Arabidopsis m6A demethylase activity modulates viral infection of a plant virus and the m6A abundance in its genomic RNAs

Mireya Martínez-Pérez1,1, Frederic Aparicio2,2, Maria Pilar López-Gresa3, Jose María Bellés4, Jesus A. Sánchez-Navarro5, and Vicente Pallás3,2

1Instituto de Biología Molecular y Celular de Plantas, Universitat Politècnica de València-Consejo Superior de Investigaciones Científicas, 46022 Valencia, Spain

Edited by George E. Bruening, University of California, Davis, CA, and approved August 22, 2017 (received for review February 23, 2017)

N6-methyladenosine (m6A) is an internal, reversible nucleotide modification present in RNAs of mammals, insects, plants, yeast, and animal viruses that participates in RNA biology through diverse mechanisms such as regulation of mRNA stability (1, 2), translation (3, 4), nuclear export (5), exon splicing (6), and protein/RNA interactions (7).

In mammals, RNA m6A methylation is catalyzed by a polyprotein complex composed of METTL3, METTL14, WAP, KIAA1429, and several cofactors not yet identified (8–10). Removal of the m6A is catalyzed by two RNA demethylases belonging to the AlkB family of nonheme Fe(II)/α-ketoglutarate (α-KG)-dependent dioxygenases, FTO, and ALKBH5 (5, 11). In addition, several proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC, eIF3, and HNRNPC) bind to the m6A-modified mRNAs to control their stability and translation (1, 2, 12, 13).

The internal m6A modification has also been found in viral RNAs (vRNAs) of animal viruses that replicate either in the nucleus or in the cytoplasm, representing a mechanism for the regulation of the viral life cycle (14–19). Silencing of METTL3/14 decreased HIV-1 replication, whereas depletion of ALKBH5 enhanced the export of vRNAs from the nucleus and protein expression, which consequently increased viral replication (15, 16). However, hepatitis C virus (HCV) and Zika virus (ZIKV) infection were positively and negatively regulated by knockdown of METTL3/14 and ALKBH5 or FTO, respectively (17, 19). Further, depletion of YTHDF proteins promoted ZIKA and HCV vRNA expression (17, 19), while in the case of HIV-1, positive (18) and negative (15) effects on HIV-1 vRNA expression have been reported. Furthermore, the host machinery that controls m6A modification detects viral infection and regulates gene expression by modulating the m6A levels of host mRNAs (16, 17).

In contrast to mammals, very few studies on the function of m6A modification have been reported in plants. Transcriptome-wide profiles in Arabidopsis thaliana detected the m6A modification in over two-thirds of the mRNAs (20). A METTL3 homolog in Arabidopsis (MTA) has been identified that plays a critical role in plant development (21, 22). In addition, the Arabidopsis FIP37 protein, a plant homolog of WTAP, interacts in vitro and in vivo with MTA and is essential to mediate m6A mRNA modification of key shoot meristem genes (21, 23). However, no demethylase or YTHFD plant activities have been described at present. The Arabidopsis genome contains 13 homologs of Escherichia coli AlkB (atALKBH1-10B) (24). Although their functional characterization has not been reported, a subcellular localization study showed that all of these proteins display a nucleocytoplasmic localization pattern except atALKBH1D, which localizes to the chloroplast as well, and atALKBH9B, which is exclusively cytoplasmic (24). Interestingly, an AlkB domain has been identified in the ORF of the replicase genes from diverse plant viruses. These domains were found to be functional in removing m6A and m1C modifications from RNA and DNA in vitro, suggesting that the replicate proteins...
cytoplasm, most probably associated with the tonoplast membrane (28). We found that an Arabidopsis knockout mutation of AtALKB9B negatively affects virus accumulation and systemic infection, correlating with increased levels of m6A of the vRNAs. Our results show that the viral genome methylation state plays a key role in the life cycle of a plant virus.

