Mapping of Functional Subdomains in the atALKBH9B m⁶A-Demethylase Required for Its Binding to the Viral RNA and to the Coat Protein of Alfalfa Mosaic Virus

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N⁶-methyladenosine (m⁶A) modification is a dynamically regulated RNA modification that impacts many cellular processes and pathways. This epitranscriptomic methylation relies on the participation of RNA methyltransferases (referred to as “writers”) and demethylases (referred to as “erasers”), respectively. We previously demonstrated that the Arabidopsis thaliana protein atALKBH9B showed m⁶A-demethylase activity and interacted with the coat protein (CP) of alfalfa mosaic virus (AMV), causing a profound impact on the viral infection cycle. To dissect the functional activity of atALKBH9B in AMV infection, we performed a protein-mapping analysis to identify the putative domains required for regulating this process. In this context, the mutational analysis of the protein revealed that the residues between 427 and 467 positions are critical for in vitro binding to the AMV RNA. The atALKBH9B amino acid sequence showed intrinsically disordered regions (IDRs) located at the N-terminal part delimiting the internal AlkB-like domain and at the C-terminal part. We identified an RNA binding domain containing an RGxxxRGG motif that overlaps with the C-terminal IDR. Moreover, bimolecular fluorescent experiments allowed us to determine that residues located between 387 and 427 are critical for the interaction with the AMV CP, which should be critical for modulating the viral infection process. Finally, we observed that atALKBH9B deletions of either N-terminal 20 residues or the C-terminal's last 40 amino acids impede their accumulation in siRNA bodies. The involvement of the regions responsible for RNA and viral CP binding and those required for its localization in stress granules in the viral cycle is discussed.

Keywords: N⁶-methyladenosine, RNA covalent modifications, RNA-binding proteins, plant viruses, alfamovirus, RNA demethylases, epitranscriptomics, ALKBH

INTRODUCTION

The addition of a methyl group to the N⁶ position of adenosine (m⁶A) is the most abundant internal modification in eukaryote mRNAs (Boccaletto et al., 2018; Covelo-Molares et al., 2018; Arribas-Hernández and Brodersen, 2020; Zhou et al., 2020). It regulates many steps of RNA metabolism, including splicing (Zhao et al., 2014), stability (Wang et al., 2014), translation
(Meyer et al., 2015), nuclear-export (Zheng et al., 2013), RNA structures (Bayoumi et al., 2020), and protein/RNA interactions (Liu et al., 2015). Also, it modulates the epigenetic effects of some non-coding RNAs (ncRNA) (Meyer and Jaffrey, 2017). Since the 1970s, m6A modification has been known to tag not only cellular RNAs but also RNAs of multiple viruses (Wei and Moss, 1975; Furuchi et al., 1976; Krug et al., 1976; Dimock and Stoltzfus, 1977), although its functional relevance has remained elusive mainly due to the lack of efficient methods of m6A detection and subsequent analysis. Recent studies have demonstrated the crucial roles of m6A in the virus–host interactions; however, most of these studies have focused on animal viruses (Dang et al., 2019; Williams et al., 2019; Arribas-Hernández and Brodersen, 2020), whereas it remains very limited in plant viruses (Martínez-Pérez et al., 2017; Li et al., 2018; Arribas-Hernández and Brodersen, 2020).

