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Plant YTHDF proteins are direct effectors of antiviral immunity against an N6-methyladenosine-containing RNA virus

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Abstract

In virus–host interactions, nucleic acid-directed first lines of defense that allow viral clearance without compromising growth are of paramount importance. Plants use the RNA interference pathway as a basal antiviral immune system, but additional RNA-based mechanisms of defense also exist. The infectivity of a plant positive-strand RNA virus, alfalfa mosaic virus (AMV), relies on the demethylation of viral RNA by the recruitment of the cellular RNA demethylase ALKBH9B, but how demethylation of viral RNA promotes AMV infection remains unknown. Here, we show that inactivation of the Arabidopsis cytoplasmic YT521-B homology domain (YTH)-containing m6A-binding proteins ECT2, ECT3, and ECT5 is sufficient to restore AMV infectivity in partially resistant alkbh9b mutants. We further show that the antiviral function of ECT2 is distinct from its previously demonstrated function in the promotion of primordial cell proliferation: an ect2 mutant carrying a small deletion in its intrinsically disordered region is partially compromised for antiviral defense but not for developmental functions. These results indicate that the m6A-YTHDF axis constitutes a novel branch of basal antiviral immunity in plants.

Keywords ECTs; IDRs; N6-methyladenosine (m6A); plant–virus interaction; RNA methylation

Subject Categories Plant Biology; RNA Biology

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Introduction

Viruses are intracellular pathogens that use their genetic code to turn cells into their multiplication factories. To defend against hostile takeover by viral genetic material, host organisms have evolved mechanisms to recognize foreign nucleic acids. For example, bacteria use a combination of restriction-modification and CRISPR-Cas systems to eliminate viral nucleic acids (Barrangou et al, 2007; Loenen et al, 2014; Egido et al, 2022), and a combination of cyclic nucleotide signaling and NAD⁺ depletion systems coupled to nucleic acid sensors to eliminate infected cells from a population (Tai et al, 2021; Koopal et al, 2022). Similar basal antiviral immune systems operating directly on viral nucleic acids have been found in animals, where at least four distinct cytoplasmic systems recognize double-stranded features of viral RNA to activate signaling pathways that lead to the induction of innate antiviral immune responses (Wu & Chen, 2014; Rehwinkel & Gack, 2020; Holleuer et al, 2021; Slavik et al, 2021). In plants, the double-strandedness of cytoplasmic viral RNA is also used to distinguish it from endogenous RNA, since plants use RNA interference (RNAi) as a potent basal antiviral defense mechanism (Lindbo, 2012). In addition, extracellular double-stranded RNA (dsRNA) can be sensed by as yet unidentified receptors to effect antiviral pattern-triggered immunity PTI (Meier et al, 2019; Niehl & Heinlein, 2019). The effectiveness of RNAi as a basal antiviral defense mechanism is illustrated by the fact that nearly all plant viruses use anti-RNAi effectors as virulence factors (Voinnet et al, 1999; Cserba et al, 2015; Jin et al, 2021). In plants, RNAi remains the most generally employed basal antiviral defense mechanism to date, but features of viral genetic material other than its double-stranded regions may be used for distinction between cellular and viral RNA, and hence, for antiviral defense. For example, in both mammals and plants, the non-sense-mediated mRNA decay (NMD) pathway may detect and repress long viral RNAs with stop codons of reading frames located towards the 5’-end of the viral transcript (Balistreri et al, 2014; Garcia et al, 2014), a feature that viruses may circumvent either through the use of anti-NMD factors or by organizing their genomes into a long poly-protein-encoding mRNA containing only a single downstream stop codon (Balistreri et al, 2014; Popp et al, 2020). Furthermore, in the
nematode Caenorhabditis elegans that also relies on RNAi for basal antiviral defense (Gammon, 2017), a terminal uridytransferase exerts antiviral immunity independently of RNAi, although the feature of viral RNA recognized in this case remains unclear (Le Pen et al., 2018).

Covalent modification of mRNA is now understood to play important roles in the regulation of gene expression, and many viruses contain modified nucleotides of importance for the outcome of the host–virus interaction (Gokhale & Horner, 2017; Courtney, 2021). The most widespread, and probably most important, modification of eukaryotic cellular mRNA is N6-methyladenosine (m^6A) (Arribas-Hernández & Brodersen, 2020; Wiener & Schwartz, 2021). m^6A is installed at DRACH (D = A/C/U, R = G/A, H = A/C/U) or GGAU motifs in pre-mRNA by a dedicated, nuclear multi-subunit methyltransferase, or “writer,” complex (Arribas-Hernández & Brodersen, 2020; Arribas-Hernández et al., 2021a). This highly conserved complex consists of a heterodimeric catalytic core of methyltransferase-like proteins known as MAC, and a large MAC-associated complex (MACOM) required for activity in vivo (Bokar et al., 1994, 1997). In Arabidopsis, MAC consists of MTA (AT4G10760, orthologous to metazoan Mett3) and MTB (AT4G09980, orthologous to metazoan Mettl14), while MACOM is composed of several factors, including FKBPI2 INTERACTING PROTEIN 37 (FIP37, AT3G41700, orthologous to metazoan WTA P), VIRILIZER (VIR, AT3G05680), the ubiquitin ligase HAKAI (AT5G01160), and the Zn-finger protein HAKAI-INTERACTING Zn-FINGER2 (HIZ2; AT5G35440, equivalent to metazoan ZC3H13/ECT2/3, AT5G01160, and the Zn-finger protein HAKAI-INTERACTING Zn-FINGER3 (HIZ3; AT4G10760), orthologous to metazoan GGAU motifs in pre-mRNA by a dedicated, nuclear multi-subunit methyltransferase, or “writer,” complex (Arribas-Hernández & Brodersen, 2020; Arribas-Hernández et al., 2021a). This highly conserved complex consists of a heterodimeric catalytic core of methyltransferase-like proteins known as MAC, and a large MAC-associated complex (MACOM) required for activity in vivo (Bokar et al., 1994, 1997). In Arabidopsis, MAC consists of MTA (AT4G10760, orthologous to metazoan Mett3) and MTB (AT4G09980, orthologous to metazoan Mettl14), while MACOM is composed of several factors, including FKBPI2 INTERACTING PROTEIN 37 (FIP37, AT3G41700, orthologous to metazoan WTAP), VIRILIZER (VIR, AT3G05680), the ubiquitin ligase HAKAI (AT5G01160), and the Zn-finger protein HAKAI-INTERACTING Zn-FINGER2 (HIZ2; AT5G35440, equivalent to metazoan ZC3H13/Flacc; Zhong et al., 2008; Shen et al., 2016; Rúžička et al., 2017; Zhang et al., 2022a). m^6A can be demethylated, or “erased,” by a set of enzymes belonging to the AlkB family of Fe(II)- and α-ketoglu tarate-dependent dioxygenases (Van den Born et al., 2009) that comprises 14 members in Arabidopsis (Mielecki et al., 2012; Kawai et al., 2014). Demethylation activity has been demonstrated experimentally for ALK89B and ALK891OB (Mielecki et al., 2012; Kawai et al., 2014; Duan et al., 2017; Martínez-Pérez et al., 2017), and recent evidence suggests its presence also in ALK89C (Amara et al., 2022).

