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Identification of factors required for m⁶A mRNA methylation in Arabidopsis reveals a role for the conserved E3 ubiquitin ligase HAKAI

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Summary
• N⁶-adenosine methylation (m⁶A) of mRNA is an essential process in most eukaryotes, but its role and the status of factors accompanying this modification are still poorly understood.
• Using combined methods of genetics, proteomics and RNA biochemistry, we identified a core set of mRNA m⁶A writer proteins in Arabidopsis thaliana.
• The components required for m⁶A in Arabidopsis included MTA, MTB, FIP37, VIRILIZER and the E3 ubiquitin ligase HAKAI. Downregulation of these proteins led to reduced relative m⁶A levels and shared pleiotropic phenotypes, which included aberrant vascular formation in the root, indicating that correct m⁶A methylation plays a role in developmental decisions during pattern formation.
• The conservation of these proteins amongst eukaryotes and the demonstration of a role in writing m⁶A for the E3 ubiquitin ligase HAKAI is likely to be of considerable relevance beyond the plant sciences.

Introduction
More than 150 nucleotide modifications of RNA have been described and of these > 10 have been reported in mRNA (Machnicka et al., 2013). N⁶-methyladenosine (m⁶A) is the most prevalent internal mRNA modification found in eukaryotes, and has received a burst of interest in recent years (Meyer & Jaffrey, 2014; Fray & Simpson, 2015; Yue et al., 2015). m⁶A appears to be involved in a broad range of biological processes including mRNA export from the nucleus (Fustin et al., 2013), regulation of splicing (Alarcón et al., 2015b; Haussmann et al., 2016; Lence et al., 2016), mRNA translatability and stability (Wang et al., 2014a,b, 2015; Bodi et al., 2015; Zhou et al., 2015), alternative polyadenylation site choice (Ke et al., 2015) and other mechanisms accompanying RNA maturation (Meyer & Jaffrey, 2014; Yue et al., 2015). m⁶A is essential for the earliest stages of pattern formation in plants (Zhong et al., 2008; Bodi et al., 2012; Shen et al., 2016) and metazoans (Meyer & Jaffrey, 2014; Geula et al., 2015; Yue et al., 2015; Haussmann et al., 2016; Lence et al., 2016), linked with diseases in humans and other mammalian species (Jia et al., 2011; Zheng et al., 2012).

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2013) and is required for meiosis in *Saccharomyces cerevisiae* (Clancy et al., 2002). Reduced levels of m^6^A also affect circadian period (Fustin et al., 2013) and are critical for stem cell differentiation in mammals (Geula et al., 2015).

Although m^6^A can be present throughout the primary transcript, it is enriched at 3’ ends, particularly within the last exon of mature mRNA (Bodi et al., 2012; Dominissini et al., 2012; Li et al., 2014; Luo et al., 2014; Ke et al., 2015; Wan et al., 2015). m^6^A is found within a conserved consensus sequence (G/A)(G/A) ACU (with a preference for (G/A)GACU) in different eukaryotes (Horowitz et al., 1984; Narayan & Rottman, 1988; Dominissini et al., 2012; Meyer et al., 2012; Luo et al., 2014; Wan et al., 2015). However, only a subset of potential sites is actually modified and the mechanisms underlying the specificity of writing are not yet understood.

The S-adenosyl methionine dependent methyltransferase METTL3 was first purified and characterized by Bokar et al. (1997), as a 70-kDa subunit of a larger protein complex. Whilst METTL3 was part of a 200-kDa complex, a further 875-kDa complex component(s) was required for its in vitro activity (Bokar et al., 1994, 1997). The identity of any of the other proteins that act together with METTL3 remained unknown until it was shown in *Arabidopsis* that FIP37 (FKBP12 INTERACTING PROTEIN 37) was a partner protein of MTA (the homologue of METTL3) (Zhong et al., 2008; Shen et al., 2016). Following this initial discovery, the homologues of FIP37, *S. cerevisiae* MUM2 (Muddled Meiosis 2), and mammalian WTAP (Wilms tumour 1 associated protein), were shown to interact with METTL3 (MTA) orthologues and to be required for mRNA methylation in their respective model organisms (Agarwala et al., 2012; Ping et al., 2014; Wang et al., 2014b; Liu et al., 2015). More recently, another human methylase, METTL14, phylogenetically related to METTL3 (Bujnicki et al., 2002), was shown to form a complex with METTL3 and WTAP, and to be required also for m^6^A formation (Ping et al., 2014; Wang et al., 2014b; Liu et al., 2015).

Other factors in addition to METTL3, METTL14 and WTAP are also involved in m^6^A writing. Human KIAA1429 (Schwartz et al., 2014), a homologue of *Drosophila melanogaster* Virilizer (Vir) (Niesen et al., 2001), associates with METTL3 and is required for m^6^A writer activity in mammals. Vir was first isolated as a factor that, together with the *D. melanogaster* orthologue of WTAP (Fl(2)D) regulated sex determination (Hilfiker et al., 1995; Niesen et al., 2001; Ortega et al., 2003). Despite sharing many common features with the mammalian methylation process, yeast does not have a homologue of KIAA1429, but an additional protein, SLZ1 (Sporulation-specific Leucine Zipper 1), absent in humans, is in the yeast complex and is necessary for mRNA methylation activity (Agarwala et al., 2012).

