Nuclear export of plant pararetrovirus mRNAs involves the TREX complex, two viral proteins and the highly structured 5′ leader region

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ABSTRACT

In eukaryotes, the major nuclear export pathway for mature mRNAs uses the dimeric receptor TAP/p15, which is recruited to mRNAs via the multisubunit TREX complex, comprising the THO core and different export adaptors. Viruses that replicate in the nucleus adopt different strategies to hijack cellular export factors and achieve cytoplasmic translation of their mRNAs. No export receptors are known in plants, but Arabidopsis TREX resembles the mammalian complex, with a conserved hexameric THO core associated with ALY and UIEF proteins, as well as UAP56 and MOS11. The latter protein is an orthologue of mammalian CIP29. The nuclear export mechanism for viral mRNAs has not been described in plants. To understand this process, we investigated the export of mRNAs of the pararetrovirus CaMV in Arabidopsis and demonstrated that it is inhibited in plants deficient in ALY, MOS11 and/or TEX1. Deficiency for these factors renders plants partially resistant to CaMV infection. Two CaMV proteins, the coat protein P4 and reverse transcriptase P5, are important for nuclear export. P4 and P5 interact and co-localise in the nucleus with the cellular export factor MOS11. The highly structured 5′ leader region of 35S RNAs was identified as an export enhancing element that interacts with ALY1, ALY3 and MOS11 in vitro.

INTRODUCTION

Nuclear mRNA export is central to gene expression and has been extensively studied and documented for many years in yeast and metazoa. To be efficiently exported transcripts must undergo several maturation steps including capping (1), splicing (2) and 3′ end formation (3). During this co-transcriptional maturation, which (except for capping) occurs in the nuclear perispeckles (2), maturation and export factors are recruited to transcripts forming large messenger ribonucleoproteins (mRNPs). These factors allow mRNPs to dock with the nuclear basket of the nuclear pore complex (NPC), transit the central channel and be released from the cytoplasmic fibrils (4–6). The major transporter for mRNPs is the protein heterodimer TAP/p15 (also known

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as NXF1/NXT1 in metazoa and Mex67/Mtr2 in yeast). Although TAP can directly bind single stem-loop structures called constitutive transport elements (CTE) (7,8), in the case of spliced mRNAs, TAP is recruited by adaptor proteins rather than by direct sequence-specific RNA binding (5,9). A key player in mRNP biogenesis, maturation and TAP/p15 recruitment is the transcription and export (TREX) complex. TREX is conserved across a wide range of organisms (5). TREX-I comprises a hexameric core called THO (10) and some important additional proteins such as the ALY adaptors (11) and their functional homologues UIF (12), UAP56 (13) and CIP29 (14). ALY and THO are rapidly recruited to nascent mRNAs by the cap-binding complex (CBC) (15–17), which associates with the 5′ cap. During splicing, exon junction complexes (EJCs) are deposited on mRNAs and additional ALY copies are then loaded upstream of the exon-exon junctions (18) or transferred from the CBC (17). The DEAD-box RNA helicase UAP56, also considered a component of the EJC (18), associates with both ALY and CIP29 in an ATP-dependent manner (19). Finally, the TREX adaptor proteins, and mostly ALY, together with the THO subcomplex, act as a binding platform for the export receptor TAP (and its associated p15 protein) (20), which directs the export-competent mRNPs to the nuclear pore (21). UAP56 and ALY are released before or during translocation through the NPC (22).

Although splicing is essential for the nuclear export of mature mRNAs, intronless or intron-retaining mRNAs can also recruit TREX components and adaptors to load the export transporters TAP/p15. In such cases the association between the CBC and TREX complex is crucial (23), as is probably the ability of the scanning spliceosomes to deposit (with lower efficiency) EJC and mRNA export factors on single-exon transcripts (17). Intronless mRNAs probably use different strategies to attract TAP/p15: histone mRNAs bind SR adaptor proteins that directly recruit TAP/p15 (24–26), whereas other intronless cellular mRNAs harbour conserved cytoplasmic accumulation regions (CARs) that interact with TREX components (27,28).

The nuclear export of mRNAs remains poorly studied in plants, where only the composition of the Arabidopsis TREX complex is well-described (29). With some minor differences, the hexameric THO subcomplex resembles that of metazoa (30). These differences may be important to allow plants to export different RNA populations and to adapt to changing environmental conditions. ALY adaptor proteins (ALY1–4) are encoded by four genes that are ubiquitously expressed in vegetative cells in Arabidopsis (31). All four ALY's localise to the nucleus, interact with UAP56, and bind to single and double-stranded RNA (32). Single and double *aly* knockout plants do not exhibit visible phenotypes. Quadruple *aly1 aly2 aly3 aly4* mutants (*4xaly*) show defects in growth, flowering and seed production, and a nuclear accumulation of mRNAs, suggesting inefficient nuclear export of mRNAs (32). Two UIF-like proteins have also been described in Arabidopsis: they bind to RNA and UAP56 and cooperate with ALY1–4 to mediate efficient nucleocytoplasmic mRNA transport (33). The DEAD-box RNA helicase UAP56 is encoded by two adjacent genes in Arabidopsis. The resulting identical proteins interact with single and double-stranded RNA, and with ALY and MOS11 proteins (34). MOS11 is the Arabidopsis orthologue of CIP29, also a nuclear protein; *mos11* knockout plants show nuclear accumulation of mRNAs (35). The Arabidopsis genome does not encode orthologues of TAP, the main mRNP transporter that is loaded via interactions with ALY-like adaptor proteins in yeast and metazoa. Plants may therefore use different exportin strategies to direct and transport mRNPs through the nuclear pore (29).

Like all living organisms, yeast, plants and metazoa are exposed to diverse viral infections. Many DNA and RNA viruses with nuclear replication steps in their life cycles export viral mRNAs by hijacking the TAP/p15 pathway (36). Herpesviruses, in which most mRNAs are intronless, have evolved highly efficient mechanisms for their maturation and export. They use a conserved multifunctional viral protein (ICP27 for herpes simplex virus 1 (HSV1) or its homologue ORF57 for herpes viruses saimiri and Kaposi sarcoma herpesvirus (KSHV), for example), which interacts with both viral transcripts and cellular adaptors such as ALY, UIF and CIP29, recruiting the whole TREX complex and enhancing viral mRNA accumulation and export (37–43). Simple retroviruses, such as Mason-Pfizer monkey virus (MPMV) use highly structured CTEs on their mRNAs that directly bind TAP with high affinity (44). Hepatitis B (pararetrovirus) (HBV) mRNAs also harbour a structured region named the posttranscriptional regulatory element (PRE), which is necessary for TREX recruitment on intronless viral mRNAs and efficient expression of viral proteins (45).

The paraphyletic group Pararetrovirus, whose members replicate their circular double-stranded DNA genomes by reverse transcription, not only includes the vertebrate Hepadnaviridae family but also plant Caulimoviridae. Caulimoviridae together with Geminiviridae, Nanoviridae (both with circular single-stranded DNA genomes) and Nucleorhabdoviruses (with single-stranded negative-sense RNA genomes) induce synthesis of viral mRNAs in the nuclei of the infected plant cells. These mRNAs are then necessarily exported into the cytoplasm to be translated and to enable viral replication. In plants, the general mechanism of the nuclear export of mRNAs is poorly characterised. Moreover, nothing is known regarding how phytoviruses export viral mRNAs from the nucleus. To address this question, we investigated the nuclear export of mRNAs of Cauliflower mosaic virus (CaMV), the type member of the family Caulimoviridae.

The CaMV replication cycle includes two main steps, one in the nucleus and one in the cytoplasm: (a) Following entry into the plant cell and disassembly of the capsid proteins on the nuclear envelope (46), the viral dsDNA is imported into the nucleus, where it associates with histones to form a minichromosome that is used as a template for transcription by the host DNA-dependent RNA polymerase II (Pol II). This produces a capped and polyadenylated polycistronic pregenomic 35S RNA (pgRNA) which comprises the complete genome and encodes six major proteins (P1 to P6). Pol II transcription also produces a monocistronic subgenomic 19S RNA encoding a single protein, P6 (47). Although splicing rarely occurs for plant viruses, about 70% of the total CaMV 35S RNA undergoes
alternative splicing, which generates four spliced isoforms and involves four splice donor sites and a single splice acceptor site (48). Alternative splicing appears to be a conserved and complex phenomenon since upon inactivation of splice sites by mutagenesis it is rescued by the activation of numerous cryptic splice donor and acceptor sites, highlighting the key role of this process in CaMV biology (49). (b) All these different mRNA isoforms are then exported to the cytoplasm, where their translation and the reverse transcription of pgRNA occur. The six viral proteins are as follows: the P1 movement protein; the P2 aphid transmission factor; the P3 capsid-associated protein; the P4 coat protein; the P5 polyprotein homologous to the retrovirus Pol protein which harbours a C-terminal reverse transcriptase P5 are important for viral mRNA export in Arabidopsis (47). The newly synthesised dsDNA is packaged into the virions to move from cell to cell and be transmitted from plant to plant.

In this study, we provide the first description of the nuclear export of mRNAs of a plant virus, CaMV. Our data demonstrate that CaMV exports its 35S polycistronic RNAs using the TREX export complex, and that MOS11, the adaptor protein ALY and the THO component TEX1 are required for efficient 35S mRNA export in Arabidopsis. We also show that the viral capsid protein P4 and the reverse transcriptase P5 are important for viral mRNA export, that they both interact with MOS11 in vitro and in protoplasts, and that they both co-localise with this export factor in the nucleus. We provide evidence that the highly structured 5′ untranslated region (5′ UTR) of the 35S RNAs, also called the leader region, acts as an export enhancing element, which can load ALY1, ALY3 and MOS11 proteins in vitro.

MATERIALS AND METHODS

Plant material and growth

For virus infection (CaMV or TuMV) Arabidopsis (Arabidopsis thaliana) Col-0 was grown on soil in a growth chamber, with 12 h photoperiod at 22°C and 12 h dark at 18°C, while for protoplast preparation, plants were grown on MS medium (52). In the latter case, after sowing, seeds were stratified in darkness for at least 24 h prior to incubation in the plant growth chamber under long-day conditions (16 h photoperiod at 22°C, 8 h dark at 18°C) for 10 ± 2 days.