In mammals, m6A methylation is catalyzed co-translationally by a multicomponent m6A methyltransferase complex (MTC, also known as “writer”) (Liu et al., 2014; Ping et al., 2014). The core component of MTC is a ~200 kDa heterodimer comprised of METTL3 and METTL14 (Huang et al., 2020). Other regulatory subunits of MTC have also been identified, including WTAP and its cofactors KIAA1429 (human protein), respectively, have been identified (ECT1-11; Evolutionarily Conserved C-Terminal Region 1–11, At4g11970 and the Cleavage and Polyadenylation Specificity Factor 30). The Arabidopsis genome has been described to encode more than 200 putative RNA-binding proteins (RBP) (Lorković and Barta, 2002; Abbasi et al., 2011; Ambrosone et al., 2012). RBPs are key factors in post-transcriptional gene regulation, protein synthesis, viral replication, cellular defense, and developmental regulation (Terribilini et al., 2006; Glisovic et al., 2008; Pallas and Gómez, 2013; Prall et al., 2019). RBPs are often modular and contain one or more conserved RNA-binding domains (RBD) (Lee et al., 2012; Re et al., 2014). RNA recognition motifs (RRM) and the K homology (KH) domain are the most abundant structural motifs in euarkocytes (Lorković and Barta, 2002; Chen and Varani, 2013). Other RBDs include the glycine-rich motif (GRM), the double-stranded RNA binding domain (dsRBD), DEAD- Box-, PUF, SAM-, ZnF-domains (Lunde et al., 2007; Re et al., 2014; Lee and Kang, 2016), and the RGG/RG motif (Thandapani et al., 2013). However, the majority of residues predicted to be in the protein-RNA interface are not part of a characterized RBDs (Terribilini et al., 2006). In many cases, intrinsically disordered regions (IDRs) have been identified in proteins that do not display characterized RNA-binding sites (Calabretta and Richard, 2015; Varadi et al., 2015). Among other roles, IDRs participate in protein-protein and protein-RNA interactions and are enriched in “disorder-promoting amino acids” such as G, P, or R (Gsponer and Madan Babu, 2009). In this context, IDRs can encompass diverse functional motives such as RNA binding motifs or low-complexity (LC) domains (Castello et al., 2012). These proteins often go through binding induced-folding. Thus, as a consequence of their structural flexibilty, the RNA-protein interactions can experiment conformational changes in the protein structure, the RNA or both (Frankel, 1999). Additionally, proteins containing IDRs promote liquid-liquid phase separation in the assembly and degradation of RNA granules such as stress granules or P-bodies (Spector, 2006; Lin et al., 2015). Similar to what was observed in mammals, some Arabidopsis m6A readers, such as ECT2, ECT3, and ECT4 present IDRs and can form cytoplasmic granules (Scutenaire et al., 2018; Arribas-Hernández et al., 2020). Moreover, ECT2 was found to undergo a gel-like phase transition in vitro (Arribas-Hernández et al., 2018).

Alfalfa mosaic virus (AMV) belongs to the Bromoviridae family and, like the rest of the members of this family, its genome consists of three single-stranded RNAs of plus polarity (Bujarski et al., 2019). RNA1 and RNA2 encode the replicase subunits (P1 and P2), whereas RNA3 encodes the movement protein (MP) and serves as a template for the synthesis of subgenomic RNA4 (sgRNA4), which encodes the coat protein (CP) (Boi, 2005; Pallas et al., 2013). We previously demonstrated that atALKBH9B is a key factor in AMV infection since the suppression of the atALKBH9B m6A demethylation activity reduces viral accumulation. Moreover, it was shown that the CP of this virus interacts with atALKBH9B, pointing to a direct subversion of an endogenous regulatory pathway by the virus (Martínez-Pérez et al., 2017). Due to the functional relevance...
of atALKBH9B, the first m^6A demethylase described in plants, we carried out a functional mapping of the protein to identify putative domains implicated in the diverse interactions of this m^6A-demethylase with both the viral RNA and the coat protein.

RESULTS

In vitro Mapping of the RNA-Binding Domain of atALKBH9B

In a previous study, we demonstrated that atALKBH9B interacts with the viral RNA, although the kinetic parameters of this interaction, as well as the identification of the RBD, were not analyzed (Martínez-Pérez et al., 2017). To estimate the capacity of the wild-type atALKBH9B to bind the viral RNA, we first conducted Electrophoretic Mobility Shift Assays (EMSA) by incubating a constant amount (5 ng) of an RNA transcript, corresponding to the 3′ untranslated region of AMV-RNA3 (3′ UTR-RNA3), with increasing concentrations of the glutathione S-transferase fusion protein (GST:atALKBH9Bwt) (Figure 1). We chose this part of the RNA3/4 molecule because it is the same as the one that specifically binds CP, allowing us to directly compare it with this specific interaction. The decrease in the chemiluminescent signal intensity corresponding to free RNA was evident at quantities exceeding 400 ng of GST:atALKBH9Bwt (lane 7, Figure 1A), suggesting the formation of a protein–RNA complex. At this point, it is important to note that the non-radioactive EMSA described here (see section “Material and Methods”) requires the transfer to a nylon membrane step to detect both free RNA and the corresponding ribonucleoprotein (RNP) complex. When a large amount of molecules binds to the RNA, as in a non-sequence specific interaction, the transfer of the RNP complex to the nylon membrane and its posterior detection is difficult or even impossible (Marcos et al., 1999; Herranz and Pallás, 2004; Salavert et al., 2020). In any case, the disappearance of the free RNA band is evidence of complex formation (Carey, 1991) and was therefore quantified by film densitometry to calculate the apparent constant dissociation (Kd) of the RNA–GST:atALKBH9Bwt interaction from the linear regression of the mean values from at least three technical replicates (Marcos et al., 1999). The Kd value of GST:atALKBH9Bwt was estimated to be 0.30 µM (Figure 1B).