Until now, no orthologues of METTL14 and KIAA1429 have been shown to be required for m^6^A writing in organisms distinct from mammals (Meyer & Jaffrey, 2014; Fray & Simpson, 2015; Yue et al., 2015). In *S. cerevisiae*, a physical association between IME4 (homologue of METTL3) and KAR4 (homologue of METTL14) has been reported (Ito et al., 2001). However, yeast KAR4 lacks a characteristic S-adenosyl methionine binding domain and thus it is likely to perform a different role to mammalian METTL14 (Bujnicki et al., 2002; Lahav et al., 2007).

Here we report the identification of a conserved set of proteins forming the m^6^A writer complex in *Arabidopsis*. They include MTA (orthologue of human METTL3), MTB (METTL14), FIP37 (WTAP) and VIRILIZER and a homologue of human HAKAI. HAKAI was first characterized in humans as a RING domain E3 ubiquitin-ligase that mediates the post-translational downregulation of E-cadherin at the plasma membrane (Fujita et al., 2002). It has recently appeared in animal proteomics interaction lists that include other m^6^A writer complex members (Horiuchi et al., 2013); however, until now, a role in m^6^A writing has not been proposed. The demonstration here – that this interaction is conserved across kingdoms and that plant HAKAI is functionally required for full mRNA methylation – may indicate that a similar role for mammalian HAKAI should also be considered.

### Materials and Methods

#### Plant growth conditions

Seeds were surface-sterilized and, after 2 d of stratification at 4°C, cultivated under a 16 h : 8 h photoperiod, 22 : 18°C, light : dark, on 0.5 × Murashige & Skoog medium with 1% sucrose, unless indicated otherwise. For anatomical, histological and reporter gene analyses, primary roots of 4–6-d-old vertically grown seedlings were used. Inducible transgene expression was controlled by germinating seeds for 6 d (together with appropriate controls) on sterile media containing 5 μM 17-β-estradiol (est) (purchased from Sigma) and documented. In adult plants, 20 μM est was sprayed every other day after rosette formation.

#### Plant strains

All *Arabidopsis thaliana* (L.) Heynh. lines were in the Columbia (Col-0) accession. The following mutants and transgenic plants were described previously: AHP6prom:GFP (Mählönen et al., 2006), 35Sprom:SR34-RFP (Lorkovic et al., 2008) and ABI3prom: MTA complemented mta SALK_074069 allele (Bodi et al., 2012). The T-DNA insertion SALK_018636 (fip37-3) line was obtained from the NASC Stock Centre (Nottingham, UK) and GABI_217A12 (hakai-1) from GABI-Kat (Bernd Weisshaar, Bielefeld, Germany) (Kleinboelting et al., 2012). The construct for the CRISPR mutagenesis of *hakai*-2 was made by Golden Gate cloning (New England Biolabs, Hitchin, UK) using the vectors and methods described previously (Nekrasov et al., 2013). It was designed to make the sgRNA GATTACGGTGGGAGTCA, which targets a site in the first coding exon. Following transformation, T1 plants were initially screened for the presence of an *Mly* resistant *HAKAI* PCR product and putative homozygous disruption lines were further confirmed by sequencing. Lines homozygous for the *hakai*-2 mutation, but lacking the Cas9 T-DNA were selected from subsequent generations.

#### DNA manipulations and transgenic work

The *vir-1* phenotype rescuing VIRprom:GFP-VIR was derived from a genomic sequence comprising 2155 bp of the VIR
promoter region which was fused with GFP in pEPA vector (Ruzicka et al., 2010), and subcloned into pML-BART binary vector (Gleave, 1992). 35Sprom:GS-VIR was constructed from VIR genomic sequence in pDONR221 recombined into pKNGSTAP (Karimi et al., 2007) by a standard Gateway procedure (Invitrogen). MTB, FIP37 and HAKAI mCherry Multisite Gateway based C-terminal fusions were made as previously described (Karimi et al., 2007) and according to the manufacturer’s instructions (Invitrogen), using 2374, 1823 and 1680 bp of their native promoter regions, respectively.

The β-estradiol inducible transgene WOLprom:XVE>>VIR RNAi and UBQ10prom:XVE>>MTB RNAi constructs were made as described previously (Mählönen et al., 2014; Siligato et al., 2016), inserting the regions detailed in Table S1 (Supporting Information) (table of primers used) in the sense and antisense orientation into entry clones with restriction enzyme-mediated cloning. The promoter UBQ10 was chosen because it directs stable, widespread expression and is resistant to silencing (Geldner et al., 2009). The empty RNAi hairpin was used as a negative control.

**Tandem affinity purification**

Cloning of transgenes encoding tag fusions under control of the constitutive cauliflower mosaic virus 35S promoter and transformation of Arabidopsis cell suspension cultures were made as described previously (Van Leene et al., 2007). Two independent tandem affinity purifications of protein complexes were performed using the GS tag (Van Leene et al., 2008) followed by the GS purification protocol as described in Van Leene et al. (2011). The protocols of proteolysis and peptide isolation, acquisition of mass spectra by a 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA), and MS-based protein homology identification based on the TAIR genomic database, have been described previously (Van Leene et al., 2010). Putative false positive interactions were subtracted based on prior experience with c. 40 TAP experiments on wild-type (WT) cultures and cultures expressing TAP-tagged mock proteins GUS, RFP and GFP (Van Leene et al., 2010).