The CA-rop2 mutant was described in (53) and kindly provided by Z. Yang. Seeds of the mos11-2 T-DNA insertion line (SAIL_266_E03) (35) were obtained from the Arabidopsis Biological Resource Center (ABRC) and characterized by PCR-based genotyping to distinguish between plants that are heterozygous or homozygous for the T-DNA insertion. PCR was performed on genomic DNA isolated from leaves, using the ABRC recommended primers specific for the T-DNA insertion and the target gene mos11 (data not shown). Arabidopsis double mutant tex1 mos11 was described in (54) while double mutants aly1 aly2 and aly3 aly4, and quadruple aly1 aly2 aly3 aly4 mutant (4xaly) were characterised in (32).

Viruses and host inoculation

All CaMV experiments were performed with the Cabb B-JI isolate (John Innes Institute, Norwich, GB), by mechanical inoculation of young rosette stage (4-leaf) plants with infectious Arabidopsis sap. TuMV-GFP (UKI isolate) (55) infectious sap from Brassica napus cv. Drakkar, was provided by Manfred Heinlein and inoculated in the same manner.

Plasmids

Details of the plasmid constructs are given in the Supplementary Data at NAR online.

Analysis of CaMV proteins and genomic DNA in infected Arabidopsis plants

Details are given in the Supplementary Data at NAR online.

Arabidopsis protoplast isolation and transfection

Protoplasts were isolated as described by (56) and adapted by Bouton (49). Briefly, 10-day-old in vitro grown Arabidopsis seedlings were finely chopped and incubated overnight, at 26°C under gentle shaking (50 rpm) in 25 ml enzyme solution (20 mM MES pH 7.5 – 0.4 M mannitol – 20 mM KCl – 10 mM CaCl₂ – 1.5% cellulase R10 – 0.4% macerozyme R10). The digestion medium was then filtered to eliminate leaf debris, washed in 20 ml 2 mM MES pH 5.7 – 154 mM NaCl – 125 mM CaCl₂ – 5 mM KCl solution and gently centrifuged to remove enzymes, and protoplasts were finally suspended in 4 mM MES pH 5.7 – 0.4 M mannitol – 15 mM MgCl₂ medium, counted and adjusted to 10⁶ protoplasts/ml.

Transfection was performed as described by (56) with modifications: 300 µl protoplasts were mixed with 300 µl 2 mM MES pH 5.7 – 0.2 M mannitol – 30% PEG4000 – 100 µM CaCl₂ – DNA transfection solution (10 µg expression plasmid and 10 µg carrier plasmid) and incubated during 15 min at room temperature. Transfection was stopped with 1 ml 2 mM MES pH 5.7 – 154 mM NaCl – 125 mM CaCl₂ – 5 mM KCl solution; protoplasts were centrifuged, suspended in 1 ml 4 mM MES pH 5.7 – 0.2 mannitol – 20 mM KCl and incubated for 20 to 26 h in the dark at 23°C. The ratio of transfected fluorescent protoplasts, expressing EGFP and/or mRFP, was estimated on Axio Zoom V.16 microscope (Zeiss) at 10-fold magnification and excitation at 488 nm (EGFP), 561 nm (mRFP) and 660 nm (chlorophyll).

Subcellular fractionation of transfected Arabidopsis protoplasts

The transfected protoplasts were centrifuged 5 min at 100 x g, cell pellets were suspended in 100 µl hypotonic buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 0.3% NP-40) and centrifuged again 10 min at 5000 x g. 90 µl of supernatants (cytoplasmic fractions) were harvested and pellets (nuclear fractions) washed 3 times in hypotonic buffer and finally...
suspected in 90 µl hypotonic buffer. Total fractions are prepared by suspending the protoplasts in 100 µl hypotonic buffer. Purity of the fractions was determined by western blot detection of nuclear histone H3 and cytoplasmic UDP-glucose pyrophosphorylase 1 (UGPase).

RNA extraction and analysis by real time RT-PCR

RNAs were extracted from total, cytoplasmic and nuclear fractions with TRI- Reagent (Sigma), applied on Phase- maker columns (Invitrogen), according to the manufacturer’s instructions, and finally suspended in RNase-free water. RNAs were further treated with TURBO DNase (Invitrogen) for 30 min at 37°C and analysed by PCR to monitor digestion efficiency. cDNAs were synthesised using the same amounts of RNAs for all samples with SuperScript IV and in presence of Oligo(dT)20 Primers and Random Hexamers, according to the manufacturer’s instructions (Invitrogen).

CaMV 35S RNAs in total, cytoplasmic and nuclear fractions were quantified through their respective cDNAs, diluted to 1/20, with LightCycler™ 480 SYBR Green I Master (Roche Life Science), and 35S RNA specific primers, targeting either the P5 coding sequence: TATAGGCCCA ATGATCGTG (FW) and CTTCTGGCTTTACCTTCG (REV) for RISP transfection efficiencies.

Protein purification

Details are given in the Supplementary Data at NAR online.

GST pull-down assays

Using plasmids pGEX-6P1, GST and GST-MOS11 were expressed in Escherichia coli BL21 (DE3) and affinity captured on glutathione sepharose 4B (GE Healthcare) as described by (57). Briefly, after 4h IPTG induction at 20°C, 500 ml bacterial cultures were harvested, suspended in 30 ml 50 mM Tris–HCl pH 7.5 – 1 M NaCl – 2 mM DTT – 0.1 mM EDTA – 0.5% NP-40 – Protease Inhibitors (Roche) – 10 U RNase-free DNase (Promega Corporation), and lysed by sonication. Soluble protein fractions, obtained after centrifugation, were incubated over night with 500 µl of 50% slurry of glutathione sepharose beads and washed 3 times with the same buffer at 150 mM NaCl. Protein capture on beads was analysed by SDS-PAGE and Coomassie Blue staining.

P3, P4, P5 and its truncated versions were in vitro transcribed from the T7 promoter present in pGADT7 (Clontech) and translated in the presence of 10 µCi 35S-methionine in TnT T7 Coupled Reticulocyte Lysate System (Promega Corporation), according to the manufacturer’s instructions. For in vitro binding analyses, 50 µl of GST or GST-MOS11 proteins on glutathione- Sepharose 4B beads (50% slurry) were incubated on a rotating device with 10 µl of each in vitro synthesized CaMV protein in a final volume of 200 µl. The beads were then washed three times, pelleted at 500 × g for 5 min, and boilled in SDS-PAGE sample buffer. GST and GST-MOS11 bound and unbound fractions were analysed by SDS-PAGE and autoradiography.

For EMSA, affinity-bound GST-MOS11 was eluted by 10 µg GST-coupled PreScission Protease (produced and provided by Nicolas Baumberger) during 2 × 16 h at 4°C. Cleaved MOS11 was harvested in the supernatants after 5 min centrifugation at 500 × g at 4°C.

Co-immunoprecipitation assays

EGFP-P4 or EGFP-P5 and MOS11-mRFP co-transfected protoplasts were incubated in the dark for 20h at 23°C. The co-transfected protoplasts were next pelleted at 1000 × g for 5 min and lysed for 15 min at 4°C, as described by (58) (Tris–HCl 50mM, pH7.5 – NaCl 150 mM – EDTA 5 mM – DTT 1mM – Triton X-100 1% – Complete Protease Inhibitors, Roche). Cellular debris were then removed by centrifugation for 5 min at 1000 × g 4°C and the supernatants were incubated with 7 µl anti-EGFP-coated magnetic beads (kindly prepared and provided by Quentin Chevalier & Vianney Poignavent) on a rotating device for 16h at 4°C. Protein capture on the beads was analysed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-EGFP (Sigma Aldrich, SAB-2702214).

Fluorescence localisation analysis

Fluorescent Arabidopsis protoplasts, transfected with expression plasmids coding for EGFP, mRFP and/or proteins fused with these fluorescent proteins, were observed at 63-fold magnification between a polysilene slide and cover slip, with a silicone spacer in-between, on a Zeiss LSM780 confocal microscope (Jena, Germany). EGFP and mRFP were viewed by excitation, respectively, at 488 nm and 561 nm, with an argon laser using an appropriate emission filter to collect the green or red signal from the optical section.

EMSA

L-VII and I-II RNAs were in vitro produced by T7 RNA Polymerase (Thermo Scientific) from 1 µg BamHI
linearized Litmus28i, according to the manufacturer’s instructions and in presence of 20 μCi [γ-32P]-UTP. Radiolabelled RNAs were purified by acidic phenol/chloroform treatment and isopropanol precipitation, and suspended in 50 μl RNase-free water with 20 U Ribolock RNase Inhibitor (Thermo Scientific).

Two μl (~5000 cpm) radiolabeled RNAs were incubated for 60 min at 25°C with increasing amounts (0–20 μM) of purified ALY1-GB1-His6, His6-GB1-ALY3, His6-GB1-EGFP or MOS11, in a final volume of 20 μl protein storage buffer. Four μl non-denaturing loading buffer 6× (30% (v/v) glycerol–0.3% bromophenol blue–0.3% xylene cyanol) were then added and protein–RNA complexes were analysed by non-denaturing 45 min gel electrophoresis at 100 V (TBE 0.5, pH 10.5–4% (v/v) acrylamide–bisacrylamide (37.5:1)–4% glycerol (v/v)), with a 30 min pre-equilibration run at 100 V and TBE 0.5 (pH 10.5), and subsequent analysis by Amersham Typhoon Biomolecular Imager.

**Luciferase assays**

Fifty μl of Arabidopsis protoplast suspensions transfected in triplicate with the luciferase reporter constructs Litmus28i-Luc were incubated for 10 min with 50 μl Promega Bright-Glo Reagent (Promega). The luciferase activities were measured in duplicate in a BMG LABTECH FLUOstar® Omega plate reader, averaged and normalised to the mRFP fluorescence levels, which acted as a transfection rate control.

**RESULTS**

**Assessment of nuclear export of CaMV 35S RNAs in Arabidopsis WT and CA-rop2 protoplasts**

To measure the 35S RNA nuclear export, we set up a protoplast transfection assay. Protoplasts were prepared from WT Arabidopsis seedlings and transfected with the 14 kbp pCaMV-GFP plasmid (60) containing an infectious CaMV genome controlled by the 35S promoter and a reporter GFP in a separate expression cassette for monitoring transfection efficiency. The detection of the different viral proteins and the newly synthesised and encapsidated viral DNA suggested that the time required for one round of replication of CaMV in transfected protoplasts is about 21 h (60).