In order to determine the region of atALKBH9Bwt with RNA-binding activity, we first designed GST:atALKBH9B deletion mutants lacking 160 amino acids at either the N- or C-terminal part (Δ160Nt and Δ160Ct, respectively), or 187 amino acids in the internal region of the protein (Δ187Int) (Supplementary Figure 1A). Northwestern blot assays using AMV-sgRNA4 labeled with digoxigenin showed a complete loss of RNA-binding capacity of GST:atALKBH9BΔ187Int and GST:atALKBH9BΔ160Ct proteins (Supplementary Figure 1B). Thus, we designed new GST:atALKBH9B deletion mutants affecting the internal and/or C-terminal regions: GST:Δ258Nt; GST:Δ258Nt/Δ80Ct; GST:Δ258Nt/Δ40Ct; GST:Δ258Nt/Δ20Ct and GST:Δ387Nt (Figure 2A). Northwestern blot assays showed that only Δ258Nt/Δ80Ct does not bind RNA, indicating that residues between positions 427 and 467 are critical for RNA-protein interaction (Figure 2B). To confirm this observation, we evaluated the RNA-binding activity of a mutant lacking residues from 427 and 467 (GST:ΔRBD) and one comprising only the atALKBH9B 427–467 residues (GST:RBD) (Figure 2A). As shown in Figure 2B, only GST:RBD retained the RNA-binding capacity.

To validate the northwestern blot assays, EMSAs were performed with GST:atALKBH9Bwt, GST:Δ160Nt, GST:Δ258Nt; GST:Δ258Nt/Δ80Ct; GST:Δ258Nt/Δ40Ct and GST:Δ258Nt/Δ20Ct by incubating the viral RNA with different protein concentrations. As expected, EMSA showed a decrease in chemiluminescent signal intensity corresponding to the free viral RNA in mutants containing the RBD (Supplementary Figures 2A,B), whereas those lacking the predicted RBD did not reveal RNA binding (GST:Δ258Nt/Δ80Ct). Additionally, we studied the affinity and specificity of the atALKBH9B RBD–RNA interaction by analyzing the shift in electrophoretic mobility of the 3′UTR-RNA3 (AMV) in the presence of GST:RBD fusion protein. As shown in Figure 3, increasing concentrations of GST:RBD diminished the amount of free RNA that becomes undetectable at 450 ng of the protein. A Hill transformation was used to analyze our data (Figure 3B). The Kd value of the RBD fused to GST was 0.64 µM, indicating slightly less RNA-binding than the full-length protein (GST:atALKBH9Bwt). From the linear regression adjustment, a Hill coefficient c of 2.4 was obtained; this value above 1 (c = 1 indicates no cooperativity) would be taken as an indication of positive cooperativity.
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Subsequently, we used PSI-BLAST (Altschul et al., 1997) to compare atALKBH9B RBD with other RNA binding proteins (RBP) from the databank, but the alignment of sequences did not reveal any significant similarities. Nonetheless, a high percentage of IDRs has been reported in viral, prokaryotic, and eukaryotic RBPs (Varadi et al., 2015). Therefore, we evaluated whether atALKBH9B presented these IDRs, using PrDOS1 (Ishida and Kinoshita, 2007). The results showed that 45.4% of all amino acids of atALKBH9B form IDRs through both N- and C-terminal regions, including most RBD (aa 427–467) (Figure 4). Remarkably, this RBD is enriched in G and R residues (20% and 15%, respectively), which are also common in the aforementioned IDRs. It is worth noting the presence of a RGxxxRGG motif and two extras RG residues that have been described in several RBPs showing characteristic disorder regions (Thandapani et al., 2013).