**m6A analysis**

The quantification of relative m6A levels was performed as described previously (Zhong et al., 2008). Briefly, 20 μg of total RNA was extracted from Arabidopsis seedling samples (or from roots of β-estradiol treated inducible RNAi lines) using the RNAqueous kit (Ambion), the poly(A)+ fraction was purified twice using the MicroPoly(A) Purist kit (Ambion) and the quality of the mRNA checked on an RNA 6000 LabChip, with an Agilent Bioanalyser (Ambion). For each sample, 50 ng of mRNA was digested with 1 μl of Ribonuclease T1 (1000 units μl⁻¹; Fermentas, Altrincham, UK) in a final volume of 10 μl (1× polynucleotide kinase buffer) for 1 h at 37°C and the exposed 5′ end of the digested mRNA fragments labelled using T4 polynucleotide kinase (10 units; Fermentas) and 1 μl [γ-32P] ATP (6000 Ci mmol⁻¹; Perkin-Elmer, Waltham, MA, USA). Following ethanol precipitation, labelled RNA was resuspended in 10 μl of 50 mM sodium acetate buffer (pH 5.5) and digested with P1 nuclease (Sigma-Aldrich) for 1 h at 37°C. 2 μl of each sample was loaded onto cellulose TLC plates (20 x 20 cm; Merck, Hertfordshire, UK) and developed in a solvent system consisting of isobutyric acid: 0.5 M NH4OH (5 : 3, v/v), for the first dimension, and isopropanol : HCl : water (70 : 15 : 15, v/v/v), for the second dimension. Spot intensities were determined using a storage phosphorscreen (K-Screen; Kodak, Rochester, NY, USA) and BioRad Molecular Imager FX in combination with QUANTITY ONE 4.6.3 software (Bio-Rad).

**Seedling phenotype analysis**

For the quantification of seedling phenotypes, the plates with seedlings were photographed and measured with IMAGEJ software (Schneider et al., 2012). Vertical growth index, as a measure of gravitropic response, defined as a ratio between the root tip ordinate and the root length, was determined as described previously (Grabov et al., 2005). Approximately 15–20 seedlings were processed for each treatment, and three independent experiments were performed, giving the same statistically significant results (representative experiments are presented).

For statistical analysis, equal variances of datasets were verified by the Levene test, and the Kruskal–Wallis nonparametric test was performed simultaneously with ANOVA. Data were evaluated with NCSS 2007. The data presented are means ± standard errors.

**Histological analysis and microscopy**

Fuchsin staining and confocal imaging were performed on the primary roots of 4–5-d-old seedlings as described previously (Mählönen et al., 2006). The quantitative analysis of protoxylem phenotypes was performed on fuchsin-stained roots as described (Bishopp et al., 2011). Lugol staining for columella starch granules was carried out as described previously (Friml et al., 2002). For the confocal laser scanning microscopy, a Zeiss LSM 780 microscope was used. Due to low signal intensity of transgenes inside the root stele, the contrasts and brightness were enhanced to reveal expression and localization patterns, unless stated otherwise. The analysis of AHP6prom:GFP on longitudinal optical sections were acquired with the same confocal settings on all lines in the experiment and the (heat map) RAINBOWRGB lookup table was applied on unprocessed images by IMAGEJ in order to demonstrate expression changes (Schneider et al., 2012).

**Genetic screening and positional cloning**

The vir-1 mutant was isolated in an EMS mutagenesis screen for altered pattern of AHP6prom:GFP expression. The 970-kb mapping window between marker nga172 and BAC F2O10 on chromosome 3 was established using 100 F2 recombinant plants. Using Illumina whole genome sequencing (CD Genomics, Shirley, NY, USA; Schneeberger et al., 2009), we identified three mutations within the mapping window in intragenic regions: one in intronic sequence unrelated to known splicing consensus elements, one that
caused a synonymous mutation and one in the 5′ splice site of VIR intron 5 that led to mis-splicing of VIR transcripts.

Sequence analysis and multiple sequence alignment

The domain composition of VIR was examined using SMART (Letunic et al., 2012) and Pfam (Punta et al., 2012) databases. For creating the multiple sequence alignments, the protein sequences were aligned using the Clustal Omega algorithm (Sievers et al., 2011) and graphically visualized by JalView v.2.8.0b1 using default ClustalX colour code (Waterhouse et al., 2009).

RNA sequencing

Three biological replicates each of 5-d-old vir-1 and the complemented mutant seedlings were harvested directly into RNA stabilizing reagent RNAlater (Ambion). Approximately 1-mm root tips were excised and used as the tissue source for RNA sequencing. Total RNA was extracted with RNAqueous kit (Ambion) and treated with DNase (Fermentas, Thermo Fisher Scientific Fermentas, Vilnius, Lithuania). 1–3 µg of total-RNA was used for isolation of poly(A)+ RNA (Dynabeads mRNA purification kit; Ambion). The poly(A)+ RNA was reverse-transcribed to cDNA (SuperScript Double-Stranded cDNA Synthesis Kit, Invitrogen Life Technologies, Carlsbad, CA, USA). Random hexamers (New England Biolabs) were used for priming the first strand synthesis reaction and SPRI beads (Agencourt AMPure XP, Beckman Coulter, Brea, CA, USA) for purification of cDNA. Illumina compatible Nextera Technology (Illumina, San Diego, CA, USA) was used for preparation of RNA-seq libraries, employing DNA fragmentation and tagging by in vitro cut-and-paste transposition; 60 ng of cDNA was used instead of DNA. After the tagmentation reaction, the fragmented cDNA was purified with SPRI beads. In order to add the Illumina-specific bridge PCR compatible sites and enrich the library, limited-cycle PCR (five cycles) was performed according to the instructions for the Nextera system with minor modifications. For bar-coded libraries, 50 X Nextera Adaptor 2 was replaced with a bar-coded Illumina-compatible Adaptors from the Nextera Bar Codes kit (Illumina) in PCR setup. SPRI beads were used for purification of the PCR products and the library QC was evaluated by Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA).