The transfection efficiency of the 14 kbp viral genome encoding plasmid in WT Arabidopsis protoplasts was not always sufficiently high to allow accurate quantification of CaMV RNAs by real-time RT-PCR. Conversely, Arabidopsis CA-rop2 protoplasts were systematically and reproducibly transfected with high efficiency with pCaMV-GFP. CA-rop2 Arabidopsis express the constitutively active GTPase ROP2 (Rho-related GTPase from plants) which is closely associated with the plasma membrane (61) and was shown to control multiple developmental processes such as seed dormancy, shoot apical dominance and lateral root initiation (53).

To exclude the possibility that the active ROP2 GTPase influences the nuclear export of viral mRNAs, we compared CaMV 35S RNA nuclear export in WT and CA-rop2 protoplasts. At 20 hours post-transfection (hpt), RNAs were isolated from the total and enriched cytoplasmic and nuclear fractions (Figure 1C), and quantified by RT-qPCR using primers targeting the P5 coding region present on all 35S RNA isoforms. As shown, no significant difference was observed in the accumulation of the total 35S RNAs (A), or of the 35S RNA cytoplasmic-to-nuclear ratios (C/N) (B). Significance was tested with a Student’s unpaired two-sample t-test (ns, not significant, P > 0.05). The purity of fractions was assessed by analysing cytoplasmic marker protein UDP-glucose pyrophosphorylase 1 (UGPase) and nuclear histone H3 by western blotting (C). Detection of EGFP acted as a loading control (LC).

![Figure 1. Total 35S RNA accumulation and nucleocytoplasmic partitioning in WT and CA-rop2 protoplasts](image-url)
pared in at least three independent experiments. In agreement, mechanically inoculated WT or mutant plants were compared to all reference Cts measured in WT, suggesting the importance of these components of the TREX complex for the nuclear export of viral mRNAs and the outcome of CaMV infection. Interestingly, although 35S RNA nuclear export is less efficient in the tex1 mos11 double mutant (Figure 2A), the viral life cycle is not more severely affected in tex1 mos11 than in mos11 (Figure 3A).

The situation was different for the ALY adaptor mutants (Figure 3B). Compared to WT, aly1 aly2 and aly3 aly4 double mutants showed no delay in symptom appearance (at 14 dpi). However, the aly1 aly2 double mutant reproducibly showed lower infection levels at 17 and 19 dpi. Infection by CaMV was severely inhibited in the 4xaly quadruple mutant (Figure 3B), with only 20% of systemically infected plants at 40 dpi versus 92% for the WT. Among all TREX-deficient mutants, 4xaly exhibited the most severe inhibition of the nuclear export of CaMV mRNAs (Figure 2B). Therefore, it is unsurprising that this significant nuclear retention of viral mRNAs blocked the viral life cycle in most of the inoculated plants.

Considering that the nuclear export of 35S RNAs was also strongly inhibited in aly1 aly2 and aly3 aly4 double mutants, although to a lesser extent than in 4xaly (Figure 2B), one might expect that both double mutants would be less susceptible to CaMV infection. However, our results show that this is not the case, suggesting that the inhibition of viral infection in 4xaly might be due not only to impaired viral mRNA export from the nucleus but also to a general nuclear retention of cellular mRNAs (as observed for housekeeping genes) some of which might encode host factors important for the virus.

To confirm that the partial resistance of TREX mutant Arabidopsis to CaMV infection was mainly due to the impairment of nuclear export of viral mRNAs, we first examined the susceptibility of the same TREX complex mutants to turnip mosaic virus (TuMV), a Potyvirus with single-stranded positive-sense RNA genome and a strictly
Figure 2. Nuclear export of 35S RNAs is inhibited in TREX export mutants. WT/CA-rop2, tex1, mos11, tex1 mos11, aly1 aly2, aly3 aly4 and 4xaly protoplasts were transfected with pCaMV-GFP. At 20 hpt, the cytoplasmic-to-nuclear ratios (A, B) or the total levels (C, D) of 35S mRNAs were determined as described in Figure 1. Cts of the cellular reference genes and of GFP, measured in WT protoplasts and the single and double mutants were averaged and used for normalisation in 4xaly. Three independent experiments were performed in triplicate. The results are expressed as the mean fold change ± SEM. Significance was tested with a Student’s unpaired two-sample t-test (ns, not significant, *P > 0.05, **P < 0.05, ***P < 0.01, ****P < 0.001).

cytoplasmic replication cycle (62). For this, we used a recombinant TuMV encoding a GFP tag (TuMV-GFP) (55), the expression of which is easily followed under UV light and reflects viral replication. As shown in Supplementary Figure S3, most TuMV-GFP infections were detected at 7 dpi indifferently on WT and mutant plants. TuMV-GFP infection spread more rapidly than that of CaMV, because the maximal number of systemic plants was reached at 10 dpi for almost all WT or mutant Arabidopsis (Supplementary Figure S3).

Additionally, no significant differences were recorded in the ratios of infected plants between WT and the six assessed single, double and quadruple mutants (Supplementary Figure S3). Our results show that the TuMV-GFP replication cycle was affected in none of the TREX mutants that we tested, which is unsurprising considering that all viral RNAs here are synthesised in the cytoplasm and do not require nuclear export. However, these observations also suggest that even in the strongest export mutant, 4xaly, the cellular mRNAs encoding host factors essential for TuMV can still be exported from the nucleus, possibly via the ALY analogue UIFs (33) or shuttling SR proteins that act as export adaptors in other organisms (5).

Finally, we also investigated in WT and TREX mutant Arabidopsis protoplasts, transfected with pCaMV-GFP, the nuclear export of four cellular mRNAs coding proteins that are either essential or important for the CaMV life cycle. We focused on three proteins that interact with the viral protein P6: TOR, whose partial depletion makes Arabidopsis resistant to CaMV (63); RISP, for which disruption of one of the two encoding genes is responsible for delayed symptom appearance and viral protein accumulation (64) and CHUP1, silencing of which results in a delay of CaMV lesion formation (65). We also analysed a protein that interacts with the viral protein P1: PDLP1, whose genetic disruption delays CaMV systemic invasion and causes milder symptoms (66).
Figure 3. TREX-deficient plants are partially resistant to CaMV. (A) WT, tex1, mos11 and tex1 mos11 plants and (B) WT, aly1 aly2, aly3 aly4 and 4xaly plants were mechanically inoculated with CaMV crude extract and monitored for symptoms. The results are means of at least three independent experiments ± SEM. Significance was tested with a Student’s unpaired two-sample t-test (ns, not significant, \( P > 0.05 \), * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \)). Representative mock- or CaMV-inoculated systemic plants with enlargement of a symptomatic leaf (on the right bottom) are shown below the corresponding histograms.
As shown in Supplementary Figure S4, despite of the differences observed in the three different experiments neither the total amounts of the above-mentioned cellular mRNAs nor their nuclear export were significantly modified in the TREX mutants compared to WT Arabidopsis, suggesting that the partial resistance of these mutant plants to CaMV infection is not due to the disrupted export of these cellular mRNAs required for the viral life cycle.

**CaMV 35S RNA nuclear export depends at least on the expression of viral protein P5**

To determine whether CaMV proteins are involved in the nuclear export of viral mRNAs, we mutagenised the CaMV 35S RNA-encoding plasmid (pCaMV-GFP) by creating two STOP codons in the P6 coding sequence, at positions 9 and 15. In the absence of P6, synthesised from the monocistronic 19S RNA, translation reinitiation on the polycistronic 35S RNA is completely abolished, and no viral protein can be expressed (67–70). We generated two additional mutant genomes: the pCaMV-P5 PR, RT, RH and RT/RH (RR). We also included viral proteins P3 (15 kDa) and P4 (56 kDa) and firefly luciferase (Luc, ca. 60 kDa) as controls. Under these *in vitro* translation conditions, P5_FL was mainly synthesised as an uncleaved polypeptide precursor, since only a ≈ 78 kDa product was detected upon autoradiography (Figure 5A, P5_FL). Larger amounts of the P5_FL (and P4) proteins synthesised *in vitro* and a longer exposure time (Supplementary Figure S5A, left panel) allow several bands of lower intensity and molecular weights to be distinguished. These could correspond to degradation products, incomplete polypeptides or other proteins endogenously produced in the rabbit reticulocyte lysates. For P5, two of these bands, indicated by asterisks (Supplementary Figure S5A, left panel), co-migrate with the RT and PR domains and could correspond to peptides cleaved by the viral PR.

The P4 capsid protein is also synthesised during viral infection as a 56 kDa precursor (pre-CP), partially processed during morphogenesis in the viral factories by the virus-encoded PR into four subspecies: p42, p39, p37 and p35, all of them lacking the Nt and/or Ct domains of pre-CP and produced in variable amounts depending on the virus preparation (46,72–74). As observed (Figure 5A), *in vitro* synthesised P4 almost co-migrates with 78 kDa P5_FL and might correspond rather to incompletely denatured dimers of the FL precursor (72,73) or to abnormally migrating monomers. We cannot exclude the possibility that this band represents dimers of shorter P4 proteins, synthesised either by initiation with an internal AUG codon or by premature translation arrest. The P4 proteins were detected using specific polyclonal antibodies, but not in the P3 and P5 translation reactions (Figure 5A, bottom).

To determine whether the viral proteins or domains produced *in vitro* interact with components of the TREX complex, we performed GST pull-down assays (Figure 5C–F) with bead-bound GST or GST-MOS11 (Figure 5B). The viral and control proteins were mostly present in the GST unbound fraction (Figure 5C) except for the RT and RH domains of P5, for which a faint signal was also detected bound to GST (Figure 5E). Luciferase control and P3 were both found exclusively in the GST-MOS11 unbound fraction, whereas P5 PR and RH domains were almost equally bound and unbound (Figure 5D, F), indicating a weak association under our assay conditions (150 mM NaCl). P4, P5_FL, P5_RT and P5_RR were enriched in the GST-MOS11-bound fraction, demonstrating that they interact with MOS11 (Figure 5F).