Results

Arabidopsis atALKB9B Interacts with the CP and the Viral RNA of AMV. The CP of AMV is a multifunctional protein that participates in replication, transcription, viral movement, and encapsidation (26, 29), which most probably implies that this protein interacts with host factors involved in diverse cellular functions (30, 31). A yeast two-hybrid (Y2H) screen, using the CP as bait and an Arabidopsis leaf-specific cDNA library as the prey, was performed to identify host proteins that interact with the AMV CP. Several clones containing part of the atALKB9B gene (at2g17970) ORF were found to grow on interaction minimal synthetic selective medium (Fig. S1A). To validate the original Y2H screening, the full-length atALKB9B ORF was fused to the activation domain (pAD plasmid) and transformed into yeast cells expressing the AMV CP fused to the binding domain (pBD plasmid). After growth at 28°C for 5 d on interaction selective medium, we found that the AMV CP specifically interacted with atALKB9B but not with the empty pBD vector or the one expressing the Gal4 binding domain fused with lamin (pBD:LAM) (Fig. 1A). To corroborate this interaction, histidine-tagged atALKB9B (His-atALKB9B) and the N-terminal fragment of YFP (His-NYFP) were expressed in E. coli and purified by Ni-NTA agarose chromatography. Before the elution step, Ni-NTA columns containing histagged proteins were incubated with purified AMV virions. Western blot analysis using anti-CP antibody of eluates after incubation with viral particles showed that virions interacted with atALKB9B but not with NYFP (Fig. S1B). To confirm that the CP–atALKB9B interaction occurs in plants we used bimolecular complementation (BiFC) analysis. Confocal laser scanning microscopy (CLSM) images showed that reconstituted YFP fluorescence formed discrete granules in cells of Nicotiana benthamiana infiltrated with atALKB9B plus AMV CP but not when the host protein was coinfiltrated with the C-terminal fragment of YFP alone (CYFP) (Fig. 1B). Finally, a Northwestern blot assay demonstrated that atALKB9B interacts with viral RNA, showing that the protein has RNA binding activity (Fig. 1C).

Arabidopsis atALKB9B Activity Modulates AMV Infection. To investigate possible roles for atALKB9B in virus infection, we searched for T-DNA insertions in atALKB9B. We identified a homozygous T1 line in the Nottingham Arabidopsis Stock Centre (N671317; SALK_015591, ecotype Col-0) with a T-DNA insertion in exon 4 (Fig. 2A). Absence of atALKB9B mRNA expression was confirmed by RT-PCR with gene-specific primers (Fig. 2B). To determine whether reduced levels of atALKB9B affect virus infection, WT Col-0 and atalkbh9b plants were inoculated with AMV viral particles. Total RNA and proteins were extracted and vRNA and CP accumulation were analyzed at 3 and 6 d post-inoculation (dpi) by Northern and Western blots using digoxigenin-labeled probe to detect the vRNAs (DigAMV) and a specific anti-CP antibody, respectively. We found that levels of both vRNAs and CP were clearly reduced in atalkbh9b compared with WT plants (Fig. 2C and D and Fig. S2), indicating that viral accumulation is impaired in atalkbh9b plants. To analyze AMV systemic movement, total RNA extracted from upper non-inoculated floral stems were blotted onto nylon membranes, and vRNAs were detected with DigAMV. Fig. 2E shows that only 11% of atALKB9B plants were systemically infected at 14 dpi, while this percentage reached 100% in WT plants. Overall, these results indicate that atALKB9B positively regulates AMV infection.