Each transriptome was loaded to occupy 1/4 of the lane capacity in a flow cell. C-Bot (TruSeq PE Cluster Kit v3, Illumina) was used for cluster generation and Illumina HiSeq2000 platform (TruSeq SBS Kit v3 reagent kit) for paired-end sequencing. Each biological replicate was sequenced once, producing 100 bp paired-end reads that were then quality trimmed to a length of 93 bp. The sequencing resulted in 123 M reads for the complemented VIR line and 112 M reads for the complemented mutant seedlings. The sequencing was done in collaboration with the Finnish Institute for Molecular Medicine, Helsinki, Finland.

Transcriptome analysis

For testing the effects of vir-1 on global splicing with rMATS (v.3.2.5; Shen et al., 2014), the obtained quality filtered reads in FASTQ format were trimmed to 80 nucleotides using the rMATS trimFASTQ Python script. The trimmed reads were then aligned to the TAIR10 genome build using STAR (Dobin et al., 2013). The WT and vir-1 data were compared using the |c parameter set to 0.0001 (0.01% splicing difference). Summary outputs filtered by FDR (Q<0.05) from rMATS are provided (Table S2).

For analyses based on differential expression, sequences were aligned against the TAIR10 genome using TopHat (v.2.0.8b) with the options −bowtie1 and −no-discordant in two runs and a mean inner distance between mate pairs of 112 to 133 (SD = 50). The junctions predicted by the first run supplied to the second run. Transcript counts were calculated with HTSeq with features marked in the ENSEMBL v.72 TAIR10 Gene Transfer Format annotation. Differential gene expression was assessed with the DESeq package (Anders & Huber, 2010). The full procedure is described in (Edgren et al., 2011). The GO analysis of complete gene list expressed in the vir-1 root tip was done using the AGrIGO tools (Du et al., 2010) with FDR-corrected P-value (Q value) as a ranking criterion and Hypergeometric test (Hochberg FDR) with 0.05 significance cut-off. The list of vascular regulators (Caño-Delgado et al., 2010) has been used as a benchmark for defining appropriate GO terms for generating manually edited and updated list of genes required for vascular formation.

Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out as described previously (Furuta et al., 2014) using a LightCycler 480 (Roche) with LightCycler 480 SYBR Green master mix (Roche) and the manufacturer’s qRT-PCR program recommendations. Four technical repeats were carried out to assess the gene expression levels. Gene expression was normalized to UBQ10, as described previously (Furuta et al., 2014).

Yeast two-hybrid system

The protein–protein interactions were tested as described previously (Zhong et al., 2008; Pékarová et al., 2011). Plasmids were constructed by Gateway-based technology using destination vectors pDEST22 (activation domain) and pDEST32 (binding domain). VIR sequence was split into two parts, corresponding to amino acid residues 1 to 2883 (part 1) and 2584 to 6417 (part 2), referring to the AT3G05680.1 gene model. The interactions were tested in three technical and three biological replicates on media lacking histidine and supplemented with inhibitor of histidine synthesis, 3-amino-1,2,4-triazole (3-AT; Sigma). The yeast growth was recorded after 4 d.

Accession numbers

Sequence data for genes described in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AHP6 (At1g80100), MTA (At4g10760), MTB (At4g09980), FIP37 (At3g54170),...
VIR (At3g05680) and HAKAI (At5g01160). The raw sequences for vir-1 transcriptome and rMATS outputs have been deposited in the Gene Expression Omnibus (GEO) database, accession GSE97174.

Results

Identification of a viable mutant orthologous to the splicing regulator/m6A writer protein VIRILIZER/KIAA1429

The histidine pseudophosphotransmitter AHP6 is a factor required for protoxylem formation in Arabidopsis and is the earliest marker of root protoxylem development (Mähönen et al., 2006). In a mutant screen designed to identify regulators of Arabidopsis vascular development, we isolated one line that exhibited reduced and irregular AHP6prom:GFP expression (Fig. 1a) accompanied by defects in protoxylem development in the primary root (Fig. 1b). We used positional cloning and whole genome sequencing to identify EMB2016 (Tzafrir et al., 2004) as the disrupted gene (Fig. 1c). This gene of unknown function was described previously as essential (Tzafrir et al., 2004) and is homologous to the D. melanogaster sex determination splicing factor, Virilizer (Hilfiker et al., 1995; Niessen et al., 2001), and to human KIAA1429, associated with m6A formation in mammals (Schwartz et al., 2014; Fig. S1). Based on the homology to the prototypical gene, we refer to this mutant (and gene) as virilizer-1 (vir-1). The vir-1 EMS-induced mutation results in a G-to-A conversion at the first nucleotide of intron 5, leading to numerous predominantly wrongly spliced VIR transcripts with minor but detectable content of the correct VIR mRNA (Figs 1c–e, S2a). The vir-1 mutant exhibited pleiotropic phenotypes, which included aberrant root cap formation, gravity response and lateral root development, as well as defective corydonal development (Fig. S2b–f). Because these aberrant phenotypes were rescued by complementation with a VIR (Figs 1f, S2g), and phenocopied by inducible VIR RNAi lines (Fig. S2h), we concluded that we had isolated a viable hypomorphic virilizer allele.