Taken together, these results indicate that atALKBH9B efficiently cooperatively binds viral RNA through an RBD that has the characteristics of the IDRs present in other viral and eukaryotic RBPs.

**In vivo Mapping of atALKBH9B Binding Domain to AMV CP**

We previously showed that the demethylase activity of atALKBH9B affected the infectivity of AMV but not of cucumber mosaic virus (CMV), correlating with the ability of atALKBH9B to interact (or not) with their coat proteins (Martínez-Pérez et al., 2017). Since this interaction is, thus, critical for the proviral function of atALKBH9B, we decided to delineate the domain of atALKBH9B involved in the host-protein/viral-protein interaction using the Bimolecular Fluorescent Complementation (BiFC). For this purpose, we first designed three mutants corresponding to the N-terminal, central and C-terminal regions, fused to the N- and C-terminal parts of the YFP (Figure 5A, left panel [1]). These constructs were co-infiltrated with the corresponding N−C−YFP:CP proteins in Nicotiana benthamiana leaves, and fluorescence was examined by confocal laser scanning microscopy (CLSM) after 48h.

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1http://prdos.hgc.jp/cgi-bin/top.cgi

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**FIGURE 2** Analysis of the RNA-binding activity of purified GST:atALKBH9B proteins by Northwestern blot assay. (A) GST fusion proteins containing Internal or C-terminal deletions of atALKBH9B were produced in a prokaryotic system. (B) Duplicated membranes with GST:atALKBH9B wt (lane 1) or its mutant variants (lanes 2–8), as depicted in (A), were resolved by 12% SDS–PAGE and, after transferring to nitrocellulose, incubated with 5 µg of AMV-sgRNA4 labeled with digoxigenin. The left panel shows a Coomassie blue-stained gel, and the right panel shows results from Northwestern blot analysis. Positions of full-length GST:atALKBH9B proteins are indicated by arrows. Asterisks indicate GST:atALKBH9B fusion proteins which interact with the AMV-sgRNA4. The positions of molecular mass markers (in kDa) are shown on the left side.
Our previous studies determined that ALKBH9B that is exclusively localized in the cytoplasm (Mielecki et al., 2012). The folding of the protein depends on the correct localization, which could be explained assuming that the proper three-dimensional folding of the protein might be a critical requirement to form the cytoplasmic granules and consequently to colocalize with siRNA bodies.

Leaves co-infiltrated with CYP-9B_{wt} plus N-YFP:CP, CYP-9B_{A160Nt} plus N-YFP:CP, and CYP-9B_{A187Int} plus N-YFP:CP rendered a strong YFP fluorescence signal in the cells, whereas no reconstituted YFP fluorescence was detected in leaves co-infiltrated with the pair CYP-9B_{A160Ct} plus N-YFP:CP (Figure 5B and Supplementary Figure 3, upper panels).

To further delimit the domain involved in atALKBH9B/AMV-CP interaction, we designed new N-CYP fusion mutants by deleting residues located at the C-terminal region of atALKBH9B (Figure 5A, right panel [II]). Leaves co-infiltrated with CYP-atALKBH9B_{A320Ct} plus N-YFP:CP, CYP-atALKBH9B_{A340Ct} plus N-YFP:CP, and CYP-atALKBH9B_{A360Ct} plus N-YFP:CP produced a reconstituted YFP fluorescence signal in the cells, whereas no YFP fluorescence was detected in leaves co-infiltrated with the pair CYP-atALKBH9B_{A320Ct} plus N-YFP:CP (Figure 5B and Supplementary Figure 3, lower panels). Finally, western blot assays using anti YFP and N-YFP antibodies confirmed that all atALKBH9B mutated versions fused to the YFP and the N-YFP:CP accumulated at detectable levels in the co-infiltrated tissues (Supplementary Figure 4). Overall, our results suggest that amino acids located between positions 387 and 427 of atALKBH9B are critical for the interaction with the AMV-CP.

**The Subcellular Localization of atALKBH9B Depends on the Correct Folding of the Protein**

atALKBH9B is the only protein of the 14 homologs of E. coli AlkB that is exclusively localized in the cytoplasm (Mielecki et al., 2012). Our previous studies determined that atALKBH9B can specifically colocalize with SGS3 protein (a component of siRNA bodies), forming biomolecular-condensates associated with stress granules (Martínez-Pérez et al., 2017).