atALKB9B Localizes to Cytoplasmic Bodies. atALKB9B is one of the 13 homologs of E. coli AlkB, and it is the only one that is uniquely located in the cytoplasm (24). To determine more precisely the atALKB9B subcellular localization, we transiently expressed translational fusions with GFP (GFP:atALKB9B and atALKB9B:GFP) by agroinfiltration in N. benthamiana leaves. CLSM images showed that atALKB9B:GFP was localized as a diffuse pattern throughout the cytoplasm, while GFP:atALKB9H was accumulated in small cytoplasmic granules and filaments (Fig. S3). We next performed a sequence similarity analysis, which showed that five Arabidopsis AlkB homologs, including atALKB9B, grouped in the same branch with human ALKBH5 (Fig. S4). Hence, these proteins might be orthologous to the human protein and other prospective m6A demethylases. It has been proposed that m6A methylation functions in the cytoplasm, serving as a reversible tag to direct mRNAs to processing bodies (P bodies) (12). Moreover, several studies have demonstrated that P bodies dynamically associate with siRNAs to processing bodies (P bodies) (12). Moreover, several studies have demonstrated that P bodies dynamically associate with siRNAs to processing bodies (P bodies) (12).
and/or P bodies. To test this hypothesis, we performed colocalization experiments in mock and AMV-infected *N. benthamiana* leaves by coexpressing atALKBH9B with DCP1 (a decapping enzyme located in P bodies) and SGS3 (a component of siRNA bodies) (32, 34) fused to fluorescent proteins. CLSM images showed that, in both healthy and infected tissues, atALKBH9B granules perfectly colocalized with siRNA bodies, whereas ~40% of the atALKBH9B granules were spatially associated with P bodies (Fig. 3A and Fig. S5). These results suggest that atALKBH9B might be a new component of siRNA bodies and P bodies.

Recently, the nonsense-mediated mRNA decay system (NMD) has been proposed to work as viral restriction mechanism in plants (35), and the m6A abundance was quantified by UPLC-PDA-TOF-MS. In this case, vRNAs extracted from AMV particles isolated from WT or atalkbh9b plants were electrophoresed in agarose gels, transferred to nylon membranes, and immunoblotted using the m6A antibody (Fig. 4C and Fig. S6). In parallel, vRNAs were digested to single nucleosides with a single specifically incorporated m6A, followed by digestion to nucleosides and ultra-performance liquid chromatography–photodiode detector–quadrupole/time-of-flight–mass spectrometry (UPLC-PDA-TOF-MS) analysis. We found that GST:atALKB9B almost completely demethylates m6A in the ssRNA substrate (Fig. 4A and B). Therefore, atALKB9B is a protein described in plants with ssRNA m6A demethylation activity.

**m6A Abundance in AMV vRNAs Correlates with Viral Fitness.** Given that atALKB9B has been shown to possess m6A demethylation activity and that its depletion influences AMV infection, we investigated the presence of the m6A modification in AMV vRNAs. Total RNA was purified from *Arabidopsis* WT AMV-infected plants, and an RNA immunoprecipitation assay (RIP) using the anti-m6A antibody and immunoglobulin-A was performed to immunoprecipitate the m6A-modified RNAs. Subsequent hybridization of RIP products with the DigAMV probe clearly detected the presence of the AMV vRNAs (Fig. 4C and D). These results demonstrate that in *Arabidopsis*, adenosine residues in the AMV genome become modified to m6A during the infection process. We next determined whether atALKB9B depletion in atalkbh9b plants modulates m6A levels in the AMV genome. In this case, vRNAs from AMV particles isolated from WT or atalkbh9b plants were electrophoresed in agarose gels, transferred to nylon membranes, and immunoblotted using the m6A antibody (Fig. 6). In parallel, vRNAs were digested to single nucleosides and the m6A abundance was quantified by UPLC-PDA-ToF-MS. The m6A/adenosine ratio (m6A/A) was reduced ~35% in WT

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**Fig. 2.** AMV infection is impaired in atalkbh9b plants. (A) Annotated genomic atALKB9B gene structure showing the exons (gray boxes) and location of the T-DNA insertion (SALK_015591). (B) Agarose gel electrophoresis of RT-PCR products produced with specific primers to amplify the full-length mRNA of the atalkbh9b gene from WT and atalkbh9b plants. The position of the mRNA is indicated on the right. (C and D) Representative Northern and Western blots from inoculated leaves at 3 and 6 dpi of three WT and atalkbh9b plants. Positions of the vRNAs and CP are indicated on the left. Ethidium bromide and Coomassie blue staining of ribosomal RNAs and total protein extracts (rRNA and cb, respectively) were used as RNA and protein loading controls. (E) Dot-blot hybridization of upper noninoculated floral stems to determine the extent of viral systemic movement. Dots in rows a and b correspond to WT plants; dots in c and d correspond to atalkbh9b plants. Samples b6 and d6 are healthy WT and atalkbh9b plants used as negative controls.