Transcriptome of vir-1 mutant

Drosophila melanogaster Vir is required for promoting certain alternative splicing events associated with female gametogenesis and X chromosome dosage compensation (Hilfiker et al., 1995; Ortega et al., 2003; Haussmann et al., 2016; Lence et al., 2016). We isolated poly(A)+ RNA from vir-1 root tips and carried out Illumina RNA sequencing. We examined the changes in alternative splicing in vir-1 using rMATS tools (Shen et al., 2014). Only 22 retained introns, one mutually exclusive exon, and eight skipped exon, one alternative 5′ and 13 alternative 3′ splice sites (FDR < 0.01) were identified (Table S2). Given the selected threshold (see the Materials and Methods section), we conclude that the vir-1 mutation does not result in extensive alternative or mis-splicing of transcripts.

Gene ontology (GO) analysis (Du et al., 2010) based on the differentially expressed genes in the vir-1 mutant (Table S3) revealed that the vir-1 mutation affects a range of processes which include those associated with response to environmental cues, metabolic processes and macromolecular localization, and also growth and development (Table S4). The root tip tissue-specific context allowed us to examine expression of early vascular genes (Table S5). Out of 138 vascular formation related genes identified in our dataset, 35 have been misexpressed, which suggests that vascular development in vir-1 is affected at multiple levels (Table S6). Altogether, the observed changes in the vir-1 root tip transcriptional profiles suggests that VIR is likely involved in regulation of gene expression, but the function of VIR is rather general than specific and knock-down of VIR does not affect overall splicing rates in Arabidopsis.

VIR closely associates with a conserved set of proteins linked with m6A writing

Although it is a relatively large protein (236 kDa), VIR lacks well-characterized protein domains and its precise molecular function remains obscure. The mammalian homologue of VIR was recently shown to associate with m6A writer proteins (Ortega et al., 2003; Horiuchi et al., 2013; Schwartz et al., 2014), we therefore decided to test experimentally whether a similar association also existed in Arabidopsis. We used tandem affinity purification (TAP) followed by proteolysis and mass spectrometry (Van Leene et al., 2011) to identify proteins that associated with the VIR-GS bait in Arabidopsis suspension cell cultures. Using this approach, we consistently identified FIP37 (Zhong et al., 2008) and HAKAI (Fig. S3; Fujita et al., 2002; Horiuchi et al., 2013) co-purifying with VIR and MTB (Bujnicki et al., 2002) also significantly enriched with a lower confidence score (Fig. 2a; Table S7).

In order to further characterize these putative members of the m6A writer complex and to examine their relationship to the known plant m6A methylase MTA, we tested several pairwise interactions between them using the yeast two-hybrid system (Y2H) (Fig. 2b). In addition to the known MTA-FIP37 interaction (Zhong et al., 2008), we observed heterodimerization of the Arabidopsis MTA and MTB, consistent with the reported interaction between their mammalian orthologues METTL3 and METTL14 (Liu et al., 2014; Ping et al., 2014; Wang et al., 2014b). We also found that MTB but not MTA formed homodimers in Y2H. These data support the findings of Liu et al. (2014) who reported that mammalian methylases METTL3 and METTL14 can form heterotramers. In addition, FIP37 also gave self-interaction and a consistent though weaker interaction was also seen between HAKAI and MTB. Due to lower interaction fidelities of very large proteins in Y2H (Koegl & Uetz, 2007), we split VIR protein into two parts and also used it for Y2H. However, no interaction was seen with the carboxy terminal fragment in our test system (Fig. 2b). In summary, we show that, in addition to MTA and FIP37 (Zhong et al., 2008), MTB, VIR and HAKAI are also associated in the Arabidopsis m6A writer complex.

Because this conserved set of associating proteins co-purify, one would expect that they would be found together in the same cell
Fig. 1 A hypomorphic mutation in the VIRILIZER (VIR) locus of Arabidopsis thaliana leads to vascular defects. (a) Wild-type (WT) expression pattern of the AHP6prom:GFP marker in the root protoxylem founder cells and its aberrant expression in vir-1, as documented by optical longitudinal and cross-section images. In the WT, the reporter is expressed in one or two files on each side inside the vascular cylinder (giving rise to protoxylem, white arrowheads) and in two accompanying cells of pericycle (blue arrowheads). (b) Fuchsin staining illustrating vir-1 protoxylem defects, such as interruptions or doubling of protoxylem (arrowheads). (c) The vir-1 mutation is caused by a single nucleotide substitution in the 5′ splice site of the 5th intron of the VIR gene. Although the vir-1 mutation does not significantly change VIR expression levels at α=0.05 (d), it affects its correct splicing as assayed by reverse transcription polymerase chain reaction (RT-PCR) around mutated site in vir-1 (e). (f) Quantification of the vir-1 protoxylem phenotype, which can be rescued by introducing a GFP-VIR transgene into vir-1 plants. Quantitative RT-PCR (d) data are represented as means ± SE. Bars, 20 μm.
Establishing a set of stable transgenic lines that perturb the expression of the proteins associated with m6A writing

In order to test the requirement for each of the associating proteins in writing m6A, we generated a collection of Arabidopsis lines defective in the expression of MTB, FIP37, VIR and HAKAI (Figs 1b–d, 4). Null mutations in MTB are embryonic lethal (Zhong et al., 2008), as are null alleles of Arabidopsis MTB, FIP37 and VIRILIZER (Tzafir et al., 2004; Vespa et al., 2004). However, we identified a viable hypomorphic allele of FIP37, caused by a T-DNA insertion within its 7th intron (fp37-4, identical to that described in Shen et al. (2016); Fig. 4a). We also identified an insertion mutant of hakai (hakai-1; Fig. 4b), and generated a deletion hakai allele using CRISPR/Cas9 to remove a single nucleotide in the coding sequence of the 1st exon (hakai-2, Fig. 4b). All of these homozygous mutants were viable in our growth conditions. qRT-PCR confirmed that expression of the respective genes was compromised in each case (Fig. 4c).