It is an important function of m^6A to generate a binding site for RNA-binding proteins specialized for the m^6A recognition via a so-called YTH domain (Imai et al., 1998; Stoilov et al., 2002; Zhang et al., 2010; Dominissini et al., 2012; Liao et al., 2018). The YTH domain contains an aromatic cage that forms a hydrophobic pocket for the methyl group in m^6A, thus resulting in 10–20-fold higher affinity for m^6A-containing RNA than for unmethylated RNA (Li et al., 2014; Luo & Tong, 2014; Theler et al., 2014; Xu et al., 2014, 2015; Zhi et al., 2014; Patil et al., 2018). For that reason, YTH domain proteins are sometimes referred to as m^6A readers. In the cytoplasm, a family of so-called YTHDF proteins binds to m^6A-containing RNA, and while genetics shows these YTHDF proteins to be of key importance for plant and animal development (Arribas-Hernández et al., 2018, 2020; Kontur et al., 2020; Lasman et al., 2020), the exact reasons for this biological importance remain undefined in plants, and heavily debated in mammals (Tsutsui & Higashiyama, 2017; Lasman et al., 2020; Murakami & Jaffrey, 2022). In the flowering plant Arabidopsis, there are 11 YTHDF proteins, named EVOLUTIONARILY CONSERVED C-TER MINAL REGION (ECT) because of the presence of the conserved YTH domain in the C-terminal part, after an N-terminal intrinsically disordered region (IDR) as in YTHDF proteins of other organisms. ECT2 and ECT3 act redundantly as m^6A readers to stimulate cellular proliferation in organ primordia, such that ect2/ect3 double mutants show delayed leaf and flower formation, slow root and stem growth, and aberrant leaf, flower, siliquae, and trichome morphology (Arribas-Hernández et al., 2018, 2020, 2021b). In nearly all cases, these phenotypes are exacerbated by additional mutation of ECT4 (Arribas-Hernández et al., 2018, 2020), and five additional Arabidopsis YTHDF proteins (ECT5, ECT6, ECT7, ECT8, ECT10) share the molecular functions of ECT2/3 required for primordial cell proliferation, as seen by complementation of ect2/ect3/ect4 phenotypes upon ectopic expression in the ECT2/3 expression domain (preprint: Flores-Téllez et al., 2023). Stimulation of growth is a recurrent function of plant YTHDF proteins because similar roles have been found for rice and tomato YTHDF proteins (Ma et al., 2022; Yin et al., 2022), and because the sole YTHDF protein of the liverwort Marchantia polymorpha can replace ECT2/3 function when expressed in Arabidopsis (preprint: Flores-Téllez et al., 2023).

m^6A is also found in viral mRNAs, first seen as early as 1975 in simian virus 40 mRNAs (Lavi & Shatkin, 1975). Subsequent studies have shown the presence of m^6A in viral RNAs from several mammalian RNA and DNA viruses (Williams et al., 2019; Wu et al., 2020; Baqueró-Pérez et al., 2021), and direct binding of YTH proteins to RNAs from some of these viruses, such as hepatitis C virus (HCV), zika virus (ZIKV) or human immunodeficiency virus-1 (HIV-1) has been demonstrated (Gokhale et al., 2016; Kennedy et al., 2016; Lichinchi et al., 2016). In plant viruses, fewer examples of the involvement of m^6A in infection cycles have been described (van den Born et al., 2008; Martínez-Pérez et al., 2017, 2021; Tian et al., 2021; Zhang et al., 2022a; He et al., 2023).

We previously showed that viral RNA of several positive-strand RNA viruses contains m^6A (Martínez-Pérez et al., 2017), among them alfalfa mosaic virus (AMV), which consists of three genomic RNA molecules (RNA1, RNA2, and RNA3) and a subgenomic RNA (RNA4; Bujański et al., 2019). Importantly, AMV infectivity of wild-type Arabidopsis plants depends on the activity of a cellular m^6A demethylase, ALK89B (Martínez-Pérez et al., 2017; Alvarado-Marchena et al., 2021), since local and systemic AMV infections are attenuated in alk89b knockout plants, including a nearly complete block of viral invasion of the floral stems that is possibly explained by the severely reduced ability of AMV to enter into the vascular tissue of alk89b mutants (Martínez-Pérez et al., 2017, 2021). Thus, methylation of the viral RNA is part of an important defense mechanism that the infectious virus adapts to by manipulation of ALK89B to act on the viral RNA (Martínez-Pérez et al., 2017, 2021). This defense mechanism seems to affect some of the steps of the viral replication cycle, because, in addition to local and systemic movement, clear differences in AMV levels were also detected upon infection of protoplasts isolated from wild-type and alk89b mutant plants (Martínez-Pérez et al., 2021). It is at present unclear whether reduced AMV titers in infected cells underlie all effects on systemic infection in alk89b mutants, or whether the hypermethylation of AMV RNA in alk89b mutants affects additional steps specifically linked to systemic movement. Most importantly, the molecular basis of m^6A-dependent antiviral defense remains undefined. It may, for example, involve m^6A-binding reader proteins or RNA structure properties related to altered base pairing properties of m^6A-U compared with A-U, the latter perhaps suggested by the observation that
adensines in a DRACH context within the 3'UTR of alfalfa mosaic virus (AMV) RNA 3 represent a key structural requirement for viral replication (Alvarado-Marchena et al., 2022).

Here, we show that mutation of the $\text{ECT2/ECT3/ECT4/ECT5}$ module in Arabidopsis reduces AMV resistance, and that the increased AMV resistance of $\text{alkb9b}$ mutants can be reverted by mutation of $\text{ECT2/ECT3/ECT5}$. We also show that the functions of $\text{ECT2}$ in the stimulation of cellular proliferation and antiviral defense can be separated by a small deletion in its N-terminal IDR. These results establish the $\text{m}^6\text{A}$-$\text{YTHDF}$ axis as a novel basal antiviral defense layer.

## Results

### AMV infection induces components of the $\text{m}^6\text{A}$-$\text{YTH}$ axis

To guide the genetic dissection of $\text{m}^6\text{A}$-mediated defense against AMV, we first analyzed transcriptome changes upon AMV infection with particular attention to transcripts encoding components of the $\text{m}^6\text{A}$ pathway. For this experiment, we selected young, emerging rosette leaves of infected plants, since $\text{m}^6\text{A}$-binding proteins and $\text{C19}$ showed a greater than 2-fold upregulation (Fig 1C). We verified the $\text{m}^6\text{A}$ readers $\text{ECT2}$ and $\text{ECT3}$ (Arribas-Hernandez et al., 2018). The viral RNA levels in inoculated mature leaves, including the leaf formation (Fig 2E). As observed for the $\text{ect2/ect3}$ mutants, $\text{ect2/ect3}$ tended to show moderately higher AMV titers systemically than $\text{ect2}$, although with some variability between experiments (Fig 2D and Appendix Fig S1C). Similar results were obtained with different CRISPR-induced $\text{ect5}$ alleles (Appendix Fig S2 and Table S1). We draw several conclusions from these initial genetic analyses. First, several ECT proteins impact the outcome of infection by Arabidopsis by AMV. Second, similar to modulation of the $\text{m}^6\text{A}$ content in AMV RNA by inactivation of $\text{ALKB9B}$, effects in both inoculated and noninoculated tissues can be observed upon mutation of $\text{ECT}$ genes, suggesting that the mechanism of $\text{m}^6\text{A}$-dependent antiviral defense may involve ECT proteins. We note that several factors may underlie the fact that much clearer differences were observed in uninoculated young tissue compared with inoculated mature leaves, including the fact that $\text{ECT2/ECT3}$ are expressed in young tissue, whereas the levels of $\text{m}^6\text{A}$ erasers did not substantially change (Fig 1C). Among YTHDF-encoding genes, only $\text{ECT5}$ showed a greater than 2-fold upregulation (Fig 1C). We verified the induction of $\text{ECT5}$, along with that of $\text{MTA}$, $\text{MTB}$, and $\text{VIR}$, using quantitative RT-PCR analysis (Fig 1D). The previously described $\text{m}^6\text{A}$ readers $\text{ECT2}$ and $\text{ECT3}$ (Arribas-Hernandez et al., 2018) were also significantly upregulated, albeit with smaller effect size (log2FC values 0.6 and 0.4 for $\text{ECT2}$ and $\text{ECT3}$, respectively; Fig 1C).