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**Fig. 3.** The atALKB9B protein colocalizes with siRNA-body/P-body components in noninfected tissues. (A and B) Confocal laser scanning microscope (CLSM) images of *N. benthamiana* leaf epidermal cells coinfected with the DNA constructs indicated above the images. Overlay panels are the superposition of images from the green and red channels. Arrowheads indicate granules with both proteins. (C) vRNAs accumulation in upf1.5 mutant with respect to WT. SD values are shown. Asterisks indicate significant differences from the WT (**P < 0.01) using the t test (n = 20).

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that the CMV genome contains m^6A as well (Fig. 5A). We then evaluated whether, similar to AMV, atALKB9B activity might influence viral infectivity and/or m^6A modification in CMV vRNAs. To address this, total RNA was isolated from upper floral stems to look for systemic viral movement, and blot hybridization with the DigCMV probe showed that 100% of atALKB9B plants were systemically infected by the virus (Fig. 5B). We also extracted vRNAs from viral particles isolated from WT and atalkbh9b plants infected with CMV. After digestion to single nucleosides, UPLC-PDA-Q/TOF-MS showed no significant differences in the m^6A/A ratios in purified vRNAs from WT compared with atALKB9B plants (Fig. 5C). Therefore, the CMV genome contains m^6A, but the methylation levels of vRNAs and viral infection are not regulated by atALKB9B. We hypothesized that our findings might correlate with a lack of interaction between atALKB9B and the CMV CP; hence, this interaction was investigated by Y2H analysis. Growth on interaction selective medium of yeast cells coexpressing both proteins showed that the viral protein fails to interact with atALKB9B in vivo (Fig. 5D). Interestingly, we found that atALKB9B interacts with the CP of Pnus necrotic ringspot virus (PNRSV) (Fig. 5D), a virus that is functionally and phylogenetically closely related to AMV (37, 38). Unfortunately, since Arabidopsis is not a host for PNRSV, we could not assay the virus infectivity in atalkbh9b plants. This finding suggests that atALKB9B may regulate the viral life cycle of other Bromoviridae family members.

**Discussion**

In the last few years, m^6A modification has emerged as an important mechanism to regulate mRNA biology (1, 2, 4–7). Components of this regulatory system (methyltransferases, demethylases, and

![Fig. 4. atALKB9B catalyzes demethylation of m^6A in ssRNA in vitro and modulates methylation of vRNAs. (A) Representative UPLC-PDA-Q/TOF-MS chromatogram showing the retention times of the nucleosides adenosine (A) and N^6^-methyladenosine (m^6A) after incubation of the m^6A-containing ssRNA substrate with GST:atALKB9B and GST as the negative control. The peak (G) corresponds to the nucleoside guanosine. The peak denoted as (*) could not be unequivocally identified although it was determined to present a molecular weight of 343 with a maximum absorption at 261 nm. (B) Graph representing the demethylation activity in three independent experiments. (C–E) Genomic AMV RNAs are m^6A hypermethylated in atalkbh9b plants. (C) RIP of vRNAs with a specific anti-m^6A antibody. Total RNA extracted from WT plants infected with AMV was incubated with anti-m^6A plus IgA or IgA alone. Dilutions of the immunoprecipitated RNAs (indicated on top) were blotted on nylon membranes and the vRNAs were detected with DigAMV. (D) Average ratios of m^6A in vRNAs obtained by quantification of m^6A on three different Northern blots from AMV-infected WT and atalkbh9b plants. (E) Graph showing the average m^6A/A ratios obtained by UPLC-Q-Tof-MS after digestion of vRNAs extracted from viros purified from AMV-infected WT and atalkbh9b plants. In B, D, and E error bars represent the SEM and asterisks indicate significant differences from the WT (*P < 0.05). Compared with atalkbh9b plants (Fig. 4E), indicating that depletion of atALKB9B correlates with hypermethylation of the AMV vRNAs.