We also constructed inducible RNAi lines (Mäkönen et al., 2014) to knockdown the expression of MTB and used RT-qPCR to confirm that expression was knocked-down upon addition of the inducer β-estradiol (Fig. 4d). In summary, we have developed a set of viable genotypes that partially or completely disrupt the expression of genes encoding each of the putative m6A writers. These provide a genetic resource to analyse the functional role of MTB, FIP37, VIR and HAKAI in writing m6A.
HAKAI and putative Arabidopsis m6A writers are required for methylation of mRNA

We next examined the requirement of each protein factor in writing m6A to Arabidopsis mRNA. We therefore measured the levels of m6A in each of the lines. Following two rounds of poly(A)+ RNA purification, mRNA was digested with RNase T1 (which cleaves after every G residue). Fragments were then end-labelled using [γ-32P]ATP and digested to mononucleotides. These were separated and the m6A:A ratios determined as described previously (Zhong et al., 2008). We found that levels of m6A were reduced to 5–15% WT levels in the fip37-4, and vir-1 lines and by 50% in the MTB RNAi line after β-estradiol treatment (Fig. 5b–e). Furthermore, in both hakai mutants m6A levels were reduced by 35% (Fig. 5f,g). We therefore conclude that each of the newly identified factors, MTB, FIP37, VIR and HAKAI, is required to write WT levels of m6A in Arabidopsis mRNA.

Genetic depletion of total m6A pools leads to similar phenotypic consequences

If MTA, MTB, FIP37, HAKAI and VIR function together to write m6A, then it would be expected that mutants defective in these components might share similar developmental defects. Indeed, the mta, mtb, fip37 and vir null alleles arrest at the globular stage of embryonic development (seedgenes.org; Tzafrir et al., 2004), but both hakai alleles are viable (Fig. 6a). Hypomorphic mutants and knockdown lines of these factors or null mta mutants rescued by embryo-specific expression of MTA driven by the ABI3 promoter (mta ABI3prom:MTA) (Bodi et al., 2012), show reduced root growth and aberrant gravitropic responses (Fig. 6a–d), whereas both hakai alleles rather resemble WT. This distinction may reflect the different impact on global m6A levels of these lines, because in each hypomorphic mutant of MTA (Bodi et al., 2012), FIP37 and VIRILIZER, m6A levels were reduced to a greater extent than in both hakai alleles (Fig. 5). In addition, the lines with reduced MTA, MTB, FIP37 and VIR expression also show delayed development and reduced apical dominance in the generative phase of development, whereas hakai mutants more closely resembled WT with respect to these traits (Fig. 6e,f). Because we isolated the viable vir-1 allele in the course of a screen for factors mediating the regulation of AHP6prom:GFP in early stages of root development, we also looked at these traits in more detail. Like the vir-1 allele which shows a misexpression of AHP6prom:GFP, we found that MTB knockdown lines and fip37-4 mutants also showed reduced expression of the AHP6prom:GFP reporter (Fig. 7a,b). Importantly, all mutant and knock-down lines show defects associated with vascular development. Similar to vir-1, each line exhibits defective protoxylem development, with increased occurrence of interruptions in, and doublings of, protoxylem strands being detected compared to WT (Fig. 7c). In conclusion, all mutants share defects in vascular development and reductions of m6A. Embryonic lethality of null alleles and other developmental defects are shared in hypomorphic lines defective in MTA, MTB,
FIP37 and VIR function, but these are generally less pronounced in *hakai* mutants.

*hakai* mutants act synergistically with other m$^6$A writer mutants

Given the relatively weak *hakai* mutant phenotypes compared to the severe developmental defects seen in the hypomorphs of the other m$^6$A writer-associated proteins, we sought to test whether HAKAI also interacted genetically with other writer components. First, we crossed both *hakai-1* and *hakai-2* to the *mta ABI3prom:MTA* line (Bodi et al., 2012) and selected double homozygous mutant plants from the subsequent filial generations. In both cases, the introduction of the *hakai* mutant into the *mta ABI3prom:MTA* hypomorph background gave rise to plants with a compromised growth phenotype that was far more severe than either parent (Fig. 8). m$^6$A levels showed a further slight reduction in m$^6$A relative to the *mta ABI3prom:MTA* parent (Fig. S5). Next we crossed the *hakai-2* mutant to the *fip37-4* hypomorph and selected plants that were homozygous for *fip37-4* and heterozygous for *hakai-2* from the F$_2$ generation. These plants were selfed and the F$_3$ seed planted on Murashige & Skoog media and the emerging seedlings genotyped with respect to the *hakai-2* mutation. Of 73 progenies, 23 (31.5%) were found to be WT with respect to *hakai-2* and 50 (68.5%) were heterozygous. No homozygous *hakai-2* seedlings were found. This suggests that the combination of the *fip37-4* and *hakai-2* mutations is lethal; indeed the WT:heterozygotes observed ratio is close to the 1:2 that would be predicted if this were the case. We did not observe an increase in the number of non-germinating seeds, suggesting that the double mutants aborted very early in their development.