### ECT2, ECT3, ECT4, and ECT5 are implicated in AMV resistance

We next analyzed single $\text{ect2-1, ect3-1, ect4-2}$ mutants (Arribas-Hernandez et al., 2018), and an $\text{ect5}$ T-DNA insertion mutant ($\text{ect5-1, SALK\_131549; Fig EV1A and B}$) for possible defects in AMV resistance (Appendix Table S1). No single mutants showed significant differences in AMV titers (Fig EV1C), perhaps due to functional redundancy as demonstrated for their growth-promoting functions (Arribas-Hernandez et al., 2020). We therefore analyzed composite mutants of $\text{ECT2, ECT3, and ECT4}$. The viral RNA levels in inoculated leaves (local infection) were slightly higher in mutant combinations involving $\text{ect2}$ but reached statistical significance only in the $\text{ect2/ect3/ect4/ect5}$-deficient triple mutant (Fig 2A). However, these differences were much more evident in noninoculated leaves (systemic infection), in which AMV titers were clearly elevated in $\text{ect2/ect3/ect4/ect5}$ (henceforth, $\text{de23}$) and $\text{ect2/ect3/ect4}$ mutant plants compared with wild-type (Fig 2B). Double mutants involving $\text{ect2}$ showed weak, if any, effects on AMV accumulation, while $\text{ect2/ect3/ect4}$ tended to have somewhat higher AMV titers than $\text{de23}$, although this effect was variable in size between experiments (Fig 2B and Appendix Fig S1B). The differences in systemic viral load between wild-type and $\text{ect2/ect3/ect4}$ double knockouts were confirmed using an $\text{ect2/ect3}$ double mutant with independent knockout alleles ($\text{ect2-3/ect3-2}$, Appendix Fig S1 and Table S1; Arribas-Hernandez et al., 2018). Next, since $\text{ECT5}$ is closely linked to $\text{ECT2}$ (Fig EV2A), we used CRISPR-Cas9 to produce the double $\text{ect2-1/ect5-2}$ (de25) and triple $\text{ect2-1/ect3-1/ect5-4}$ (te235) mutants (Fig EV2B). Importantly, we observed increased AMV titers in noninoculated tissues of $\text{de25}$ compared with wild-type (Fig 2C and D), pointing to an important involvement of $\text{ECT5}$ in limiting systemic AMV infection. In contrast to $\text{de23}$ mutants, $\text{de25}$ mutants exhibited no obvious leaf morphology defects or delay in leaf formation (Fig 2E). As observed for the $\text{te234}$ mutants, $\text{te235}$ tended to show higher AMV titers systemically than $\text{de23}$, although with some variability between experiments (Fig 2D and Appendix Fig S1C). Similar results were obtained with different CRISPR-induced $\text{ect5}$ alleles (Appendix Fig S2 and Table S1). We draw several conclusions from these initial genetic analyses. First, several ECT proteins impact the outcome of infection by Arabidopsis by AMV. Second, similar to modulation of the $\text{m}^6\text{A}$ content in AMV RNA by inactivation of $\text{ALKB9B}$, effects in both inoculated and noninoculated tissues can be observed upon mutation of $\text{ECT}$ genes, suggesting that the mechanism of $\text{m}^6\text{A}$-dependent antiviral defense may involve ECT proteins. We note that several factors may underlie the fact that much clearer differences were observed in uninoculated young tissue compared with inoculated mature leaves, including the fact that $\text{ECT2/ECT3}$ are expressed in young tissue, whereas the levels of $\text{m}^6\text{A}$ erasers did not substantially change (Fig 1C). Among YTHDF-encoding genes, only $\text{ECT5}$ showed a greater than 2-fold upregulation (Fig 1C). We verified the induction of $\text{ECT5}$, along with that of $\text{MTA}$, $\text{MTB}$, and $\text{VIR}$, using quantitative RT-PCR analysis (Fig 1D). The previously described $\text{m}^6\text{A}$ readers $\text{ECT2}$ and $\text{ECT3}$ (Arribas-Hernandez et al., 2018) were also significantly upregulated, albeit with smaller effect size (log2FC values 0.6 and 0.4 for $\text{ECT2}$ and $\text{ECT3}$, respectively; Fig 1C).
phylogenetically related to AMV, is silenced predominantly by the RDR6-dependent viral siRNAs (Dalmay et al., 2000; Mourrain et al., 2000; Wang et al., 2011; Martínez-Pérez et al., 2017). Even in the rdr6 mutant background, inactivation of ECT2/ECT3 led to increased AMV titers (Fig EV3B), and, thus, we conclude that the action of m6A-ECT system is independent of RNAi. These results do not, however, rule out that RNAi may affect the outcome of AMV infection in some settings of the virus–plant interaction, for instance with different strengths of inoculum or at different times postinoculation.

The m6A-binding activity of ECT2 is required for its antiviral activity

The amino acid residues of human YTHDF proteins implicated in RNA and m6A binding are highly conserved in Arabidopsis ECT proteins (Fray & Simpson, 2015; Scutenaire et al., 2018). This includes in particular the three tryptophan residues that form the m6A-binding aromatic cage. Mutational studies of these tryptophan residues have demonstrated that integrity of the aromatic cage is required for ECT2 binding to m6A-modified RNAs (Scutenaire et al., 2018) and for ECT2, ECT3, and ECT4 function in vivo (Arribas-Hernández et al., 2018, 2020). Thus, to test whether ECT-mediated antiviral resistance requires the m6A-binding activity, we used de23 mutants expressing either ECT2WT-mCherry or the m6A-binding deficient point mutant ECT2W464A-mCherry (Appendix Table S1). The ECT2W464A-mCherry-expressing lines were clearly defective in AMV resistance, while expression of ECT2WT-mCherry in de23 restored resistance to levels comparable to that of wild-type or ect3-1 (Fig 3A, upper and lower panels show two independent complementing transgenic lines).

Figure 1. Differential gene expression analysis between mock and AMV-infected Arabidopsis plants at 8 days postinoculation.