To identify the region of the protein involved in this subcellular localization, we performed localization experiments in N. benthamiana leaves by expressing a series of deletion mutants of atALKBH9B fused to the GFP (Figure 6A).

Thus GFP:atALKBH9B mutants were transiently co-expressed by agroinfiltration with mCherry:SGS3. As expected, full length atALKBH9B co-localized with SGS3 (Figure 6B, upper panels) whereas that only atALKBH9B with a deletion of the C-terminal 20 residues (Figure 6B panel GFP:atALKBH9B_{A20Ct}) accumulated in cytoplasmic granules colocalizing with SGS3. In contrast, deletion of the N-terminal 20, C-terminal 40 or internal 186 residues (Figure 6A; GFP:atALKBH9B_{A20Nt}, GFP:atALKBH9B_{A40Ct} and GFP:atALKBH9B_{A186Int}) resulted in proteins showing a diffuse pattern throughout the cytoplasm (Figure 6B). These results indicated that different regions of the atALKBH9B sequence are involved in its subcellular localization, which could be explained assuming that the proper three-dimensional folding of the protein might be a critical requirement to form the cytoplasmic granules and consequently to colocalize with siRNA bodies.

**DISCUSSION**

The study of RNA methylation in plants has emerged as an important cellular process of gene regulation in development (Wan et al., 2015; Ržička et al., 2017), response to abiotic stress (Li et al., 2014), and antiviral defense (Martínez-Pérez et al., 2017). Recently, atALKBH9B, atALKBH10B, and atALKBH6 were described as m^6^A erasers involved in AMV infection, flowering time in *Arabidopsis*, and growth and abiotic stress responses, respectively (Duan et al., 2017; Martínez-Pérez et al., 2017; Huong et al., 2020).

To dissect the functional activity of atALKBH9B in plant-virus infection, a protein-mapping analysis was carried out to identify putative domains required to regulate this process. In this context, by mutagenesis and northernwestern analysis, we analyzed the ALKBH9B RNA binding activity and delimited the region between residues 427 and 467 as critical for binding *in vitro* of sgRNA4 (AMV). Moreover, EMSA analysis led us to determine that the K_d of the binding of the RBD alone (0.64 µM, Figure 3) was slightly higher than the one obtained with the full-length protein (0.30 µM, Figure 1), evidencing that other domains of the protein favor the binding to the viral RNA. Nonetheless, the RBD K_d value obtained is within the range reported for other plant viruses RBPs, such as Nla from Tobacco etch virus -TVE-, Pst MP of Carnation mottle virus (0.7 µM) (Marcos et al., 1999), AMV CP (0.5 µM) (Baer et al., 1994), or the MP of prunus necrotic ringspot virus (Herranz and Pallás, 2004). Additionally, Huong et al. (2020) showed the RNA-binding capabilities of atALKBH6, although their biochemical parameters were not determined.
Visual inspection and computational analysis of the atALKBH9B sequence revealed no obvious structured RBD that could justify the RNA-binding properties described above. However, recently Varadi et al. (2015) demonstrated the prevalence of IDRs in RNA binding proteins and domains. Among other functions, IDRs are implicated in protein–protein and RNA–protein interactions (Castello et al., 2012). In fact, IDRs are a type of domain that is frequently found in proteins that undergo liquid–liquid phase separation (LLPS), a process that likely contributes to the formation and stability of RNA granules (Alberti et al., 2019). This has been demonstrated in YTHDF1, YTHDF2, and YTHDF3, m$^6$A-binding proteins, which undergo LLPS in the presence of poly methylated mRNAs. The resulting mRNA-YTHDF complexes form P-bodies and stress granules (Ries et al., 2019). Additionally, IDRs have been found to encompass diverse functional motives, e.g., well-established RNA binding activity such as RGG/RG and YGG motives, or low-complexity (LC) domains (Castello et al., 2012; Alberti et al., 2019). Both the conformational flexibility and the establishment of extended conserved electrostatic interfaces with RNAs have been proposed to provide the capability of the IDRs to specifically target different RNAs (Varadi et al., 2015). Furthermore, many proteins localized in RNA granules contain IDRs encompassing prion-like LC domains required for RNA granules assembly (Gilks et al., 2004; Reijns et al., 2008). The atALKBH9B RBD identified here is enriched with G and R (20% and 15%, respectively) and presents an RGxxxRGG motif between positions 469 and 465 (Figure 1A) or a Tri-RG motif present in proteins implicated in various cellular processes like RNA biogenesis, DNA damage signaling, and mRNA translation (Thandapani et al., 2013). Interestingly, we found that 45.4% of atALKBH9B amino acid sequence forms IDRs, located at the N-terminal part, delimiting the internal AlkB-like domain located between positions 216 and 411 and at the C-terminal part. In fact, around 77.5% of the RBD is contained in the C-terminal disordered region (Figure 4). Furthermore, the C-terminal IDR
of atALKBH9B exhibits two additional RG residues at positions 473–474 and 466–497, respectively (Figure 4).