Finally, we performed a methylated RNA immunoprecipitation sequencing (MeRIP-seq) experiment to map m^6A sites within the AMV genome. For this, vRNAs extracted from viral particles isolated from atalkbh9b AMV-infected plants were immunoprecipitated with an m^6A-specific antibody and RNAs from input, bead-only control and MeRIP-seq samples were used to generate RNA sequence libraries. We identified six discrete peaks distributed along the AMV genome, which contained three of the common m^6A consensus motifs [(G,A,U)/(G,A)/AC/(A,C,U)], [(A,C)/G/A/C/(G,U) and [UGAC] (Fig. S7).

m^6A is Present in the Genomic RNAs of Other Members of the Bromoviridae Family. Since m^6A appears to regulate AMV infection, we asked whether other viruses in the Bromoviridae family might also be influenced by this modification. We chose cucumber mosaic virus (CMV), the type member of the genus Cucumovirus, which infects Arabidopsis and is not closely related to AMV. First, the presence of m^6A was examined in CMV vRNAs by RIP from total RNA of WT CMV-infected plants using the m^6A antibody. Hybridization of RIP products with the DigCMV probe showed
protein effectors) have been identified in mammals and yeast (12). Several recent studies have shown that m6A modification is also a conserved feature of mRNA in plants (20, 39), and that it plays a critical regulatory role in plant development (21–23). However, as far as we know, only two components of the methylase complex, MTA and FIP37, have been identified at present (21, 23). In this work, using a biochemical test, we demonstrate that atALKBH9B possesses m6A demethylase activity toward single-stranded RNA in vitro, and similar to the results of a previous study, we found that this protein localizes exclusively to the cytoplasm (24). Several studies have previously reported the presence of the cellular machinery controlling m6A modification in the cytoplasm of mammalian cells (17, 19). Thus, atALKBH9B may be a new component of the cellular machinery controlling N6 methylation of adenosine in plant mRNAs, and similar to the situation in mammals, m6A modification could take place in the cytoplasm after the export of mRNAs from the nucleus.

A detailed examination showed that atALKBH9B forms discrete granules either in healthy or infected tissues that colocalize with SG3 and UPF1, and some of these granules presented a spatial association with DCPI. SG3 and DPC1 are components of siRNA bodies and P bodies, respectively (33, 34), whereas that modification could take place in the cytoplasm after the export of viruses belonging to the Flexiviridae family, the AMV genome lacks the AlkB domain, so the virus may have the ability to usurp this host function for its long-term accumulation.

We found that the CMV genome also contains m6A, but differs from AMV in that neither m6A vRNA abundance nor virus infection is modified in atalklkh9b plants compared with WT plants. Remarkably, the CMV CP did not interact with atALKBH9B in vivo. An important difference between AMV and CMV is that, whereas the latter can replicate in the absence of its CP (43), the CP of AMV is a multifunctional protein that interacts with a variety of host factors and is indispensable for replication and translation (26–29). Thus, it may be possible that the interaction between atALKBH9B and the CP is essential to usurp atALKB9B activity.

But on the other hand, the Arabidopsis genome encodes five putative demethylase orthologs, so a protein different from atALKB9B could very well participate in the CMV m6A regulation process. In fact, it has been reported that depletion of FTO negatively affects HCV infection, while depletion of ALKBH5 has no effect on the HCV cycle (19). Finally, we cannot rule out the possibility that m6A modification does not influence CMV viral infection per se.