A Volcano plot depicting differential expression of nearly 18,000 unique genes (minimum expression of 1 read per kilobase per million, RPKM) in response to AMV infection. In red, upregulated genes (log2FC ≥ 1, adjusted P-value ≤ 0.05); in blue, downregulated genes (log2FC ≤ −1, adjusted P-value ≤ 0.05).
B Most highly enriched gene ontology (GO) terms in the set of differentially expressed genes.
C Filtered volcano plot visualizing the expression of genes involved in the m6A pathway in response to AMV infection.
D RT-qPCR analysis of expression of four upregulated m6A pathway-related genes in response to AMV infection (three biological replicates). Error bars represent the standard error of the mean (SEM). Asterisks indicate a P < 0.05 applying the Student’s t-test for ΔCt mean values (n = 3).

Source data are available online for this figure.
We note that the loss of AMV resistance in both knockout and ECT2\(^{\text{W464A}}\) mCherry-expressing lines could in principle be an indirect consequence of the developmental defect of ect mutants that may render the plant more amenable to viral replication, rather than a direct effect of missing or defective association with m\(^6\)A-containing viral RNA. We disfavor this possibility for two reasons. First, in contrast to de23, de25 mutants display no obvious rosette phenotype (Fig 2E), yet the increase in AMV titers compared with wild-type is robust (Fig 2D). Second, the mutation of the nuclear m\(^6\)A writer component VIR causes a rosette phenotype similar to, but slightly stronger than ect2/ect3/ect4 (Růžička et al., 2017), yet does not lead to the same degree of loss of antiviral resistance (Fig 3B).

**ECT2 associates with AMV RNA in vivo**

We next sought to establish whether ECTs associate with AMV RNA, focusing specifically on ECT2 due to its higher expression levels compared with ECT3 and ECT5 (Arribas-Hernández et al., 2021b). We used the proximity-labeling method HyperTRIBE (Targets of RNA-binding proteins Identified by Editing; McMahon et al., 2016), in which the catalytic domain of the fly Adenosine Deaminase Acting on RNA (ADAR) is fused to ECT2 and expressed under the control of the ECT2 promoter. Because adenosine-to-inosine editing by ADAR is detected as A-to-G transitions by RNA-Seq, RNA bound by ECT2 can be identified by virtue of its higher A-G editing proportions compared with control lines expressing unfused ADAR (McMahon et al., 2016; Xu et al., 2018; preprint: Rennie et al., 2021). Using an experimental set-up similar to our previous HyperTRIBE analysis, which faithfully identified direct endogenous mRNA targets of ECT2 and ECT3 (Arribas-Hernández et al., 2021a, 2021b), we employed five independent transgenic lines expressing either the ECT2-FLAG-ADAR fusion or the free FLAG-ADAR (Appendix Table S1), the latter acting as negative controls. The negative control lines showed on average higher FLAG-ADAR expression levels than the ECT2-FLAG-ADAR fusion lines (Fig 4A), contributing to higher stringency in the analysis. In contrast, viral titers were generally lower in these same lines, thus providing fewer sequenced reads for the viral RNAs (Fig 4B). Nearly all detected AMV sequence reads mapped to the positive strand, as expected (Fig 4C). We first selected 395 candidate positions showing A-G changes compared with the reference AMV sequence in at least two of five lines. Since the viral RNA-dependent RNA polymerase introduces random mutations, and since free ADAR has a nonzero background editing activity, we sought to formally test whether significantly higher editing proportions were observed in ECT2-FLAG-ADAR lines compared with FLAG-ADAR control lines. For this purpose, we employed our recently published method for the analysis of HyperTRIBE data (preprint: Rennie et al., 2021). This statistical analysis identified two sites in the positive strand of RNA2 (RNA2\(_{701}\) and RNA2\(_{2271}\)) showing consistent A-G changes across FLAG-ADAR fusion lines, with modeled editing

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**Figure 2. Combined inactivation of ECT2/3/4/5 enhances titers of AMV in systemic tissues.**

A–D RNA blots were hybridized to AMV-specific probes to reveal accumulation of viral RNA in double (de) and triple (te) ect2/3/4 (A, B) or ect2/3/5 (C, D) mutants compared with wild-type (WT) plants. A and C show local infection at 3 days postinoculation (dpi); B and D show systemic infection in aerial tissue at 7 dpi. Each panel shows a representative RNA blot displaying AMV RNAs 1–4 (left) and its quantification histogram (right). Dashed lines indicate noncontiguous samples analyzed on the same membrane. Ethidium bromide staining of ribosomal RNAs (EB) was used as RNA loading control. Error bars indicate standard deviations. Asterisks indicate significant differences from the WT (\(^*\), \(P < 0.05\); \(^{**}\), \(P < 0.01\)) using the Student’s t-test (\(n = 4\)). AU, arbitrary units.

E Phenotypes of rosettes of the indicated genotypes at 19 days after germination. Scale bars: 1 cm.

Source data are available online for this figure.
The m^6A-ECT axis constitutes a basal layer of antiviral defense

The observations that inactivation of ECT2/3/4/5 leads to higher AMV infectivity, that ECT2 associates with AMV RNA in vivo, and that its antiviral effect requires an intact m^6A-binding site are consistent with a model in which binding of ECTs to methylated viral RNA causes inhibition of viral replication. However, the results reported thus far suffer from the limitation that they do not analyze the true effect of ECT association with AMV RNA, because they are carried out with a wild-type virus in an otherwise wild-type Arabidopsis genetic background. Since AMV requires the endogenous demethylase ALKBH9B, probably recruited via its capsid protein (CP) to AMV RNA, for infectivity (Martínez-Pérez et al., 2017), these experiments address the influence of ECTs on infection in a setting in which a virulence mechanism is diminishing the impact of adenine methylation of viral RNA. Because the CP has multiple essential functions in the infection cycle, including RNA encapsidation and initial translational control (Herranz et al., 2012), informative genetic manipulation of the CP is not straightforward. By contrast, our previous study showed that the systemic infection in the floral stems is nearly fully blocked upon disruption of the host ALKBH9B gene. We therefore constructed alkhhb9b/te235 mutants (Appendix Table S1) to test the impact of ECT inactivation in a setting in which AMV infectivity is strongly incapacitated due to hypermethylation. The AMV titers in alkhhb9b/te235 were intermediate between alkhhb9b and te235 at early stages of infection (7 dpi; Fig 5A) but were identical to te235 (and Col-0) at late stages (20 dpi; Fig SB), where little to no AMV was detectable in alkhhb9b. We interpret these results to mean that m^6A-ECTs efficiently limit the speed of systemic spread of AMV to the point where nearly no virus accumulates in stems upon leaf inoculation in alkhhb9b (Fig SB; Martínez-Pérez et al., 2017, 2021), even at late stages of infection. At such late points, additional aspects of the host–AMV interaction are likely to limit AMV titers to a maximal value reached by both Col-0 and te235. Although spreading and systemic accumulation is lower in alkhhb9b/te235 than in te235, the virus eventually reaches these same maximal titers systemically, in marked contrast to what is observed in alkhhb9b. Importantly, therefore, the inactivation of ECT2/3/5 is sufficient to break the strong m^6A-based protection against systemic infection in the absence of ALKBH9B, clearly showing that ECT2/3/5 are requisite effectors of the m^6A-based defense against AMV. These results argue that for AMV, and perhaps other viruses whose RNA is N6-adenosine methylated, the m^6A-ECT axis constitutes a basal layer of antiviral defense that must be overcome for the virus to be infectious. Indeed, the results conceptually parallel the restoration of infectivity of RNA virus strains with defective anti-RNAi effectors by inactivation of the RNAi machinery (Deleris et al., 2006; Wang et al., 2011).