Our results can be explained considering that the RGxxxRGG motif between positions 459 and 466 plays a critical role in the protein–RNA interface. This interaction might induce the formation of a flexible structure permitting additional contacts through RG residues at positions 473–474 and 496–497 and YG residues at 506–507, enhancing the binding affinity and specificity of the interaction. For example, the splicing factor Tra2-β1 presents IDRs in the N- and C-terminal regions of the RRM. In the interface protein–RNA, this region adopts a folded structure, forming extensive contacts (Cléry et al., 2011). On the other hand, we previously reported that atALKBH9B is exclusively cytoplasmic, forming discrete granules which colocalize with siRNA bodies, and some are associated with P bodies (Martínez-Pérez et al., 2017). Here we show that deletion of the first N-terminal 20 residues or the C-terminal last 40 amino acids impedes its accumulation in siRNA bodies rendering a diffuse cytoplasmic pattern (Figure 6B). Interestingly, the deleted atALKBH9B N- and C-terminal parts are predicted to form IDRs, and the C-terminal is rich in Y and S residues, which would participate in the RNA granules formation. In this sense, mutation of LC domains in hnRNPA2 and FUS reduced the efficiency of their recruitment in hydrogel polymers in vitro (Xiang et al., 2015) and stress granules (SG) in cells (Kato et al., 2012), respectively. Moreover, phosphorylation of the SG-nucleating protein G3BP within its IDR (Ser 149) impaired its ability to induce the formation of SGs (Kedersha et al., 2016).

Finally, we found that amino acids located between positions 387 and 427 of atALKBH9B are critical for CP–AMV interaction (Figure 5 and Supplementary Figure 3). Interestingly, this region is located next to the RNA-binding site, but it is
**FIGURE 6** | Subcellular localization of atALKBH9B proteins fused with GFP in infiltrated leaves (A) Schematic representation of the different GFP:atALKBH9B fusion proteins used. (B) CLSM images of N. benthamiana leaf epidermal cells co-infiltrated with agrobacterium expressing constructs indicated in (A) plus the SGS3 protein fused to the mCherry fluorescent protein (mCherry:SGS3). Scale bar = 20 µm.
may be possible that the CP binds (Bol, 2005; Guogas et al., 2005; Herranz et al., 2012), it protein indispensable for the viral replication and translation (Martínez-Pérez et al., 2017), and the CP is a multifunctional like binding are located at the protein C-terminal, the former partly for its localization in stress granules. CP-binding and RNA responsible for RNA and viral CP binding and those required in benefit of the virus.

modulate the vRNA binding and the m$^6$A at at

FIGURE 7 | atALKBH9B multi-domain structure. Predicted IDRs, between positions 1–12, 48–101, 104–187, 280–288, and 437–507, are shown in red. AlkB-like domain, located at 216–411, is shown in gray. Black boxes in the AlkB-like domain delimit the 2-OG stabilizing motif (NkY: 324–326), motif involved in binding to iron -Fe H- (H: 335–340) and the substrate specificity motif (RxxooxF: 335–411) [Alignment between the atALKBH9B and hsALKBH5 proteins, using the BLAST tool]. The AMV CP binding domain (CP-bd: 387–427) and RNA binding domain (RBD: 427–467) are shown in yellow and blue. RGxxxxRGG motif between positions 469 and 466 within the RBD characterized here is shown in green.

not part of the C-terminal IDR (Figure 4). Considering that atALKBH9B binds RNA to remove m$^6$A-modification (Martínez-Pérez et al., 2017), and the CP is a multifunctional protein indispensable for the viral replication and translation (Bol, 2005; Guogas et al., 2005; Herranz et al., 2012), it may be possible that the CP binds atALKBH9B in order to modulate the vRNA binding and the m$^6$A demethylase activity in benefit of the virus.