In the GST-MOS11-bound fraction, we detected two forms of ‘full-length’ P5: the ca. 78 kDa translation product, corresponding to the P5_FL input (Figure 5A, FL), and a second polypeptide migrating slightly higher than RT and probably comigrating with the RR doublet (a second band of extremely weak intensity was also visible on Figure 5A (RR)) where the electrophoresis conditions were slightly
Figure 4. Nuclear export of 35S RNAs is inhibited in the absence of viral protein P5. WT/C4-rop2 Arabidopsis prooplasts were transfected pCaMV-GFP (1), pCaMV-P6_STOP (2), pCaMV-P5/P6_STOP (3) or pCaMV-P5_STOP (4–8). In the three STOP plasmids (in light grey) one (for P5) or two (for P6) STOP codons were introduced after the START AUG of viral proteins P5, P6 or both. In 5 to 8 (dark grey) pCaMV-P5_STOP was co-transfected with a plasmid supplementing in trans with myc-P5_STOP (5), full-length myc-P5 (myc-P5_FL, 6), P5 protease domain (amino acids (aa) 1–201, P5_PR, 7) or P5 reverse transcriptase/RNase H domain (aa 202–680, P5_RTRH, 8). At 20 hpt, the cytoplasmic-to-nuclear ratios (A) or the total levels (B) of 35S mRNAs were determined by P4-specific real-time RT-PCR and normalized to four cellular housekeeping genes, GFP (transfection indicator for pCaMV-P5_STOP) and mRFP (transfection indicator for the P5-expressing plasmids). Three independent experiments were performed in triplicate. The results are expressed as the mean fold change ± SEM of the 35S RNA cytoplasmic-to-nuclear ratios (C/N) (A) or of the total 35S RNAs (B). Significance was tested with a Student’s unpaired two-samples t-test (ns, not significant, \( P > 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \)). (C) Detection of P4, myc-P5 and EGFP (as loading control, LC) 20 hpt by western blot with, respectively, anti-P4, anti-myc or anti-EGFP antibodies in prooplasts transfected with pCaMV-GFP (1), pCaMV-P6_STOP (2), pCaMV-P5/P6_STOP (3), pCaMV-P5_STOP (4), pCaMV-P5_STOP + myc-P5_STOP (5), pCaMV-P5_STOP + myc-P5_FL (6), or pCK-EGFP + myc-P5_FL, and corresponding, respectively, to bars 1 to 6 in (A). Positions of molecular size standards, in kilodaltons, are shown on the left and on the right. The asterisks indicate the bands corresponding to P4 and to myc-P5_FL.
Figure 5. Viral proteins P4 and P5 interact with MOS11 in vitro. In vitro translated and [35S] methionine-labelled viral proteins P3, P4 and P5 (FL: full-length, PR: protease domain (aa 1–200), RT: reverse transcriptase domain (aa 201–547), RH: RNase H domain (aa 548–680), RR: reverse transcriptase/RNase H domains (aa 201–608)) and firefly luciferase (Luc) as control (A, autoradiography) were incubated with bacterially expressed and glutathione bead-bound GST or GST-MOS11 (B, Coomassie blue staining). The inputs of P4, P5FL and P3 were subjected to a western blot with specific anti-P4 antibodies (A, bottom). The beads were washed and the GST- and GST-MOS11 unbound (C, D) and bound (E, F) fractions were analysed by SDS-PAGE followed by autoradiography after 24h exposure. The results are representative of four independent experiments. The inputs in (A) and (B) represent 1/9 of the amounts used in the GST pull-down assay (C–E). 1/10 of the total unbound fractions were analysed by SDS-PAGE (C, E). LC: loading control. Positions of molecular size standards, in kilodaltons, are shown on the left in (B) and on the right in (A), (D) and (F).

different). This observation was confirmed in all four GST pull-down assays we performed (with various stringency conditions, Supplementary Figure S5B, right panel), suggesting that the interaction of full-length P5 with MOS11 might activate the P5 Nt protease domain, which then partially cleaves itself but is not retained on the GST-MOS11 beads, unlike the second cleavage product generated, (RR), which can still interact with MOS11. The cleaved PR peptide was not recovered in the GST-MOS11 unbound fraction, either because the analysed amounts were insufficient to reveal its presence, or because, since it weakly interacts with MOS11, and is in competition with FL and RTRH, it was released during the extensive washing steps.

The in vitro interaction results indicate that two viral proteins interact with nuclear export factor MOS11: P4 and P5, via its RT domain. Capsid protein P4 harbours, close to its N terminus, a nuclear localisation signal (NLS), which is exposed on the surface of mature virions when the acidic N-terminus (aa 1 - 77) is deleted (46). As MOS11 displays an exclusively nuclear localisation in Arabidopsis (35,54),
one could imagine that the interaction between P4 and MOS11, observed \textit{in vitro}, also occurs \textit{in vivo}. However, a critical point remained concerning the apparent inability of full-length P4 to localise in the nucleus \cite{46}, necessitating further investigations. The situation of P5 was even more complex, since among all viral proteins, P5 remains the least well-characterized and its nuclear localisation has not been unambiguously confirmed, although Pfeiffer and colleagues \cite{75} described CaMV DNA polymerase activity in infected nuclear extracts.

Both EGFP-tagged CaMV P4 and P5 proteins co-localise in cell nuclei and interact with MOS11-mRFP

We addressed the subcellular localisation of P4 and P5 by transfecting Arabidopsis protoplasts with plasmids expressing EGFP-P4 or EGFP-P5 under the control of a 35S promoter and also performed co-transfections with a MOS11-mRFP coding vector (Figure 6).

Because of its small size, EGFP (27 kDa) can enter nuclear pores; thus free EGFP is found in both the cytoplasm and nucleus in plant cells \cite{76,77} (Figure 6B, panels 1). As illustrated in Figure 6A, EGFP-P4 displayed three main distribution patterns in transfected protoplasts: 1. forming numerous exclusively cytoplasmic aggregates of different sizes (Figure 6A, line 4) as described by Karsies et al. \cite{46} for uncleaved P4 precursor; 2. forming one or several cytoplasmic aggregates but also slightly accumulating in the nucleus (6A, line 1); 3. forming cytoplasmic aggregates and strongly accumulating in the nucleus (6A, lines 3 and 4). Under co-expression conditions with strictly nuclear MOS11-mRFP (6B, panels 1; 6D, line 2) the subpopulations of EGFP-P4 forming only cytoplasmic aggregates did not move to co-localise with MOS11-mRFP (Figure 6B, panels 4). Conversely, those significantly accumulating in the nucleus merged with MOS11-mRFP but also remained as cytoplasmic aggregates (6B, panels 2 and 3).

To confirm that the observed green nuclear fluorescence was due to the fusion protein EGFP-P4 and not to cleaved EGFP, we performed fractionation experiments and western blotting with anti-EGFP polyclonal antibodies with the cytoplasmic and nuclear fractions of the transfected protoplasts (Figure 6E, left panel). As shown, three forms of EGFP-P4 of approximately 60, 80 and more than 100 kDa, were detected in both fractions. Similar observations have been described for untagged P4 expressed in cis, between GFP and GUS, P5 predicted bipartite NLSs (either the first motif (NLS1, aa 276–305), or the second (NLS2, aa 308–339) or both (NLS1+2, aa 276–339). Arabidopsis protoplasts were transfected with the respective constructs, together with the one encoding MOS11-mRFP that we used as a nuclear indicator (Supplementary Figure S6B). Used individually (Supplementary Figure S6C, D) or together (Supplementary Figure S6E), the two P5 putative NLSs allowed GFP-GUS to move into the nucleus, where it merged with MOS11-mRFP, in the same way as the previously described bipartite NLS of CaMV protein P6 (Supplementary Figure S6F) \cite{81}.

To provide additional evidence supporting the interactions between MOS11-mRFP and EGFP-P4 or EGFP-P5, we performed co-immunoprecipitations in the transfected protoplasts, with immunocapture of the EGFP-tagged viral proteins and controls (Figure 6F, right panels). Again, we observed the typical P4 \textit{in vivo} expression pattern comprising three bands of > 100, > 70 and > 55 kDa. Importantly, MOS11-mRFP could be co-precipitated with EGFP-P5 and EGFP-P4 but not with the controls (EGFP and mock) (Figure 6F, left panel) suggesting that the \textit{in vitro} observed interactions also occur \textit{ex vivo}.

The leader region of CaMV 35S RNAs is an export-enhancing element

To seek a potential \textit{cis}-acting element on 35S RNA we performed competition assays for the nuclear export machinery between CaMV 35S RNAs and shorter RNA fragments (289 to 865 nt) corresponding to the 35S 5′ end (nt 1–3000) or to its 3′ UTR (Figure 7A).