The growth-promoting and antiviral activities of ECT2 are genetically separable

The model of basal antiviral defense mediated by m^6A-ECT2/3/4/5 may be at odds with the described endogenous function of these factors in promoting cellular proliferation (Arribas-Hernández et al., 2020), as a highly metabolically active state favors viral replication. Thus, it is difficult to imagine how the same biochemical activity would promote both antiviral defense and cellular proliferation. We
Since Tyr residues may promote IDR-driven phase separation, four had no effect on leaf formation (preprint: Tankmar et al, 2023). Of six positions that were originally designed to identify regions of importance for biophysical properties to achieve both effects, To analyze this possibility, we reasoned that ECTs may use different biochemical and/or biophysical properties employed to achieve different goals: one that stimulates cellular proliferation by binding to endogenous m^6^A-containing mRNA, and one that effects basal antiviral resistance when ECT2 binds to hypermethylated viral RNA. We note that since the antiviral activity was not fully abolished in te234 expressing ECT2-^N^S^-^m^C^herry, we cannot formally exclude the possibility that the different impacts of this mutation on antiviral defense and developmental phenotypes are caused by a threshold effect, such that the same reduction in molecular activity would give rise to a measurably reduced antiviral, but not developmental, function of ECT2. A complete resolution of this issue requires a full understanding of the precise molecular nature of both ECT2 functions, a task not achieved for the molecular activity would give rise to a measurably reduced antiviral, but not developmental, function of ECT2.

Therefore reasoned that ECTs may use different biochemical and/or biophysical properties to achieve both effects. To analyze this possibility, we made use of a series of small deletions engineered in the long N-terminal intrinsically disordered region (IDR). These deletions were originally designed to identify regions of importance for the developmental function of ECT2. Of six ~50–80 aa deletions, four had no effect on leaf formation (preprint: Tankmar et al, 2023). Since Tyr residues may promote IDR-driven phase separation (Wang et al, 2018; Martin et al, 2020; Bremer et al, 2022), we selected the ECT2^N^S^N^ mutant carrying a deletion in a Tyr-rich region present in many ECTs for analysis of AMV resistance (Figs 6A, and EVS ^A^ and B). We observed a reduction in AMV resistance in transgenic lines expressing ECT2^-^m^Cherry compared with ECT2^WT^-m^Cherry in the te234 mutant background (Fig 6B). This effect was reproducible, albeit somewhat variable, across several transgenic lines with similar levels of ECT2-mCherry or ECT2^N^S^-m^Cherry (Fig 6C). Because the rate of leaf formation and leaf morphology in te234 lines expressing ECT2^N^S^-m^Cherry were indistinguishable from those expressing ECT2^WT^-m^Cherry (Figs 6D and E, and EVSC), yet AMV resistance was measurably compromised, these results suggest that the IDR of ECT2 harbors two separable activities employed to achieve different goals: one that stimulates cellular proliferation by binding to endogenous m^6^A-containing mRNA, and one that effects basal antiviral resistance when ECT2 binds to hypermethylated viral RNA. We note that since the antiviral activity was not fully abolished in te234 expressing ECT2^N^S^-m^C^herry, we cannot formally exclude the possibility that the different impacts of this mutation on antiviral defense and developmental phenotypes are caused by a threshold effect, such that the same reduction in molecular activity would give rise to a measurably reduced antiviral, but not developmental, function of ECT2. A complete resolution of this issue requires a full understanding of the precise molecular nature of both ECT2 functions, a task not achieved for functions of any YTHDF protein to date. Nonetheless, regardless of the exact interpretation of the reduced antiviral resistance in te234 lines expressing ECT2^N^S^-m^Cherry, this result reinforces the conclusion reached from infections of dx25 and vir-1 mutants that compromised antiviral resistance in ect mutants is not simply an indirect effect of developmental defects caused by dysfunction of the m^6^A-ECT axis.

Figure 4. HyperTRIBE identifies in vivo interactions between ECT2 and AMV RNAs.

A ADAR expression (TPM—Transcripts Per Kilobase Million—mapping) in five samples of ECT2-FLAG-ADAR (S1–S5) and FLAG-ADAR (S6–S10) transgenic lines. B Percentage of total reads mapping to the AMV RNAs in the different samples. C Percentage of reads mapping to either the positive or negative AMV strand. D Positions preferentially edited in AMV RNAs of the ECT2-FLAG-ADAR compared with FLAG-ADAR lines. %edit and %ctrl show the mean editing proportion (G/(A + G)) in the ECT2-FLAG-ADAR and FLAG-ADAR lines, respectively. Significant sites were determined using hyperTRIBER pipeline (preprint: Rennie et al, 2021). To be formally tested, an A-to-G change had to be present in at least two of the five ECT2-FLAG-ADAR samples. E Correlation between ADAR log TPM and editing proportions across individual ECT2-FLAG-ADAR transgenic lines for the three most highly significantly edited sites. F Schematic representation of the positions of the two high-confidence ECT2-dependent editing sites in AMV RNAs. G Exact position of the two high-confidence ECT2-depentence editing sites in AMV RNA2 (light blue). The DRACH motives located nearby editing sites are highlighted in gray.

Source data are available online for this figure.
We next investigated the possibility that the relevance of the region deleted in ECT2ΔNS for AMV resistance may be linked to the propensity to engage in higher-order assemblies for two reasons. First, ECT2 clearly has a propensity to form condensate-like structures in vitro and in vivo (Arribas-Hernández et al., 2018), with localization to cytoplasmic stress granules upon abiotic stress (Scutenaire et al., 2018). Second, as noted above, the region deleted in ECT2ΔNS is enriched in Tyr residues that may be drivers of phase separation (Wang et al., 2018; Martin et al., 2020; Bremer et al., 2022). Thus, we carried out molecular simulations to probe and quantify the propensity of the IDR of ECT2 and ECT2ΔNS to self-associate in a phase-separated state using a recently published coarse-grained model that accurately captures the phase-separation behavior of IDRs (Tesèi et al., 2021). These simulations confirmed that the IDR of ECT2 has a clear propensity to phase separate, similar to, albeit less pronounced than, the well-studied IDRs of the human RNA-binding proteins hnRNP A1 and FUS (Fig 7). Importantly, the IDR of ECT2ΔNS showed substantially reduced propensity to phase separate compared with that of ECT2, with clear signs of dispersion over time in the simulations (Fig 7A and B), and a thermodynamically less favorable transition to the condensed phase (Fig 7C). A simulation of the behavior of an IDR of ECT2 in which all Tyr residues in the ΔNS region had been substituted by Ala (ECT2ΔNS→) further indicated the essential contribution of the Tyr residues in this region for phase separation (Fig 7), potentially explaining their conservation in ECT2 orthologs and ECT paralogs more broadly. Taken together, our genetic dissection of the IDR of ECT2, and our simulation of its biophysical properties suggest that the ability to phase separate or form other higher-order structures may be of particular importance for the antiviral function of ECT2.