**Discussion**

Role of m$^6$A in plant development

A regulatory role of N6-adenosine methylation (m$^6$A) in development and in determining cell fate has been demonstrated in major model systems such as mouse, *Drosophila melanogaster* and *Saccharomyces cerevisiae* (Clancy et al., 2002; Hongay & Orr-Weaver, 2011; Geula et al., 2015; Haussmann et al., 2016). m$^6$A has recently been shown to have a crucial role during murine stem cell development, where it prompts stem cell differentiation from their naïve stage (Geula et al., 2015). *Arabidopsis* full knockouts of MTA, MTB, FIP37 and VIR also do not progress past the embryonic globular stage (Tzafrir et al., 2003; Vespa et al., 2004; Zhong et al., 2008). We have circumvented this embryo lethality by establishing a collection of hypomorphic, mutants or RNAi lines for each of the proteins associated with the m$^6$A writer complex in order to study the effects of reduced m$^6$A in postembryonic development. In a recently published study, (Shen et al., 2016) reported that reduced FIP37 expression resulted in increased proliferation of the shoot apical meristem.
Our findings independently expand this, further revealing that impaired expression of any of the five m\textsuperscript{6}A writer components we identify results in a range of developmental defects, including vascular formation, implying a role for m\textsuperscript{6}A in establishing and maintaining these processes.

Conserved Eukaryotic writer proteins and role of VIRILIZER

Based on our genetic and biochemical data, we propose that the core constituents of the plant writer complex are nuclear localized proteins MTA, MTB, FIP37, VIR and HAKAI. Orthologues of MTA and MTB interact with each other and with orthologues of FIP37 in \textit{S. cerevisiae} (Agarwala \textit{et al.}, 2012) and human cells (Liu \textit{et al.}, 2014; Ping \textit{et al.}, 2014; Schwartz \textit{et al.}, 2014; Wang \textit{et al.}, 2014b). In \textit{S. cerevisiae}, writing of m\textsuperscript{6}A requires orthologues of MTA (IME4), FIP37 (MUM2) and the yeast-specific SLZ1 (Agarwala \textit{et al.}, 2012). In mammals, in addition to the orthologues of MTA (METTL3) and FIP37 (WTAP), MTB (METTL14) and the splicing/methylation factor VIR (KIAA1429) are also present in the complex and required for m\textsuperscript{6}A writing. \textit{Arabidopsis} MTA and MTB are nonredundant and, similar to METTL3 and METTL14 (Liu \textit{et al.}, 2014; Ping \textit{et al.}, 2014), they also interact directly in Y2H.

In contrast to \textit{S. cerevisiae}, where the m\textsuperscript{6}A writers reside in nucleoli (Agarwala), their plant orthologues occupy the nucleoplasm, similar to animal systems (Bokar \textit{et al.}, 1997; Niessen \textit{et al.}, 2001; Zhong \textit{et al.}, 2008; Liu \textit{et al.}, 2014; Ping \textit{et al.}, 2014). Subnuclear domains, so called nuclear speckles, correspond to sites with active transcription in the interchromatin regions. They are connected with the presence of splicing factors, but less with the accumulation of other factors participating on RNA processing in microscope localization studies (Spector & Lamond, 2011; Reddy \textit{et al.}, 2012). Analogously to their animal orthologues (Liu \textit{et al.}, 2014; Ping \textit{et al.}, 2014), plant writers also...
show a similar punctate pattern (Zhong et al., 2008) and co-localize with the splicing factor SR34-RFP. This splicing factor, as well as the co-localizing writers, shows a more diffuse pattern in the meristematic cells, whereas in the cells above the elongation zone the speckle pattern is more prevalent. This likely coincides with differential transcriptional activities in rapidly dividing and differentiated cells, similar to observations from animal systems (Tillemans et al., 2006; Spector & Lamond, 2011; Reddy et al., 2012). Because m6A affects splicing in mammals and D. melanogaster (Liu et al., 2014; Ping et al., 2014; Alarcón et al., 2015a; Haussmann et al., 2016; Lence et al., 2016) possibly by recruiting splicing factors (Xiao et al., 2016), it is interesting to speculate whether m6A could also regulate splicing in plants. The rather normal splicing patterns observed in vir-1 root tip suggests that m6A is not involved in large-scale regulation of splicing in plants or that splicing regulation only occurs at the transcript or tissue-specific level, which is below the detection limit of the root-tip RNA-Seq.

In yeast, mRNA methylation only occurs naturally under very specific conditions where cells must be diploid and starved for both a nitrogen and fermentable carbon source (Clancy et al., 2002). In plants and mammals, mRNA methylation amounts vary between different developmental stages or organ types (Zhong et al., 2008; Meyer et al., 2012), indicating that regulation in these multicellular organisms is likely to be more subtle than a simple on or off state. It seems that variations of the writer complex evolved in other eukaryotes. According to our in silico analysis, VIR and HAKAI are missing in the genomes of Fungi, including S. cerevisiae. VIR has been implicated in the regulation of sex-specific alternative splicing in D. melanogaster (Hilfiker et al., 1995; Niessen et al., 2001), but more recently it was also found to be required for m6A formation in human cells (Schwartz et al., 2014). In D. melanogaster, the FIP37 homologue Fl(2)D closely associates with VIRILIZER (Ortega et al., 2003) and interacts genetically with methylation pathways to regulate sex-specific splicing events (Haussmann et al., 2016; Lence et al., 2016). In human cells their orthologues WTAP and KIAA1429 are required for m6A formation (Schwartz et al., 2014).
the conserved role of VIRILIZER, although whether this large protein is performing a scaffolding role or is catalytically active (and/or carries out a regulatory function) remains to be tested.