In summary, we have mapped the atALKBH9B regions responsible for RNA and viral CP binding and those required for its localization in stress granules. CP-binding and RNA binding are located at the protein C-terminal, the former partly overlapping the AlkB-like domain, whereas the RBD is partially embedded in the predicted IDR located at the C-terminal (Figure 7). Thus, as found in other proteins (Protter and Parker, 2016), atALKBH9B IDRs and the RBD could act cooperatively to promote the formation of RNA granules. This follows the role of both IDRs and folded domains in mediating RNA binding and oligomerization by acting together with RNAs to produce and maintain these granules (Jonas and Izaurralde, 2013; Protter and Parker, 2016). Therefore, although our results reinforce the existence of this cooperativity in RNA granules formation, the mechanisms underlying IDR-folded domain cooperativity and their potential regulation need further examination.

MATERIALS AND METHODS

Protein Expression and Purification in Bacteria

Full-length atALKBH9B ORF and deletion mutants were subcloned into pGEX-KG (GE Healthcare Life Sciences) to generate a construct with 9B merged to the C-terminal part of the GST. GST and GST:atALKBH9B fusion proteins were expressed in BL21 (DE3) E. coli cells and purified with glutathione Sepharose 4B beads (GE Healthcare Life Sciences) according to the manufacturer’s recommendations. All protein purification procedures were performed at 4°C.

Nucleic Acid-Binding Assay by Northwestern Blot and EMSA

Dilutions of GST or GST:atALKBH9B purified proteins were electrophoresed in 12% SDS/PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C in Renaturing Buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl, 0.05% Triton X-100, 1X Blocking Reagent, Roche). After this, membranes were incubated with 20 µL of the same buffer containing 50 ng/µL of the AMV sgRNA 4 labeled with digoxigenin for 3 h at 25°C. For the EMSA assay, 5 ng of the 3′UTR of AMV RNA 3 transcripts were heated for 5 min at 85°C and cooled at room temperature for 15 min. Different amounts of purified GST:atALKBH9B fusion proteins were added and incubated for 30 min at 4°C in a 10-µL final volume of Union Buffer (100 mM Tris-HCl pH 8.0, 1 M NaCl, 8 units of Ribolock RNase inhibitor, Thermo Fisher Scientific). Following incubations, the samples were separated through 1.2% agarose. RNAs were transferred to positively charged nylon membranes (Roche). RNAs were visualized on blots using DIG-labeled riboprobes corresponding to the 3′UTR of AMV RNA 3. Synthesis of the digoxigenin-labeled riboprobes, hybridization and digoxigenin-detection procedures were carried out as described in (Pallás et al., 1998).

Bimolecular Fluorescence Complementation and Subcellular Localization Study

N–CYFP:atALKBH9B (wild-type and deletion mutants); GFP:atALKBH9B and mCherry:SGS3 fusion proteins were cloned using the Gateway System (Invitrogen) according to the manufacturer’s recommendations. Plasmid expressing the CP$_{AMV}$ merged to the N, or C-terminal part of the YFP (N–C–YFP:CP$_{AMV}$), was previously described in Aparicio et al. (2006). All binary vectors were transformed into Agrobacterium tumefaciens C58 cells. Pairs of cultures carrying specific fusion proteins were mixed at an optical density of 0.5 each in infiltration solution (10 mM MES, pH 5.5, and 10 mM MgCl$_2$) and co-infiltrated into N. benthamiana leaves. Laser-scanning confocal images were taken 48 h after agroinfiltration. Excitation and emission wavelengths were 488 and 508 nm for GFP, 514 and 527 nm for YFP, and 545 and 572 nm for mCherry. Expression of fusion proteins was corroborated by western blot analysis using anti-N$^+$GFP or anti-C$^-$GFP (Roche) antibodies were conducted following the recommendations of the manufacturer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.
AUTHOR CONTRIBUTIONS
VP and FA conceived the project and designed the experiments. LA-M conducted the experiments with assistance from JM-M and MM-P. LA-M, FA, and VP wrote the manuscript. All authors analyzed and discussed the results.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.701683/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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