**Discussion**

**A new basal antiviral defense mechanism**

Our analysis of the AMV–Arabidopsis interaction provides a clear case of the m’A-YTHDF axis acting as a basal antiviral defense layer: when the infection mechanism targeting the m’A-YTHDF axis is hampered by host ALKBH9B mutation, AMV systemic infection is severely disrupted and practically blocked in floral stems. However, systemic infectivity is restored upon additional mutation of ECT2, ECT3, and ECT5. These genetic data resemble the key arguments for
the importance of RNAi as a basal antiviral defense mechanism in plants and insects (Deleris et al., 2006; Ding & Voinnet, 2007). Apart from obvious mechanistic questions that we touch on below, this discovery raises the important question of how widespread the use of this defense mechanism may be. AMV may be a special case, as it contains m6A, what the effect of inactivation of genes encoding the m 6A pathway is more generally implicated in plant–virus interactions. Clearly, it will be of key importance to analyze the degree to which RNA of other plant viruses contain m\(^6\)A, what the effect of inactivation of genes encoding the major YTHDF proteins is on their infectivity, and whether they too employ mechanisms to interfere with the m\(^6\)A-YTHDF axis. We note in this regard that studies on mammalian viruses have shown the widespread use as an antiviral defense mechanism should not be disregarded, however. A study comparing the Arabidopsis transcriptome in response to 11 plant viruses pointed out that differential expression of ECT3 is a common response to all viruses, while the genes encoding the methylase components MTA and HAKAI1 and the YTHDF proteins ECT5 and ECT6 were differentially expressed upon infection with at least one virus (Postnikova & Nemchinov, 2012). This suggests that the m\(^6\)A pathway is more generally implicated in plant–virus interactions. Clearly, it will be of key importance to analyze the degree to which RNA of other plant viruses contain m\(^6\)A, what the effect of inactivation of genes encoding the major YTHDF proteins is on their infectivity, and whether they too employ mechanisms to interfere with the m\(^6\)A-YTHDF axis. We note in this regard that studies on mammalian viruses have shown the...
widespread implication of the m^6A-YTHDF axis in animal–virus interactions, albeit not with the rigorous genetics employed here to demonstrate function as a basal layer of defense. For example, the depletion of mammalian YTHDF proteins was found to enhance viral replication of ZIKV (Lichinchi et al., 2016) and HIV-1 (Tirumuru et al., 2016; Jurczyszak et al., 2020), and to increase stability and protein expression of hepatitis B virus (HBV; Imam et al., 2018) and endogenous retroviral mRNAs (Chelmicki et al., 2021). The m^6A-YTHDF system is not universally employed for antiviral defense in animals, however, as viruses also appear to manipulate it for their benefit. For example, mammalian YTHDF proteins stimulate HIV-1 Gag processing and viral release (Tirumuru et al., 2016), and enhance viral translation and replication via binding to m^6A sites located in the 3’UTR of the viral RNAs (Kennedy et al., 2016). The difficulty of generalizing the role of m^6A in viral RNA in host–virus interactions is well illustrated by several examples. In HBV, m^6A in the 5’-UTR of viral pregenomic RNA enhances reverse transcription, while m^6A sites located in the 3’-UTR promote RNA destabilization (Imam et al., 2018). In addition, the recent characterization of the wheat m^6A writer MTB as a susceptibility factor in the interaction with wheat yellow mosaic virus shows that, also in plants, m^6A in viral RNA cannot automatically be assumed to be antiviral (Zhang et al., 2022b).

Are all m^6A effects on AMV infectivity mediated by YTHDF proteins?

The marked restoration of AMV infectivity of alkbb9b mutants upon inactivation of ECT2, ECT3, and ECT5 strongly argues that YTHDF proteins are the major, if not the exclusive, effectors of m^6A-mediated basal AMV defense. In general, at 7 dpi, knockout of ECT2/3/5 in the alkbb9b background leads to intermediate viral loads compared with alkbb9b and ect2/ect3/ect5 mutants, although the outcome shows some variability. However, the most plausible explanation for this is that, in a hypermethylation context—where ALKBH9B is not functional—residual contributions by some of the remaining ECT proteins become relevant. Nonetheless, YTHDF-independent effects of m^6A cannot be formally excluded at this point. For example, m^6A may act as a secondary structure switch caused by altered m^6A-U compared with A-U base pairs (Kierzek & Kierzek, 2003; Liu et al., 2015), a property that may either benefit or impede viral replication. An evaluation of this potentially important possibility will require mapping of m^6A sites in AMV to single-nucleotide resolution in alkbb9b and wild-type backgrounds, as well as in vivo secondary structure analysis now feasible via sequencing-adapted methods (Poulsen et al., 2015).

Source of adenosine methylation of AMV RNA

The observation that the infectivity of AMV is not drastically enhanced in vir-1 mutants, in contrast to te234, constitutes strong evidence that the hypersusceptibility of composite ect mutants is not simply an indirect consequence of developmental defects in plants with a disabled m^6A-YTHDF pathway. This important conclusion is reinforced by the hypersusceptibility of ect2/ect5 mutants and of te234 mutants expressing ECT2 with the ΔN5 deletion, as both of these backgrounds do not show developmental delay and aberrant leaf morphology. VIR is required for adenosine methylation of endogenous mRNA (Rüžička et al., 2017), but if it were also the case for AMV RNA, a hypersusceptibility phenotype would be expected, thus arguing that methylation of AMV RNA is largely VIR-independent. Such a scenario would be consistent with the cytoplasmic replication cycle of AMV, but nuclear localization of MAC/MACOM subunits translocate to the cytoplasm during AMV infection, as has been reported in different infection contexts in mammalian cells (Hao et al., 2019; Srinivas et al., 2021; Sacco et al., 2022), and, more recently, also in plants (Zhang et al., 2022b).

Distinction between cellular and viral m^6A-containing RNA and the basis of ECT-mediated antiviral defense

At first glance, it is a surprising result that ECT2/ECT3 carry out functions in both antiviral defense and in the stimulation of cellular
proliferation, especially given that endogenous mRNA targets of ECT2/ECT3 are enriched in factors involved in central metabolic processes such as oxidative phosphorylation and translation (Arribas-Hernández et al., 2021b). Thus, ECT2/ECT3 binding to endogenous targets presumably increases their expression, consistent with the predominant downregulation of ECT2/ECT3 mRNA targets in root meristem cells devoid of ECT2/ECT3/ECT4 activity (Arribas-Hernández et al., 2021b). By contrast, AMV RNA is repressed by ECT2/ECT3/ECT4 in plants subjected to oxidative stress (Scutenaire et al., 2021). Identification of ECT2/ECT3 mRNA targets presumably increases their expression, consistent with the observed repression in AMV RNA-expressing plants. In this setting, the YTHDF proteins may use short linear motifs in their IDRs to recruit other cellular factors to enhance protein expression, but such activities would be overridden upon condensation into a separate phase. Although admittedly speculative at this point, this model is consistent with a number of observations.

First, abiotic stress indeed causes ECT2, ECT3, and ECT4 to condense into regular hydrogel-like particles in vitro (Arribas-Hernández et al., 2018), identified as stress granules in the case of ECT2 in plants subjected to heat stress (Scutenaire et al., 2018), and purified ECT2 can separate into regularly shaped hydrogel-like particles in vitro (Arribas-Hernández et al., 2018). Thus, ECT2 has a marked propensity to self-associate. Second, our simulations show that the phase-separation propensity of the IDR of ECT2 is substantially reduced in the ECT2<sub>ΔNS</sub> mutant. Third, mammalian YTHDF2 has only weak phase-separation properties in vitro, but they are markedly stimulated upon binding to RNA that contains multiple, but not single, m<sup>6</sup>A sites (Gao et al., 2019; Ries et al., 2019; Fu & Zhubang, 2020).