For this purpose, Arabidopsis protoplasts were co-transfected with pCaMV-GFP and an eight-fold molar
Figure 6. EGFP-P4 and EGFP-P5 both co-localise and interact with nuclear MOS11-mRFP. WT/CA-rop2 Arabidopsis protoplasts were transfected (or co-transfected) with (A, E) pCK-EGFP-P4, (B) pCK-EGFP + Litmus-35S-MOS11-mRFP or pCK-EGFP-P4 + Litmus-35S-MOS11-mRFP, (C, E) pCK-EGFP-P5 (line 1) or Litmus-35S-MOS11-mRFP (line 2) and (D) pCK-EGFP-P5 + Litmus-35S-MOS11-mRFP. Observations of EGFP and mRFP fusion proteins were made 20 hpt by LSCM. The LSCM settings and acquisition conditions of the images were identical in all panels. In (B) and (D) panels 1 to 4, and 1 to 3, respectively are projections while all other images show single sections. G: EGFP; R: mRFP; M: merge; D: differential interference contrast (DIC) images that indicate the localisation of chloroplasts and nuclei. Scale bars: 5 μm. In (E) the pCK-EGFP-P4 or pCK-EGFP-P5 transfected protoplasts were subjected to nucleocytoplasmic fractionation and analysed by western blotting for the presence of full-length EGFP-P4, EGFP-P5, cytoplasmic UGPase and nuclear H3. LC: loading control. Interaction of fusion proteins EGFP-P4 and EGFP-P5 with MOS11-mRFP was analysed in transfected protoplasts by co-immunoprecipitation (F) with anti-EGFP magnetic beads. Pulled-down proteins were detected by western blot with anti-EGFP and anti-mRFP antibodies. Positions of molecular size standards in kilodaltons are shown on the left in (E) and (F).
Figure 7. Co-expression of 5′ UTR inhibits the nuclear export of 35S RNAs. (A) Structure of the capped and polyadenylated 35S RNA: the 5′ UTR (or leader (L) in red), 608 nt long, folds into a stable multibranched stem–loop (SL), and precedes the 7 ORFs (VII, I, III, IV, V and VI) and the 3′ UTR (nt 7,928–8,216). The five RNA fragments used in the competition assays and overlapping the leader and ORF VII (L–VII), ORF I and II (I–II), ORF II, III and IV (II–IV) and the 3′ UTR are shown with double-headed arrows. The position of the primer (tRNAMeti) binding site (PBS, nt 596–609) is indicated by a black arrow. The coat protein P4 binding site is shown with a black circle. (B) to (E) WT/CA-rop2 Arabidopsis protoplasts were transfected with pCaMV-GFP (black), or co-transfected with pCaMV-GFP and a 10-fold molar excess of Litmus-mRFP-35S-(L–VII) (light grey), Litmus-mRFP-35S-(I–II), Litmus-mRFP-35S-(II–IV) or Litmus-mRFP-35S-(3′ UTR) (dark grey). At 20 hpt, the cytoplasmic-to-nuclear ratios (B) or the total levels (C) of 35S mRNAs were determined by P5-specific real-time RT-PCR and normalised to a set of four cellular housekeeping genes, GFP (transfection indicator for pCaMV-GFP) and mRFP (transfection indicator for the RNA fragments expressing plasmids). Three independent experiments were performed in triplicate. The results are expressed as the mean fold change ± SEM. Significance was tested with a Student's unpaired two-sample t-test (** P < 0.01).

(D) Detection of the five competition RNA fragments by RT-PCR in total RNA fraction, with primers specific to their common short 5′ and 3′ sequences: (L–VII): 765 bp, (VII–I): 674 bp, (I–II): 865 bp, (II–III): 696 bp and (3′ UTR): 289 bp. (E) PCR positive controls, directly amplified with the same primers from the respective Litmus-mRFP-35S plasmids. Positions of molecular size standards, in bp, are shown on the left.

While the co-expression of RNAs VII–I, I–II, II–IV or of the 3′ UTR did not significantly influence the nuclear export of 35S RNAs, that of the L–VII fragment induced a strong nuclear retention of 35S viral RNAs (Figure 7B), suggesting a possible hijacking of the export machinery by the overexpressed RNA fragment. This mainly corresponds to the 35S leader region, and was detected by RT-PCR in total RNA fractions of transfected protoplasts, as for the four other RNA fragments (Figure 7D, E). The total 35S RNAs accumulation was unaffected by the co-expression of any of the RNA fragments (Figure 7C), indicating excess of Litmus-mRFP-35S plasmid encoding one of the five short RNA fragments: (i) RNA L–VII corresponding to nt 1–765 of 35S RNA and encompassing the 5′ UTR, or leader, and the first 157 nt of ORF VII; (ii) RNA VII–I (nt 766–1439) overlapping ORFs VII and I; (iii) RNA I–II nt (1440–2304) overlapping ORFs I and II; (iv) RNA II–IV (nt 2305–3000) overlapping ORFs II, III and IV and (v) 3′ UTR corresponding to the terminal nt 7928–8216 (Figure 7A). Total RNAs were then isolated from nuclear, cytoplasmic or total fractions, and 35S RNAs were quantified by real-time RT-PCR (Figure 7B, C).
that transcription efficiency was not modified under these conditions.

The L–VII RNA fragment (Figure 7A) that we propose to target the nuclear export machinery, contains in 5′ a 74-nucleotide unstructured sequence harbouring a short ORF (sORF A, nt 57–68), which is essential for ribosome shunting during translation (82). It is followed by a stable helical section of a large stem-loop secondary structure of 482 nt (83) containing five additional sORFs (for the Cabb B-JI isolate) but also the poly(A) signal and the first splicing donor (SD) site located, respectively, on the ascending and descending arms of a middle section of the stem-loop structure (84). The 35S pregenomic RNA packaging signal that interacts with the P4 coat protein is exposed on the uppermost section of the stem-loop structure (nt 288–355) (85). The 209 nt following the stem-loop and preceding ORF VII are probably unstructured and end with the tRNA\textsuperscript{Met} primer binding site (PBS) (nt 596–609), where the P5 RT is loaded (86).

To further investigate whether some sections of the L–VII fragment are more specifically involved in the nuclear export, we split the sequence into eight different sections (Supplementary Figure S7C, upper part): the stem–loop (SL), its left (SL\_Left) and right (SL\_Right) arms, the bottom (SL\_Bottom) and top (SL\_Top) sections, the 5′ (L\_5′) and 3′ ends (L\_3′) of the leader, and the single-stranded sequence preceding ORF VII (SS\_3′). The respective plasmids were then co-transfected, together with pCaMV-GFP, in protoplasts, and the nuclear export of 35S RNAs was analysed as previously described (Supplementary Figure S7A). The important nuclear retention of 35S RNAs was confirmed in this assay. Moreover, to a lesser extent, a significant inhibition effect was observed only with the co-expression of the SL.

These results suggest that two elements might be recognised by the export machinery, the highly structured SL and an additional element located at sequences upstream or downstream. Disrupting the SL structure in separate arms (SL\_Left with the poly(A) signal, and SL\_Right with the SD site) or expressing only sections of it (SL\_Bottom, SL\_Top with the packaging signal, L\_5′, L\_3′ and SS\_3′ both containing the PBS) completely abolished the competition for the export proteins, between these short RNA fragments and the 35S RNAs that were here efficiently exported into the cytoplasm (Supplementary Figure S7A). The total 35S RNA accumulation was again unchanged by the expression of these additional RNA fragments (Supplementary Figure S7B), detected in transfected protoplasts by RT-PCR (Supplementary Figure S7C, D).

To further confirm that the 5′ NTR of CaMV is involved in nuclear export, we used an unspliced reporter RNA assay (87). The reporter RNA contains the luciferase coding sequence within an inefficiently spliced intron derived from the HIV-1 env gene and harbourises, besides the inefficient splice donor and acceptor sites, the Rev response element (RRE) (Figure 8A). In human cells, the few spliced RNAs are exported but lose the luciferase gene. The unspliced RNA still has the luciferase sequence but is actively retained in the nucleus and does not allow luciferase expression either. To adapt the reporter RNA, we placed the env-luciferase-RRE expression cassette under the control of the CaMV 35S promoter and the NOS terminator, and cloned within the RRE either (i) the CaMV 5′ UTR (L–VII) or (ii) a mutated variant (L–VII\textsubscript{mut}), in which the inefficient splice donor site D4 (nt 481–482) and the cryptic Db (nt 456–457) (49) were inactivated or (iii) the 35S (I–II) fragment, or (iv) the CTE of MPMV (Figure 8A). The resulting plasmids were transfected in WT or TREX mutant Arabidopsis protoplasts and luciferase activity was measured at 20 hpt. Luciferase was expressed even with the original Luc-RRE construct (Figure 8B, white bars), at different levels in WT or TREX mutants, indicating that, unlike in human cells, the unspliced reporter RNA is exported in Arabidopsis. This result can be explained by the fact that intron retention (IR) is the most prevalent alternative splicing event in plants, with observed frequencies as high as 64% in Arabidopsis, and that IR transcripts often escape nonsense-mediated decay (NMD), and are exported into the cytoplasm (88). The addition of the WT or mutant CaMV 5′ UTR (Figure 8B) significantly increased the luciferase activity both in WT and in TREX mutant Arabidopsis. The detection of the different reporter RNAs by RT-PCR (Figure 8C) shows that both their total amounts and splicing were unaffected, since only the unspliced isoforms (>2700) were amplified. This suggests that the enhanced luciferase activity was probably due to an enhanced nuclear export of its mRNA. Conversely, in the presence of 35S RNA fragment (I–II) or the CTE, the luciferase activity was lower, indicating that the RNA export was less efficient and that the MPMV CTE is probably not functional in plants.

Our combined results demonstrate that the 5′ UTR of 35S RNA, and particularly its stable SL secondary structure, behaves as an export-enhancing element that might be recognised by the export proteins. Therefore, we investigated whether this region can effectively interact with some RNA-binding proteins of the TREX complex, such as ALY1, ALY3 (32) or MOS11 (54).

MOS11 and export adaptors ALY1 and ALY3 can interact with CaMV 35S RNA in vitro

It was shown that both MOS11 (54) and ALY1 (32) bind in vitro 25-nucleotide RNA duplexes and single-stranded RNAs. We investigated whether these proteins would also interact with the complex secondary structure of the 35S RNA leader region, which folds into a large stem interrupted by several loops, short hairpins and four side stems (83,89).

To test the putative RNA-protein interactions, we performed in vitro binding EMSA assays with RNA L–VII (765 nt) and I–II (865 nt), produced and radiolabelled in vitro (Figure 9A), and ALY1, ALY3, MOS11 or EGFP (as control), all expressed in bacteria and affinity-purified (Figure 9B and Supplementary Figure S8). Both ALY1, ALY3 and EGFP were His\textsubscript{6}-tagged and expressed fused to the GB1 polypeptide to increase protein solubility in E. coli (32).