Materials and Methods

Analysis of GST atALKB9B-Viral RNA Interaction by Northwestern Assay. Dilutions of GST or GST:ALKB9B purified proteins were electrophoresed in 12% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated overnight at 4 °C (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl, 0.0005% Triton X). After two washes of 5 min each with the same buffer, membranes were incubated with 20 mL of buffer B (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl) containing 50 ng/mL of the AMV RNA 4 labeled with digoxigenin for 2 h at 25 °C. Then digoxigenin detection procedures were carried out as detailed in SI Materials and Methods.

BIFC and Subcellular Localization Study. atALKB9B and atUPF1 ORFs were amplified with specific primers designed for cloning using the Gateway System (Invitrogen) and recombined into binary destination vectors expressing the fluorescent proteins mCherry or GFP for subcellular localization studies and the N-terminal part of the YFP for BIFC analysis, following manufacturer recommendations (GFP atALKB9B, atALKB9B:GFP, NYPF atALKB9B, atUPF1: GFP). For details, see SI Materials and Methods.

Virus Isolation and Viral Genomic RNA Purification and Rip Using m6A Antibody. Virus purification was performed following PEG protocol with some modifications (SI Materials and Methods). For vRNAs purification, pellets were directly resuspended in 1 mL of Ribozol reagent (Ambroset) and RNA extraction was carried out following manufacturer recommendations. RNAs with m6A modification were immunoprecipitated as previously reported with some modifications as detailed in SI Materials and Methods.

Purification of atALKB9B Protein. atALKB9B was subcloned into pGEX-KG (GE Healthcare Life Sciences) to generate a construct with atALKB9B fused to the C-terminal part of the GST. GST and GST atALKB9B proteins were expressed in BL21 (DE3) E. coli cells and purified with glutathione sepharose 4b beads (GE Healthcare Life Sciences) following manufacturer recommendations. All of the protein purification procedures were performed at 4 °C.

In Vitro m6A Demethylation Assays. The m6A demethylase activity assay was performed by incubating 2.5 μg of GST or GST:ALKB9B proteins and 1 μg of m6A monomethylated siRNA (Dharmacon, Inc.) oligonucleotide for 3 h at 25 °C in a reaction mixture containing 50 mM of Hepes buffer (pH 7.0), 10 μM α-ketoglutarate, 100 μM l-ascorbic acid ascorbate, 20 μM α-ketoglutarate, 100 μM l-ascorbic acid ascorbate, 20 μM α-ketoglutarate, 100 μM l-ascorbic acid ascorbate, 20 μM α-ketoglutarate, 100 μM l-ascorbic acid ascorbate, 20 μM

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PNAS  October 3, 2017  vol. 114  no. 40  10759

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MeRip-Seq. Immunoprecipitation of m^6^A RNA fragments was performed using 200 μg of purified vRNAs as previously described (44). For details, see SI Materials and Methods.

Acknowledgments. We thank L. Corachan for her excellent technical assistance, Dr. Emilio Martínez de Alba and Dr. Christophe Rizenthaler for kindly providing GFP-PSG33 and DCP2:mcCherry plasmids, Prof. John W. S. Brown for providing a plasmid containing the ORF of atUP1, and the Bioinformatics Core Service at the Instituto de Biología Molecular y Celular de Plantas (IBiMP) for the support provided in the data analysis. UPLC-PDA-Q/TOF-MS analyses were performed by the Metabolite Analysis Department of the IBiMP. P.C. and M.M.-P. were recipients of Contract RYC-2010-06169 from the Ramón y Cajal Program of the Ministerio de Educación y Ciencia, and Pre-doctoral Contract FPI-2015-072406 from the Subprograma FPI-MINECO (Formación de Personal Investigador–Ministerio de Economía y Competitividad), respectively. This work was supported by Grant BIO2014-54862-R from the Spanish granting agency Dirección General de Investigación Científica y Técnica and the Prometeo Program (GV2015/010) from the Generalitat Valenciana.