Fourth, a recent computational approach to infer RNA modifications in plant-pathogenic viruses using high-throughput annotation of modified ribonucleotides (HAMR), a software that predicts modified ribonucleotides using high-throughput RNA sequencing data, revealed a higher proportion of RNA chemical modifications in comparison with mRNAs of Arabidopsis (Marquez-Molins et al., 2022). Finally, we note that although multiple m<sup>6</sup>A sites have been found in many endogenous mRNA targets in Arabidopsis, it is unclear whether they co-exist on the very same mRNA molecules. We stress that despite the existence of evidence consistent with the proposed model for the basis of m<sup>6</sup>A-YTHDF-mediated basal AMV defense, we view this model as a conceptual framework of value in the design of future experiments to test its validity.

**Materials and Methods**

**Plant growth conditions, virus inoculation, and northern blot analysis**

The source and background of each *Arabidopsis thaliana* (Columbia-0) transgenic line are described in Appendix Table S1. Plants were grown in 6 cm diameter pots in a growth chamber with a photoperiod of 25°C-16 h light/20°C-8 h dark. The mechanical inoculations of 15–19 days old plants were carried out using carbendazim and purified virions (1 mg/ml) of AMV PV0196 isolate (Plant Virus Collection, DSMZ) in PE buffer (30 mM sodium phosphate buffer, pH 8). Next, the detection of vRNAs was performed by northern blot analysis. Inoculated leaves and noninoculated aerial tissue were harvested at the corresponding dpi. Systemic infection was evaluated in single plants, while local viral accumulation was analyzed by pooling two plants per sample. Tissues were grounded in liquid nitrogen with mortar and pestle and total RNA was extracted, following EXTRAzo reagent protocol (Blirt; Gdansk, Poland), from 0.1 g leaf material. For northern blots, 500 ng of total RNA was denatured by formaldehyde treatment and after agarose gel electrophoresis in MOPS buffer, RNAs were transferred to positively charged nylon membranes (Roche; Basel, Switzerland) by capillarity in SSC buffer as previously described (Sambrook et al., 1988) and hybridized with digoxigenin-labeled riboprobes to detect AMV RNA 1, RNA 2, RNA 3 and sgRNA 4. Synthesis of the digoxigenin-labeled riboprobes, hybridization, and digoxigenin-detection procedures was performed as previously described (Pallás et al., 1998). Hybridization intensity signal was measured on files from Fujifilm LAS-3000 Imager using Fujifilm Image Gauge V4.0. Every infection experiment was repeated at least twice.

**Generation of Arabidopsis CRISPR/Cas9 transgenic lines**

*Arabidopsis* lines carrying CRISPR/Cas9-mediated gene knockout were generated using the pKAMA-ITACHI Red (pKIR1.1) vector as previously described with some modifications (Tsutsui & Higashiyama, 2017). Different oligonucleotides (Appendix Table S2) were designed inside the ORF of ECT5 (AT3G13060), searching for protospeacer adjacent motif (PAM) sequences (NGG), to work as sgRNAs. Then, ligations of the hybridized DNA oligomers with the vector, previously digested with AarI enzyme, were performed. Agrobacterium tumefaciens GV3101 was transformed with the resulting vectors, and cultures expressing two different sgRNAs were used to generate Arabidopsis stable transgenic lines by floral dip transformation (Clough & Bent, 1998) in etr2-1 and de23 backgrounds. Selection of T1 transgenic plants was carried out by hygromycin resistance and a first assortment of Cas9-free T2 seeds was performed by the absence of red fluorescence. Final genotyping by PCR with specific primers (Appendix Table S2) was realized from T2 plants and confirmed in T3 transformants. Finally, an RT–PCR with specific primers (Appendix Table S2) of total RNA extraction from plants of selected lines and the sequencing of the cDNA products verified the frameshift mutations (Fig EV2C and D).

**Generation of Arabidopsis ECT2<sub>ΔNS</sub>-mCherry lines**

Cloning and line selection of ECT2<sub>ΔNS</sub>-mCherry was performed by USER cloning and agrobacterium-mediated transformation as described in Tankmar (preprint: Tankmar et al., 2023). In brief, USER-compatible primers (LA336, MT5, MT6, LA337) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018). The fragments were purified and inserted into pCAMBIA3300U (pCAMBIA3300 with a double PacI USER-compatible primers (LA336, MT5, MT6, LA337)) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018). The fragments were purified and inserted into pCAMBIA3300U (pCAMBIA3300 with a double PacI USER-compatible primers (LA336, MT5, MT6, LA337)) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018). The fragments were purified and inserted into pCAMBIA3300U (pCAMBIA3300 with a double PacI USER-compatible primers (LA336, MT5, MT6, LA337)) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018). The fragments were purified and inserted into pCAMBIA3300U (pCAMBIA3300 with a double PacI USER-compatible primers (LA336, MT5, MT6, LA337)) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018). The fragments were purified and inserted into pCAMBIA3300U (pCAMBIA3300 with a double PacI USER-compatible primers (LA336, MT5, MT6, LA337)) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018). The fragments were purified and inserted into pCAMBIA3300U (pCAMBIA3300 with a double PacI USER-compatible primers (LA336, MT5, MT6, LA337)) were used to amplify fragments from ECT2Pro:ECT2gDNA-mCherry:ECT2Ter; a construct previously generated by Arribas-Hernández et al. (2018).
after which the plasmid was transformed into Escherichia coli DH5α (NEB). Kanamycin-resistant colonies were selected and their plasmids purified followed by restriction digestion and sequencing prior to introduction into Agrobacterium tumefaciens GV3101 for plant transformation. Arabidopsis stable transgenic lines were generated by floral dip transformation (Clough & Bent, 1998) of ect2-1/ect3-1/ect4-2 (te234) (Arribas-Hernández et al., 2018), and selection of primary transformants (T0) was done on MS-agar plates supplemented with glucosinate-ammonium (Merc, Darmstadt, Germany; 10 mg/l). T2 generation was used for the infection experiments.

**RNA differential expression analysis between Arabidopsis Col-0 WT mock and AMV samples**

Two-week Arabidopsis Col-0 wild-type (WT) plants were mechanically inoculated with 30 mM sodium phosphate buffer pH7 (mock treatment) or AMV PV0196 isolate viral particles in the same buffer. Tissues from the young, emerging rosette leaves were harvested at 8 dpi and ground in liquid nitrogen with mortar and pestle. Total RNA was extracted from 0.1 g leaf material using EXTRAzol reagent (Milenyi Biotec, Germany) and treated with DNase I for 30 min at 37°C. The experiment consisted of three biological replicates (8 individual plants/replicate) of mock and AMV WT inoculated plants. Generation and sequencing of the cDNA libraries were performed by the Genomic Service (SCSIE) of the Universidad de Valencia. Six TruSeq Stranded cDNA libraries (three for healthy and three for AMV-infected plants) were sequenced by non-paired-end sequencing (75 bp) in a NextSeq 550 (Illumina; San Diego, CA, USA). The bioinformatics analysis was carried out by the Bioinformatic Service of IBMCP. Deseq2 package was the tool used for the differential expression analysis and an expression threshold of 1RPMK was applied.

