct or indirect effect of the loss of methylation, as the plant morphology is so severely affected. However, there are parallels with yeast, where methylation of mRNA is induced by nutritional stress and subsequently leads to new developmental pathways (meiosis and sporulation).

The GO term analysis of the genes down regulated in the low m6A plants indicates an enrichment of genes associated with “phospholipid,” and nitrate biosynthesis, as well as with transport and localization. Since gene expression changes in a methylation deficient plant are likely to be a complex overlay of direct and indirect results of the lack of m6A in mRNA it is not realistic to pick single genes or a gene list as potential methylation candidates from this dataset. If the observed levels of m6A found in wild type plants is distributed evenly between transcripts, then more than 50% of all messages should be methylated (Zhong et al., 2008). Thus, it is likely that the phenotypes seen in the low methylation plants are a result of global changes in mRNA processing, transport, or translatability rather than the result of changes to just a few key transcripts.

The location of m6A within a transcript could give an indication of the possible role of the modification. Methylation has been found within the body of transcripts from *Rous sarcoma* virus, but this may not be representative of the modifications occurring in the endogenous messages of the host cell. In an *in vitro* assay a methylation competent cell extract was found to methylate a synthetic mouse dihydrofolate reductase transcript at three unmapped sites toward the 5′ end (Rana and Tuck, 1990). However, as similar extracts have been shown to be capable of efficiently methylating any RNA oligonucleotide containing a GGACU or AGACU consensus (Harper et al., 1990), such experiments may not reflect the true distribution of m6A within messages *in vivo*. Precise (Horowitz et al., 1984) or partial (Bodi et al., 2010) mapping of methylation in endogenous cellular transcripts has only been reported for two transcripts (bovine prolactin and yeast IME2 respectively). In bovine prolactin the methylation was located in the 3′ UTR (Horowitz et al., 1984), and in the yeast IME2 the m6A is located within the 3′ half of the message (Bodi et al., 2010).

Using a controlled alkali fragmentation approach followed by 3′ and 5′ fragment enrichment, we show a general preferential localization of m6A toward the 3′ end, of messages, probably within the last 100–150 nt before the poly(A) tail. We found an overrepresentation amongst the differentially expressed genes of the low methylation plants for transcripts containing a GGACU site within 200 nt before the end of their 3′ UTR. This may indicate that in plants as in mammals the extended RRACH methylation consensus is preferred and would support the preferential location for methylation that we observe. This enrichment could be even more significant as our gene set overlap search used sequences only which contained the extended consensus in the 3′ UTRs. Therefore genes without 3′ UTRs were not considered in the overlap search.
We have previously suggested that in yeast the methylation in the 3′ half of the IME2 transcript may prevent the binding of Khd1 protein (Bodi et al., 2010), which is a translational suppressor, and is involved in message localization and asymmetric translation in yeast (Hasegawa et al., 2009). The global 3′ enrichment of m6A in an mRNA population is consistent with a similar general role in the regulation of translatability and localization.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at http://www.frontiersin.org/plant_genetics_and_genomics/10.3389/fpls.2012.00048/abstract

Table S1 | Differentially expressed genes.

Table S2 | GO enrichment in upregulated gene set.

Table S3 | GO enrichment in down regulated gene set.

Table S4 | Differentially expressed genes in GO:0008173 RNA methyltransferase activity.

Table S5 | Differentially expressed genes with GGACU in the last 200 nt region of their 3′ UTR.

Table S6 | Differentially expressed genes with GGACU in the last 200 nt region of their 3′ UTR.

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APPENDIX

FIGURE A1 | Measuring levels of MTA in ABI3 promoter driven transgenic plants. Reverse transcription was carried out using SuperScript II reverse transcription kit (Invitrogen). Real-time PCR was carried out using the MX3005P qPCR machine and the Brilliant SYBR Green qPCR master mix (Stratagene). MAXPro software was used for data analysis. Primers used were, MTA primers: 5′-GGAACCTTTGGAGTTGTTATG-3′ and 5′-CAAAGCTCCAAACATTCACG-3′, and the β-actin2 (normalizer gene) primers: 5′-GTACAACCGGTATTGTGCT -3′ and 5′-ATCAGTAAGGTCACCA-3′. Total RNA was isolated from young buds of three wild type plants, and three MTA homozygous T-DNA insertion lines complemented with the ABI3 promoter driven construct. The error bars are representing SD of three replicates.

FIGURE A2 | Quality control of fragmented mRNA. The poly(A) RNA used for the fragmentation experiments was purified using oligo(dT) at least three times, the quality of the mRNA was confirmed by RNA6000 LabChip (Agilent) (A). After fragmentation the average fragment size was confirmed by RNA6000 LabChip (Agilent) (B). A dot-blot was used for confirming that the fragments after fractionation were enriched in the predicted parts of the mRNA pool. Probes were made from the 5', the middle and the 3' regions of the ATPC mRNA and it was hybridized to membrane bound RNA pools of the different fractionated fragments (C).