In the presence of 10 or 20 μM of the control protein His\textsubscript{6}-GB1-EGFP, the electrophoretic mobility of neither RNA L–VII nor RNA I–II was reduced (Figure 9C), demonstrating that this protein, harbouring the same tags as ALY1 and ALY3, does not interact with any of the 35S RNA fragments. On the contrary, increasing amounts of
Figure 8. The 5′ UTR of CaMV 35S RNA enhances the nuclear export of a reporter luciferase mRNA. (A) Schematic representation of the Luc-RRE reporter and the resulting constructs obtained by cloning of 35S RNA fragments L–VII, L–VII\textsubscript{mut} (the two red asterisks show the positions of the two mutated splice donor sites Db and D4) and I–II, and MPMV CTE in the Bst\textsubscript{API} restriction site (p35S: 35S RNA promoter, Env: HIV-1 ENV coding sequence, Luc: firefly luciferase, RRE: REV response element, NOS\textsubscript{r}: NOS terminator, SD: splice donor site, SA: splice acceptor site). (B) WT/CA-rop2, mos11, aly1 aly2 and aly3 aly4 protoplasts were transfected with the above described reporter plasmids and 20 hpt luciferase activities (RLU: relative light units) were measured in triplicate in three different experiments, normalised to the transfection efficiency by assaying for RFP fluorescence and averaged. Significance was tested with a Student’s unpaired two-sample t-test (ns, not significant, \( P > 0.05 \), \( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \)). (C) The reporter mRNAs expression in the transfected protoplasts was analysed by RT-PCR with specific primers, the position of which is indicated by black arrows in (A).
Figure 9. CaMV 35S RNA can bind ALY1 and MOS11 in vitro. (A) 35S RNA fragments L–VII and I–II were in vitro synthesised by T7 RNA polymerase, labelled with [$\alpha$-32P]-UTP, and analysed by denaturing agarose electrophoresis. (B) ALY1 and EGFP (as control), and MOS11 were expressed in E. coli as GB1-His$_6$ (ALY1 and EGFP) or GST (MOS11) fusion proteins, purified by affinity chromatography, and examined by SDS-PAGE. (C) EMSA with $\approx$ 5000 cpm L–VII or I–II RNA and 0, 10 or 20 $\mu$M purified His$_6$-GB1-EGFP, analysed by non-denaturing electrophoresis and autoradiography. EMSA with $\approx$ 5,000 cpm RNA L–VII (D, E) or I–II (F, G) and increasing amounts (0 to 20 $\mu$M) of purified ALY1-GB1-His$_6$ (D, E) or MOS11 (F, G), analysed by non-denaturing electrophoresis and autoradiography. Positions of molecular size standards in nt (A) or kDa (B) are shown on the left and right, respectively.

ALY1-GB1-His$_6$ (0–20 $\mu$M) progressively reduced RNA L–VII migration (Figure 9D): compared to free RNA L–VII (0 $\mu$M ALY1), the band-shift occurred in the presence of 5 $\mu$M ALY1, and its intensity increased at 10, 15 and 20 $\mu$M protein suggesting that ALY1 interacts with RNA L–VII and that multiple copies of the proteins are progressively loaded on the 765 nt long fragment. Since RNA L–VII is composed of 5' and 3' unstructured sequences (74 and 209 nt, respectively), and the 482 nt SL, it is probable that ALY1 interacts with both structured and unstructured RNA sections, forming increasingly larger ribonucleoproteins, the migration of which is progressively shifted. This observation is consistent with the results of Pfaff et al. (32) who demonstrated that Arabidopsis ALY1 behaves as a typical RNA-binding protein that interacts via its central RNA recognition motif (RRM) with both single-stranded (ss) and double-stranded (ds) RNA, and even with ssDNA. However, it exhibits a preference for ssRNA. The RRM alone binds weakly, and binding is strongly enhanced by the termini, particularly the N-terminus (32).
Consistent with these findings, it is unsurprising that we observed almost the same gel-shift of RNA I–II in the presence of increasing amounts (0 to 20 μM) of ALY1 (Figure 9E), starting, as for L–VII, at 5 μM protein. Although unnecessary for 35S RNA nuclear export, this RNA interacted with export adaptors ALY1, as did the cis-acting fragment L–VII.

We also tested ALY3 for its ability to interact with 35S RNA, since Pfaff et al. (32) had shown that ALY1 and ALY2 (54% amino acid sequence identity) as well as ALY3 and ALY4 (70% amino acid sequence identity) share a high degree of sequence similarity, whereas the similarity of ALY1/2 versus ALY3/4 is clearly lower (less than 42% amino acid sequence identity). Moreover, ALY3 and ALY4 are strongly enriched in nucleoli, whereas ALY1 and ALY2 are clearly less prominent in nucleoli relative to the nucleoplasm. As shown in Supplementary Figure S8, ALY3 also binds to both the L–VII and I–II RNA fragments but with less affinity, since the single band-shifts only occurred with 15 and 20 μM ALY3 for the L–VII RNA and with 20 μM ALY3 for the RNA I–II.

We observed the same RNA-binding properties of ALY1 and ALY3 towards a CaMV-unrelated but also highly structured RNA, the HIV-1 RRE element (87) (data not shown). Together, these results demonstrate that the ALY adaptor proteins can interact with complex secondary structures.

MOS11 RNA-binding was markedly different. As shown (Figure 9F), MOS11 associated with the 35S L–VII RNA fragment in a concentration-dependent manner. However, contrary to ALY1, RNA migration was not further delayed, while increasing the protein concentration (10 to 20 μM). This observation suggests that MOS11 cannot interact randomly, as does ALY1, with several binding sites on the 765 nt long L–VII RNA.

Moreover, MOS11 did not associate with the I–II RNA fragment (Figure 9G), which, according to mfold (90), is presumably lacking a stable secondary structure. These findings can be correlated with previous work, which showed that this RNA-binding protein associates better with dsRNA than with ssRNA (54), further suggesting that MOS11 preferentially interacts with the 482 nt SL of the 35S RNA leader region.

DISCUSSION

Host cellular machineries play indispensable roles in virus life cycles. Among them is the nuclear export machinery that allows mRNAs to reach the cytoplasm to be translated. The export receptor TAP, which is conserved in yeast and metazoans (91), does not exist in Arabidopsis, despite the TREX adaptor complex being conserved in plants. The main role of this TREX complex is to recognise and interact with cargo mRNAs before recruiting the export proteins.

In this study, we described for the first time the mechanisms used by a plant virus, CaMV, to export its 35S mRNAs out of the nucleus. We demonstrated that this export is mediated by the TREX complex and involves the THO subunit and splicing factor TEX1 (54), together with MOS11 (35,54) and the ALY adaptor proteins (32). In mutant protoplasts, deficient for the expression of one or more of the export factors, 35S RNAs were retained in the nucleus. This export inhibition explains the partial resistance of the respective Arabidopsis mutants to CaMV infection.

It has been shown that, among the six THO components (HPR1, THO2, TEX1, THO5–7), TEX1 and HPR1 are required for efficient cellular mRNA export in Arabidopsis (54,92,93). However, in our experiments, CaMV 35S RNA nuclear export was unaffected in the single mutant tex1, as well as in hpr1 or thob protoplasts, and the respective mutant plants displayed the same susceptibility to CaMV (data not shown). These results suggest that the particular composition and assembly of the THO core are probably less important for the export of viral mRNAs than of cellular mRNAs and that differences exist in the TREX-dependent export of these different mRNA populations.

The export adaptor MOS11 proved essential for 35S RNA export, because nuclear export was reduced by almost 50% in the absence of this protein. This observation is consistent with the strong nuclear retention of cellular mRNAs described for mos11 plants (54), and, like for cellular mRNAs, the export inhibition of 35S RNA increased in the double mutant mos11 tex1. One can imagine that TEX1 enhances the effect of MOS11 and is important for CaMV because of its role in splicing: TEX1 interacts with several splicing factors and co-localises in a speckled pattern in the nucleoplasm with SR proteins, and its absence in tex1 and tex1 mos11 mutants is responsible for quantitative changes in alternatively spliced transcript variants (54). According to these findings, it cannot be excluded that in the absence of TEX1, CaMV 35S splicing is modified leading to the accumulation of viral RNA isoforms that are better retained in nuclei of tex1 mos11 protoplasts. However, when examining by RT-PCR the splicing profile of 35S RNA in tex1 single and double mutants, we did not detect significant differences: the four spliced and the unspliced isoform were present at levels comparable to those in WT protoplasts (data not shown). This suggests that the potentiating effect of TEX1 on export is not linked to splicing and the mechanism thus requires further clarification.

The most important proteins for CaMV 35S RNA nuclear export are ALY1–4, since the export was strongly inhibited in aly1 aly2 and aly3 aly4 double mutants, and especially in the 4xaly quadruple mutant, where this inhibition exceeded 90%. Consistent with this observation, 75% of 4xaly plants displayed resistance to CaMV infection. All ALYs are ubiquitously expressed in vegetative tissues but display different subnuclear localisations. ALY3 and ALY4 are present at higher levels in nucleoli than ALY1 and ALY2 (32). Considering the disruption in viral mRNA export in both ALY double mutants, it is surprising that these plants did not show more resistance to CaMV. Our results suggest that there must be a functional compensation in planta for the lack of ALYs by the two other ALY paralogues in each case. It is also surprising that, despite the strong indisputable role of ALYs in viral and cellular mRNA export, 4xaly plants are severely affected but still viable and not completely resistant to CaMV infection. Conversely, yeast cells lacking Yra1, the orthologue of ALY, are not viable (94,95). These findings can be explained with the recently demonstrated existence, in Arabidopsis, of two additional ALY functional homologues, the UAP56 interacting export factors UIEF1 and 2, which can also bind RNA.
The role of P5 in nuclear export can be easily understood when considering several previous findings. As genome encapsidation probably occurs during reverse transcription of 35S unspliced (pregenomic) RNA (112,113), at least three copies P5 must be present inside the virions (114), which are required to perform the negative-strand synthesis and the discontinuous synthesis of the positive-strand DNA (86,115). After capsid dissociation on nuclear pores, some capsid and P5 proteins are probably imported into the nucleus in association with the viral genome. Pfeiffer et al. (75) also described an increase in a DNA polymerase of ca. 75 kDa, present in infected turnip nuclear extracts. During the viral life cycle, P5 together with P6, which is independently expressed from its 19S RNA, and P1 synthesised from unspliced 35S RNAs, accumulate early in turnip protoplasts (116). This observation is surprising considering that the main functions of P5 (genome replication and capsid maturation) occur late in the viral cycle, and suggests other potentially early role(s) for P5. Therefore, we investigated whether P5, important for viral mRNA export, can interact with export factors. We demonstrated by GST pull-down assays, that P5, and especially its central RT domain, associates with MOS11 in vitro.

In the same GST pull-down experiments, we found that a second viral protein, P4, probably unprocessed and as a dimer, interacts with MOS11 in vitro. This interaction suggests that P4 is also involved in TREX-mediated viral mRNA export. The simultaneous absence of P5 and P4 in protoplasts transfected with pCaMV-GFP-P6,STOP could explain the strong nuclear retention of 35S RNA. In mammals, the HBV core protein involvement in the nuclear export of some viral mRNAs by direct loading, not of CIP29 but of transporter TAP/p15, was described (117).