**Quantitative reverse transcription PCR (qPCR)**

Four microliter of the reaction-containing DNA-free RNA were mixed with 0.5 µg (1 µl) of oligo(dT)18 (ThermoFisher Scientific; Waltham, MA, USA), denatured for 5 min at 65°C and added to a total of 20 µl of first-strand cDNA synthesis reaction containing 1 U of RevertAid H Minus Reverse Transcriptase, 0.5 U of Ribolock, 4 mM dNTP mix and 1× RT buffer (ThermoFisher Scientific). The RT reaction proceeded at 42°C for 1 h, followed by 10 min at 70°C to deactivate the enzyme. qPCR reactions were carried out using PyroTaq EvaGreen mix Plus (ROX; CultiK Molecular Bioline, Madrid, Spain) according to the manufacturer’s instructions. All analyses were performed in triplicate on an ABI 7500 Fast-Real Time qPCR instrument (Applied Biosystems, Waltham, MA, USA). Specific oligonucleotides efficiencies were tested by qPCR using 10-fold serial dilutions of the corresponding cDNA (Appendix Table S2). Expression analysis was performed by calculating the relative quantification (RQ) values with respect to the endogenous genes F-BOX and PDF2 (Lilly et al., 2011) of three biological replicates. The Student’s t-test was applied to ΔCt values for statistical analysis.

**Fluorescence microscopy**

Imaging of plants at the rosette stage was done using a Leica MZ16 F fluorescence stereomicroscope, as described by Arribas-Hernández et al. (2018).

**Analysis of ECT2–RNA<sub>AMV</sub> interaction by HyperTRIBE**

Five independent lines expressing ECT2 fused to the catalytic domain of the adenosine deaminase acting on RNA (ADAR) on the ect2-1 background and five control lines expressing only ADAR on wild-type background (Arribas-Hernández et al., 2021a) were inoculated at 16 days with AMV (McMahon et al., 2016; Xu et al., 2018). Tissue from the innermost, young rosette leaves of each line (10 plants per sample) was selected at 7 dpi and grounded in liquid nitrogen with mortar and pestle. After total RNA extraction, precipitation of nucleic acids was carried out with 3.75 M LiCl (–20°C, overnight) to avoid RNA precipitation. Next, these samples were submitted to a ribosomal RNA depletion treatment and cDNA libraries were prepared with CORALL. Total DNA-Seq Library Prep Kit. The libraries were sequenced by paired-end sequencing (150 bp) on an Illumina NEXTseq 550 platform with v2.5 flow cell (300 cycles). Libraries were first quality checked before being merged across lanes, then trimmed, duplicated, and mapped using the CORALL Total RNA-Seq integrated Data Analysis Pipeline. Quantifications of reads mapping to AMV and ADAR were done using Salmon (Patro et al., 2017) using paired-end stranded mode. Mapped .bam files were converted to reflect forward and reverse strands before processing using hyperTRIBER (preprint: Rennie et al., 2021): briefly, base counts were aggregated from reads for all positions containing at least one mismatch, which were subsequently filtered for viral positions containing putative A-G edits in at least two of five lines expressing ECT2-FLAG-ADAR. Three hundred ninety-five candidates were formally tested for the differential occurrence of the base G while adjusting for differences in both read coverage at the position for each condition and variation in the levels of the ECT2-FLAG-ADAR. The results were subsequently FDR corrected in R, and the R function cor.test was used to calculate Pearson’s correlation between ADAR and editing proportions (A/(A + G)) in ECT2-FLAG-ADAR samples. All plots were produced in R (R Core Team, 2021).

**Western blot analysis**

Total protein was extracted homogenizing 0.1 g of tissue from 7 dpi infected plants (same samples used for northern blot analysis in Fig 6B) with three volumes of extraction buffer (0.4 M Tris–HCl pH 8.8, 2% SDS, 15% glycerol, 0.1 M DTT), and heat at 95°C for 10 min. After centrifugation, 5 µl of Laemmli 6× (0.3 M Tris–HCl pH 6.8, 10% SDS, 0.05% xylene cyanol/bromophenol blue, 15% β-mercaptoethanol) were added to 25 µl of each sample and loaded into a 10% SDS-PAGE gel. The electrophoresis was run at 100 V for around 2 h, and proteins were transferred to a PVDF membrane, previously activated with methanol, at 30 V and 4°C overnight. ECT2-mCherry protein was detected using the anti-mCherry antibody (ab183628, Abcam; Cambridge, UK) at dilution 1:10,000. Secondary antibody and detection procedure was carried out following the manufacturer’s instructions (ECL™ Prime Western Blotting System, Merck; Darmstadt, Germany).

**Molecular simulations**

We performed implicit-solvent coarse-grained simulations of IDR solutions using the CALVADOS 2 forcefield (Tesei et al., 2021; Tesei & Lindorff-Larsen, 2023) which uses a representation of the protein
with one bead per residue. Beads of neighboring residues were linked by harmonic bonds with an equilibrium distance of 0.38 nm and force constant of 8,033 kJ/mol/nm². Nonbonded interactions were modeled with a hydrophobic amino acid “stickiness” model, truncated at 2 nm (Tesei & Lindorff-Larsen, 2023). In addition, we used a truncated (rc = 4 nm) and shifted Debye–Hückel potential to model salt-screened electrostatic interactions. Simulations were performed using a Langevin integrator (γ = 0.01 ps−1) with a 0.01 ps time step.

We used a previously described procedure (Dignon et al., 2018; Tesei & Lindorff-Larsen, 2023) to perform slab simulations of IDRs of ECT2 WT, ECT2 A5, ECT2 9x Y→A, hnRNPA1, and FUS. The sequence “ECT2 9x Y→A” has nine substitutions of tyrosine residues to alanine in region N5 of ECT2 IDR. All protein sequences are listed at https://github.com/KULL-Centre/_2023_martinez-perez-AMV-ECT. For each of the five different IDR sequences, we ran a slab simulation with 100 copies of the IDR, with simulation box sizes of 25 × 25 × 300 nm (ECT2 WT and variants) and 15 × 15 × 150 nm (hnRNPA1 and FUS). We carried out all simulations for 7,500 ns at T = 293 K and saved configurations every 1 ns. We considered the first 2,000 ns of each simulation as equilibration which was not used for analysis. In a postprocessing step, we centered the protein condensates in the z-dimension of the simulation box. We obtained dense phase and dilute phase concentrations (Cdense and Cdlute, respectively) by fitting the time-averaged concentration profiles to a hyperbolic function (Tesei et al., 2021). We calculated excess transfer free energies via ΔGtrans = RT ln (Cdlute/Cdense) (Benayad et al., 2021).

Data availability

RNA-Seq and HyperTRIBE RNA-Seq data: PRJEB56577 (https://www.ebi.ac.uk/ena/browser/view/PRJEB56577).

Code to perform and analyze the molecular simulations is available via https://github.com/KULL-Centre/_2023_martinez-perez-AMV-ECT and https://github.com/KULL-Centre/CALVADOS.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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