HAKAI is a new element required for the function of the Arabidopsis m\(^6\)A writer complex and directly interacts with its core components

Mammalian HAKAI, also known as Casitas B-lineage lymphoma-transforming sequence-like protein 1 (CBLL1), was initially identified as an E3 ubiquitin ligase that facilitates endocytosis of E-cadherin at cell–cell contacts, thus regulating epithelial integrity (Fujita et al., 2002). More recently, it has also been implicated with influencing RNA–protein interactions in animal systems (Fujita et al., 2002; Figueroa et al., 2009) and has been reported in association with m\(^6\)A writers (Horiuchi et al., 2013), but a role in m\(^6\)A writing has not been proposed until now. Although believed to act on E-cadherin complex at the plasma membrane, human and D. melanogaster HAKAIs are localized predominantly in the nucleus and in lower amounts at the plasma

Fig. 7 Reduced expression of proteins associating with the Arabidopsis thaliana m\(^6\)A writers results in vascular defects. (a, b) Similar to vir-1, AHP6prom:GFP fluorescence signal is reduced in the weak fip37-4 allele (a) and following induction of inducible UBQ10prom:XVE>>MTB RNAi (b). (c) Mutants with reduced m\(^6\)A levels show vascular defects, which includes interruptions or duplications of protoxylem files across the primary root. Bars, 50 μm. For the MTB RNAi line and the corresponding control, plant material was grown on media supplemented with 5 μM β-estradiol.

Fig. 8 Arabidopsis thaliana plants homozygous for either (a) hakai-1 or (b) hakai-2 crossed with mta ABI3prom:MTA show more severe developmental defects than either parent on its own (double mutants magnified in inset). Bars, 5 cm.
membrane and in the cytoplasm (Figueroa et al., 2009; Kaido et al., 2009). Arabidopsis HAKAI-mCherry resides mainly in the nucleus (Fig. 3g). Plants lack cadherins and the mechanisms determining cell polarity are generally different in metazoans and plants (Kania et al., 2014). Thus, the RNA-associated m’A-forming function of HAKAI that we identify here is likely to prevail in Arabidopsis. As phenotypes of hakai knockouts are rather subtle compared to the embryo lethality of other complex member knockouts, it is possible that HAKAI may have a more complex role. However, the extreme severity of the hakai mta ABI3prom:MTA and the lethality of the hakai fip37-4 double mutants supports the conclusion that HAKAI is a bona fide functional member of the methylation complex. It also suggests that as methylation amounts drop below ~10% of wild-type (WT), the severity of growth phenotypes increases dramatically. The mta ABI3prom:MTA line was generated by complementing an MTA insertion knockout with an MTA coding sequence under control of the ABI3 promoter, which drives high expression, and full m’A methylation, in seeds, but has very low activity post-germination (Bodi et al., 2012). Thus, the combination of this genotype with hakai knockout would only be expected to show a double mutant phenotype post-germination. By contrast, the fip37-4 hypomorph shows low levels of FIP37 expression and has poor seed set even before combining with other mutant genotypes. Thus, selling a plant that is homozygous for fp37-4 and heterozygous for hakai-2 would be expected to give a further, possibly developmentally lethal, reduction in m’A levels in the early stages of embryonic development.

HAKAI has been extensively studied in mammals due to its connection with oncogenesis (Aparicio et al., 2012), primarily attributed to its controlling of cell–cell adhesion (Fujita et al., 2002). However, it has been reported that this protein, through an uncharacterized mechanism, interacts with pyrrole intermediate tract binding protein associated splicing factor (PSF) and promotes its ability to bind specific (particularly tumourogenesis related) transcripts (Figueroa et al., 2009). Mammalian HAKAI specifically ubiquitinates phosphoryrosine modified E-cadherin (Fujita et al., 2002) and it may be that a similar mechanism is required for its activity within the m’A writer complex. Given the association of this protein with the writer complex in both plants and animals, and considering that in plants it is required for normal m’A methylation amounts, we propose that HAKAI is likely to also play a similar role in mRNA methylation in mammals. In this context, our findings could improve understanding of the mechanistic role of HAKAI in promoting tumorigenesis and regulating the binding of PSF with cancer-associated mRNA transcripts.

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Author contributions

K.R., M.Z., A.C., Z.B., M.K., M.S., H.L. and S.Z. conducted experiments; A.C. performed genetic screen; D.E. and G.D.J. made the TAP experiment; S.E-S. and N.P.M. carried out bioinformatics analyses; K.R., J.H., R.G.F. and Y.H. conceived research and designed experiments; and K.R. and R.G.F wrote the manuscript. All authors read and commented on the final version of the manuscript.

References


Fig. S4 Expression of VIR and other proteins associated with m^6A writing in the root tip.

Fig. S5 m^6A levels are reduced in adult hakai-2, mta ABI3prom:MTA and their double mutant combination.

Table S1 Summary of oligonucleotides used in this study

Table S2 Summary of splicing events altered in the vir-1 background

Table S3 List of genes with significantly changed expression in the vir-1 root tips as determined by the DESeq software package

Table S4 Summary of gene ontology (GO) analysis of genes misexpressed in the vir-1 mutants.

Table S5 Establishing list of genes required for vascular formation

Table S6 List of genes involved in vascular formation, which show altered expression in the vir-1 root tips

Table S7 TAP-VIR proteomics data

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