CaMV coat proteins that are proteolytically matured (by P5 and unidentified cellular proteinases) to remove N- and C-termini, can enter the nucleus via their Nt NLS, which becomes accessible only on cleavage (46). However, using EGFP fusion proteins, we observed that not only full-length P4 protein but also uncleaved P5, in which we discovered two functional bipartite NLSs, displays a nucleocytoplasmic distribution, with typical cytoplasmic aggregates coherent with the cytoplasmic functions of both proteins. Additionally, they perfectly co-localised with MOS11-mRFP in the nucleus. This nuclear co-localisation and the related co-immunoprecipitation assays suggests that the physical interaction observed in vitro between the two viral proteins and the export factor occurs in the nucleus and promotes the nuclear export of viral mRNAs.

We finally investigated the presence of potential cis-acting elements in the 35S RNAs that are important for the loading of the export machinery. We demonstrated by competition experiments and reporter RNA assays in protoplasts that an RNA fragment corresponding to the 5′ leader region followed by the first third of ORF VII (L–VII) efficiently outcompetes full-length viral RNAs for export factors and enhances the nuclear export of a reporter RNA. The overexpression of the highly structured SL of the leader (83), which harbours several important elements, such as five regulatory sORFs, the conditional poly(A) site, the coat protein P4 binding site and the first splice donor site D4, and which is bypassed during translation by ribosome shunting (82), also inhibited viral RNA nuclear export but to a lesser extent. This observation suggests that the short 5′ and/or the longer 3′ regions that flank the SL are important for export. Consistent with our previous results on the role of P5, we can expect that this additional cis-acting element is the tRNA_Met PBS, which precedes ORFVII and where P5 is loaded on 35S RNA (86).

Finally, we showed that the RNA-binding adaptors ALY1 and ALY3 (32) can interact, probably without specificity, with the 35S RNA 5′ leader (L–VII) and/or with a downstream coding sequence (I–II) unrelated to export. Most important, however, was the observation that MOS11 preferentially interacts with the structured 35S RNA leader but not with the I–II control RNA, consistent with previous work showing that this RNA-binding protein associates better with dsRNA than with ssRNA (54). One can imag-
ine that the TREX complex can be further preferentially enriched on the 35S 5′ secondary structure by the interactions existing between MOS11, P4 loaded on its binding site on the top of the SL, and/or P5 recruited to the downstream located PBS, in the presence or absence of tRNA_{Met} primer.

Based on the results presented in this study, we can predict the following model for CaMV 35S mRNA nuclear export. When the viral DNA is released from the virion into the nucleoplasm, host DNA repair enzymes repair the gaps left by RT during the previous replication cycle to create covalently closed molecules. This closed viral DNA is then transcribed by Pol II into progenomic polycistronic 35S RNA, 70% of which is alternatively spliced, and into subgenomic 19S RNA, which is the mRNA for the viral protein P6. The unspliced 35S RNA and three out of the four spliced isoforms contain the highly structured 5′ leader, whereas the last isoform and 19S RNA do not. During the first round of viral transcription only a few copies of coat protein P4 and RT P5 are present in the nucleus. Thus, a first minimal round of nuclear export might occur, during which TREX complexes might be loaded on viral mRNAs mainly by CBCs and EJCs, probably more efficiently on the leader-containing mRNA species than for the others, which can be considered as regular cellular mRNAs. In the cytoplasm, these mRNAs are then translated, and viral proteins progressively accumulate. P5 polyprotein and P4 coat precursor enter the nuclei via their NLSs. There, they bind their respective recognition sites on the leader-containing 35S RNAs and, interacting with MOS11, might promote the recruitment of TREX and enhance the nuclear export of these viral RNAs.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Ramanathan, A., Robb, G.B. and Chan, S.-H. (2016) mRNA capping: biological functions and applications. Nucleic Acids Res., 44, 7511–7526.
2. Girard,C., Will,C.L., Peng,J., Makarov,E.M., Kastner,B., Lemm,L., Urlaub,H., Hartmuth,K. and Lührmann,R. (2012) Post-transcriptional splicingosomes are retained in nuclear speckles until splicing completion. Nat. Commun., 3, 994.
3. Stewart,M. (2019) Polyadenylation and nuclear export of mRNAs. J. Biol. Chem., 294, 2977–2987.
4. Delaeye,M. and Borden,K. L. B. (2015) Multiple export mechanisms for mRNAs. Cells, 4, 452–473.
5. Heath,C.G., Viphakone,N. and Wilson,S.A. (2016) The role of TREX in gene expression and disease. Biochim. J., 473, 2911–2935.
6. Williams,T., Ngo,L.H. and Wickramasinghe,V.O. (2018) Nuclear export of RNA: different sizes, shapes and functions. Semin. Cell Dev. Biol., 75, 70–77.
7. Kang Y., Bogerd,H.P., Yang J. and Cullen,B.R. (1999) Analysis of the RNA binding specificity of the human tap protein, a constitutive transport element-specific nuclear RNA export factor. Virology, 262, 200–209.
8. Teplova,M., Wohlbold,L., Khin,N.W., Izaurralde,E. and Patel,D.J. (2011) Structure-function studies of nucleocytoplasmic transport of retroviral genomic RNA by mRNA export factor TAP. Nat. Struct. Mol. Biol., 18, 990–998.
9. Björk,P. and Wieslander,L. (2017) Integration of mRNP formation and export. Cell. Mol. Life Sci., 74, 2875–2897.
10. Masuda,S., Das,R., Cheng,H., Hurt,E., Dorman,N. and Reed,R. (2005) Recruitment of the human TREX complex to mRNA during splicing. Genes Dev., 19, 1512–1517.
11. Hautbergue,G.M., Hung,M.-L., Golovanov,A.P., Lian,L.-Y. and Wilson,S.A. (2008) Mutually exclusive interactions drive handover of mRNA from export adaptors to TAP. Proc. Natl. Acad. Sci. U.S.A., 105, 5154–5159.
12. Hautbergue,G.M., Hung,M.-L., Walsh,M.J., Snijders,A.P.L., Chang,C.-T., Jones,R., Ponting,C.P., Dickman,M.J. and Wilson,S.A. (2009) UIF, a new mRNA export adaptor that works together with REF/ALY, requires FACT for recruitment to mRNA. Curr. Biol., 19, 1918–1924.
13. Shen,J., Zhang,L. and Zhao,R. (2007) Biochemical characterization of the ATPase and helicase activity of UAP56, an essential pre-mRNA splicing and mRNA export factor. J. Biol. Chem., 282, 22544–22550.
14. Fukuda,S., Wu,D.W., Stark,K. and Pelus,L.M. (2002) Cloning and characterization of a proliferation-associated cytokine-inducible protein, CIP29. Biochem. Biophys. Res. Commun., 292, 593–600.
15. Cheng, H., Dufu, K., Lee, C.-S., Hsu, J.L., Dias, A. and Reed, R. (2006) Human mRNA export machinery recruited to the 5’ end of mRNA. Cell, 127, 1389–1400.
16. Chi, B., Wang, Q., Wu, G., Tan, M., Wang, L., Shi, M., Chang, X. and Cheng, H. (2013) Aly and TOT are required for assembly of the human TREX complex and association of TREX components with the spliced mRNA. Nucleic Acids Res., 41, 1294–1306.
17. Vipakone, N., Sudbery, I., Griffith, L., Heath, C.G., Sims, D. and Wilson, S.A. (2019) Co-transcriptional loading of RNA export factors shapes the human transcriptome. Mol. Cell, 75, 310–323.
18. Le Hir, H., Gatfield, D., Izaurralde, E. and Moore, M.J. (2001) The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. EMBO J., 20, 4987–4997.
19. Dufu, K., Livingstone, M.J., Seebacher, J., Gygi, S.P., Wilson, S.A. and MacFarlane, S.A. (2004) Functional analysis of RNA and TAP binding to SF2/ASF. Mol. Cell Biol., 24, 2043–2053.
20. Vipakone, N., Hautbergue, G.M., Walsh, M., Chang, C.-T., Holland, A., Folco, E.G., Reed, R. and Wilson, S.A. (2012) TREX exposes the RNA-binding domain of Nxf1 to enable mRNA export. Nat. Commun., 3, 1006.
21. Wickramasinghe, Y.O. and Laskey, R.A. (2015) Control of mammalian gene expression by selective mRNA export. Nat. Rev. Mol. Cell Biol., 16, 431–442.
22. Kiesler, E., Miralles, F. and Visa, N. (2002) HEL/UPAP6 binds cotranscriptionally to the Balbiani ring pre-mRNA in an intron-independent manner and accompanies the BR mRNP to the nuclear pore. Curr. Biol., 12, 859–862.
23. Nojima, T., Hirose, T., Kimura, H. and Hagiwara, M. (2007) The interaction between Cap-binding complex and RNA export factor is required for intronless mRNA export. J. Biol. Chem., 282, 15645–15651.
24. Huang, Y. and Steitz, J.A. (2001) Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Mol. Cell, 7, 899–905.
25. Hargous, Y., Hautbergue, G.M., Tang, Y., Golovanov, A.P., Stevenin, J., Li, S.-Y., Wilson, S.A. and Allain, F.H.-T. (2006) Molecular basis of RNA recognition and TAP binding by the SR proteins SRp20 and 9G8. EMBO J., 25, 5126–5137.
26. Tintraru, A.M., Hautbergue, G.M., Hounsaw, A.M., Hung, M.-L., Lian, L.-Y., Craven, C.J. and Wilson, S.A. (2007) Structural and functional analysis of RNA and TAP binding to SF2/ASF. EMBO Rep., 8, 756–762.
27. Lei, H., Dias, A.P. and Reed, R. (2011) Export and stability of naturally intronless mRNAs require specific coding region sequences and the TREX mRNA export complex. Proc. Natl. Acad. Sci. U.S.A., 108, 17985–17990.
28. Lei, H., Zhai, B., Yin, S., Gygi, S. and Reed, R. (2013) Evidence that a consensus element found in naturally intronless mRNAs promotes mRNA export. Nucleic Acids Res., 41, 2517–2525.
29. Ehrnsberger, H.F., Grasser, M. and Grasser, K.D. (2019) Nucleocytoplasmic mRNA transport in plants: export factors and their influence on growth and development. J. Exp. Bot., 70, 3757–3763.
30. Yellin, N.E., Smith, L.M., Jones, A.M.E., Patel, K., Kelly, K.A. and Balcombe, D.C. (2010) Putative Arabidopsis TOT/TREX mRNA export complex is involved in transgene and endogenous sRNA biosynthesis. Proc. Natl. Acad. Sci. U.S.A., 107, 13948–13953.
31. Uhrig, J.F., Canto, T., Marshall, D. and MacFarlane, S.A. (2004) Relocalization of nuclear ALY proteins to the cytoplasm by the tomato bushy stunt virus P19 pathogenicity protein. Plant Physiol., 135, 2411–2423.
32. Pfaff, C., Ehrnsberger, H.F., Flores-Tornero, M., Sorensen, B.B., Schubert, T., Längst, G., Griesenbeck, J., Sprunck, S., Grasser, M. and Grasser, K.D. (2018) ALY RNA-Binding proteins are required for Nucleocytoplasmic mRNA transport and modulate plant growth and development. Plant Physiol., 177, 226–240.
33. Ehrnsberger, H.F., Pfaff, C., Hachani, I., Flores-Tornero, M., Sorensen, B.B., Längst, G., Sprunck, S., Grasser, M. and Grasser, K.D. (2019) The UPAP6-Interacting export factors UIE1F1 and UIE1F2 function in mRNA export. Plant Physiol., 179, 1525–1536.
34. Kamel, C., Thomaier, M., Sorensen, B.B., Schubert, T., Längst, G., Grasser, M. and Grasser, K.D. (2013) Arabidopsis DEAD-box RNA helicase UPAP6 interacts with both RNA and DNA as well as with mRNA export factors. PLoS One, 8, e60644.
35. Samer, H., Chao, M., Stahl, J., Hohn, T., Hohn, T. and Arribere, C. (2010) MOS11: a new protein involved in the constitutive spliceosome. PLoS Genet., 6, e1001230.
36. Gales, J.P., Kubina, J., Geldreich, A. and Dimitrova, M. (2020) Strength in diversity: nuclear export of viral RNAs. Viruses, 12, 1014.
37. Koffa, M.D., Clements, J.B., Ameer, E., Sorensen, B.B., Mattaj, I. and Kuersten, S. (2001) Herpes simplex virus IC27 protein provides viral mRNAs with access to the cellular mRNA export pathway. EMBO J., 20, 5769–5778.
38. Malik, P., Blackbour, M.J. and Clements, J.B. (2004) The evolutionarily conserved Kaposis sarcoma-associated herpesvirus ORF57 protein interacts with REF protein and acts as an mRNA export factor. J. Biol. Chem., 279, 33001–33011.
39. Boyne, J.R., Colgan, K.J. and Whitehouse, A. (2008) Herpesvirus saimiri ORF57: a post-transcriptional regulatory protein. Front. Biosci. J. Virtual Lib., 13, 2928–2938.
40. Tunnicliffe, R.B., Hautbergue, G.M., Walsh, M., Chang, C.-T., Wilson, S.A. and MacFarlane, S.A. (2012) TREX exposes the RNA-binding domain of Nxf1 to enable mRNA export. Nat. Commun., 3, 1006.
41. Sørensen, B.B., Ehrnsberger, H.F., Esposito, S., Pfaff, A., Bruckmann, A., Hauptmann, J., Meister, G., Merkl, R. and Schubert, T.,
62. Revers, F. and García, J. A. (2015) Molecular biology of potyviruses. *Plant Biol.*, 17, 1046–1051.

63. Kim, G.-D., Cho, Y.-H. and Yoo, S.-D. (2017) Regulatory functions of the 35S RNA 5’-encoded p26 protein: evidence for structural pathogenicity determinants. *J. Gen. Virol.*, 98, 2115–2125.

64. Lellis, A. D., Kasschau, K. D., Whitham, S. A. and Carrington, J. C. (2005) Functional characterization of the Beta necrotic yellow vein virus RNA silencing factor TAV, whose genes are trans activated by the product of gene VI. *Virology*, 324, 295–308.

65. Angel, C. A., Lutz, L., Yang, X., Rodriguez, A., Adair, A., Zhang, Y., Amari, K., Boutant, E., Hofmann, C., Schmitt-Keichinger, C., Kasschau, K. D., Singh, K. B., D dodds, P. N., and Taylor, J. M. (2017) LOCALIZER: subcellular localization prediction of both plant and effector proteins in the plant cell. *Sci. Rep.*, 7, 44598.

66. Haas, G., Azevedo, J., Moisissiard, G., Geldreich, A., Himber, C., Bureau, M., Fukuhara, T., Keller, M. and Voinnet, O. (2008) Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. *EMBO J.*, 27, 2102–2112.
95. Zenklusen, D., Vinciguerra, P., Strahm, Y. and Stutz, F. (2001) The yeast hnRNP-Like proteins Yra1p and Yra2p participate in mRNA export through interaction with Mex67p. Mol. Cell. Biol., 21, 4219–4232.

96. Kang, Y. and Cullen, B.R. (1999) The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences. Gene Dev., 13, 1126–1139.

97. Cullen, B.R. (2003) Nuclear RNA export. J. Cell Sci., 116, 587–597.

98. Tian, X., Devi-Rao, G., Golovanov, A.P. and Sandri-Goldin, R.M. (2013) The interaction of the cellular export adaptor protein Aly/REF with ICP27 contributes to the efficiency of herpes simplex virus 1 mRNA export. J. Virol., 87, 7210–7217.

99. Sakuma, T., Davila, J.I., Malcolm, J.A., Kocher, J.-P.A., Tonne, J.M. and Ikeda, Y. (2014) Murine leukemia virus uses NXF1 for nuclear export of spliced and unspliced viral transcripts. J. Virol., 88, 4069–4082.

100. Pessel-Vivares, L., Houzet, L., Lainé, S. and Mougel, M. (2015) Insights into the nuclear export of murine leukemia virus intron-containing RNA. RNA Biol., 12, 942–949.

101. Mougel, M., Akkawi, C., Chamontin, C., Feuillard, J., Pessel-Vivares, L., Socol, M. and Laine, S. (2020) NXF1 and CRM1 nuclear export pathways orchestrate nuclear export, translation and packaging of murine leukemia retrovirus unspliced RNA. RNA Biol., 17, 528–538.

102. Flint, S.J., Huang, W., Goodhouse, J. and Kyin, S. (2005) A peptide inhibitor of exportin1 blocks shuttling of the adenoviral E1B 55 kDa protein but not export of viral late mRNAs. Virology, 337, 7–17.

103. Yatherajam, G., Huang, W. and Flint, S.J. (2011) Export of adenoviral late mRNA from the nucleus requires the Nxf1/Tap export receptor. J. Virol., 85, 1429–1438.

104. Johnson, L.A., Li, L. and Sandri-Goldin, R.M. (2009) The cellular RNA export receptor TAP/NXF1 is required for ICP27-mediated export of herpes simplex virus 1 RNA, but the TREX complex adaptor protein Aly/REF appears to be dispensable. J. Virol., 83, 6353–6346.

105. Tian, X., Devi-Rao, G., Golovanov, A.P. and Sandri-Goldin, R.M. (2013) The interaction of the cellular export adaptor protein Aly/REF with ICP27 contributes to the efficiency of herpes simplex virus 1 mRNA export. J. Virol., 87, 7210–7217.

106. Heise, T., Sommer, G., Reumann, K., Meyer, J., Will, H. and Schaal, H. (2006) The hepatitis B virus PRE contains a splicing regulatory element. Nucleic Acids Res., 34, 353–363.

107. Lee, G.H., Wasser, S. and Lim, S.G. (2008) Hepatitis B pregenomic RNA splicing—The products, the regulatory mechanisms and its biological significance. Virus Res., 136, 1–7.

108. Sommer, G. and Heise, T. (2008) Posttranscriptional control of HBV gene expression. Front. Biosci. J. Virtual Libr., 13, 5533–5547.

109. Hernandez, F.P. and Sandri-Goldin, R.M. (2010) Head-to-tail intramolecular interaction of herpes simplex virus type 1 regulatory protein ICP27 is important for its interaction with cellular mRNA export receptor TAP/NXF1. mBio, 1, e00268-10.

110. Diot, C., Fourrier, G., Dos Santos, M., Magnu, J., Komarova, A., van der Werf, S., Munier, S. and Naffakh, N. (2016) Influenza A virus polymerase recruits the RNA helicase DDX19 to promote the nuclear export of viral mRNAs. Sci. Rep., 6, 33763.

111. Ren, X., Yu, Y., Li, H., Huang, J., Zhou, A., Liu, S., Hu, P., Li, B., Qi, W. and Liao, M. (2019) Avian influenza A virus polymerase recruits cellular RNA helicase eIF4A3 to promote viral mRNA splicing and spliced mRNA nuclear export. Front. Microbiol., 10, 1625.

112. Marsh, L.E. and Guilfoyle, T.J. (1987) Cauliflower mosaic virus replication intermediates are encapsidated into virion-like particles. Virology, 161, 129–137.

113. Himmelbach, A., Chapdelaine, Y. and Hohn, T. (1996) Interaction between cauliflower mosaic virus inclusion body protein and capsid protein: implications for viral assembly. Virology, 217, 147–157.

114. Menissier, J., Laquel, P., Lebeurier, G. and Hirth, L. (1984) A DNA polymerase activity is associated with Cauliflower Mosaic Virus. Nucleic Acids Res., 12, 8769–8778.

115. Haas, M., Bureau, M., Geldreich, A., Yot, P. and Keller, M. (2002) Cauliflower mosaic virus: still in the news. Mol. Plant Pathol., 3, 419–429.

116. Kobayashi, K., Nakayashiki, H., Tsuge, S., Mise, K. and Furusawa, I. (1998) Accumulation kinetics of viral gene products in cauliflower mosaic virus-infected turnip protoplasts. Microbiol. Immunol., 42, 65–69.

117. Li, H.-C., Huang, E.-Y., Su, P.-Y., Wu, S.-Y., Yang, C.-C., Lin, Y.-S., Chang, W.-C. and Shih, C. (2010) Nuclear export and import of human hepatitis B virus capsid protein and particles. PLoS Pathog., 6, e1001162.