Inferring protein function in an emerging virus: detection of the nucleoprotein in Tilapia Lake Virus

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Running head: Discovering the nucleoprotein of an emerging virus

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Abstract

Emerging viruses impose global threats to animal and human populations and may bear novel genes with limited homology to known sequences, necessitating the development of novel approaches to infer
and test protein functions. This challenge is dramatically evident in tilapia lake virus (TiLV), an emerging orthomyxo-like virus that threatens the global tilapia aquaculture and food security of millions of people. The majority of TiLV proteins have no homology to known sequences, impeding functionality assessments. Using a novel bioinformatics approach, we predicted that TiLV’s Protein 4 encodes the nucleoprotein - a factor essential for viral RNA replication. Multiple methodologies revealed the expected properties of orthomyxoviral nucleoproteins. A modified yeast three-hybrid assay detected Protein 4-RNA interactions, which were independent of the RNA sequence, and identified specific positively charged residues involved. Protein 4-RNA interactions were uncovered by R-DeepP and XRNAX methodologies. Immunoelectron microscopy found that multiple Protein 4 copies localized along enriched ribonucleoproteins. TiLV RNA from cells and virions co-immunoprecipitated with Protein 4. Immunofluorescence microscopy detected Protein 4 in the cytoplasm and nuclei, and nuclear Protein 4 increased upon CRM1 inhibition, suggesting CRM1-dependent nuclear export of TiLV RNA. Together, these data reveal TiLV’s nucleoprotein and highlight the ability to infer protein functionality, including novel RNA-binding proteins, in emerging pathogens. These are important in light of the expected discovery of many unknown viruses and the zoonotic potential of such pathogens.

**KEYWORDS:** Nucleoprotein, Tilapia Lake Virus, RNA-binding protein, emerging virus

**Importance**

Tilapia is an important source of dietary protein, especially in developing countries. Massive losses of tilapia were identified worldwide, risking the food security of millions of people. Tilapia lake virus (TiLV) is an emerging pathogen responsible for these disease outbreaks. TiLV’s genome encodes ten major proteins, nine of which show no homology to other known viral or cellular proteins, hindering...
functionality assessment of these proteins. Here we describe a novel bioinformatics approach to infer
the functionality of TiLV proteins, which predicted Protein 4 as the nucleoprotein - a factor essential for
viral RNA replication. We provided experimental support for this prediction by applying multiple
molecular, biochemical, and imaging approaches. Overall, we illustrate a strategy for functional
analyses in viral discovery. The strategy is important in light of the expected discovery of many unknown
viruses and the zoonotic potential of such pathogens.

Introduction

In recent decades, more and more emerging viruses have become evident both in the human population
and in wildlife, particularly in domestic animals or in wild stock raised by humans in dense conditions.
The impact of such emerging viruses on global health and the economy may be devastating and
necessitates a rapid response. Tilapia lake virus (TiLV) is an emerging pathogen that threatens the global
tilapia aquaculture and the food security of millions of people, in particular in developing countries.
Since its discovery in 2014 (1), and the first reports from Israel and Ecuador of the disease it causes (1,
2), TiLV has been detected in 16 countries across four continents (3). Moreover, a more significant global
spread of the virus is suspected due to fish export from TiLV-infected hatcheries to over 40 countries (4).
TiLV infections may result in extremely high mortality rates in both experimentally infected fish (1, 5, 6)
and farmed tilapia (2, 7–9), ranging between 70 to 90%. As tilapia comprise the second most important
group of farmed fish worldwide (references in (1, 3, 10)), several international agencies, including the
Food and Agriculture Organization (FAO) of the United Nations, have issued urgent warnings regarding
the global threat that TiLV imposes to food security (3).
The RNA genome of TiLV (1) consists of ten segments, numbered according to their length: Segment 1 is the longest (1641 nt), and Segment 10 is the shortest (465 nt) (10). All ten segments have conserved, complementary sequences at their 5' and 3' termini, and each segment contains one primary open reading frame (ORF). We identified peptides from all ten predicted proteins by mass spectrometry (10 and unpublished data). While Segment 1 ORF shows weak sequence homology to the influenza C virus (ICV) PB1 subunit, remarkably, the other nine ORFs completely lack sequence homology to any other sequence, viral and cellular alike. Nevertheless, several features suggested that TiLV is an "orthomyxovirus like" virus (10). These include an enveloped virion; a single-stranded, negative-sense, segmented RNA genome; the presence of similar, complementary sequences at the 5' and 3' noncoding termini of all TiLV segments; a short (3 to 5 bases long), uninterrupted uridine stretch present at all of the 5' ends of TiLV genomic RNA segments; nuclear and cytoplasmic localization of TiLV mRNA, implying a nuclear site for transcription (1, 10). A later classification has assigned TiLV as a new species (Tilapia tilapinevirus), under the genus Tilapinevirus, family Amnoonviridae and order Articulavirales (11). Recent metatranscriptomic and data mining analyses have expanded the Amnoonviridae family by identifying transcripts derived from 12 unknown vertebrate viruses, which match multiple genomic segments of TiLV (12, 13).

The nucleoprotein (NP) of negative-sense RNA viruses is essential for their replication. Multiple copies of NP bind the single-strand RNA genome and antigenome to form ribonucleoprotein (RNP) complexes (also called nucleocapsids). In addition to their function in genome encapsidation, NPs interact with multiple viral and cellular factors to enable genome transcription, replication, packaging, and intracellular trafficking (14–22). Several facts hinder the identification of TiLV’s NP: (i) no apparent homology is detected among known viral NPs and any of TiLV ORFs. (ii) there is greater diversity in NP structure for viruses with a segmented RNA genome, compared to viruses with non-segmented RNA genomes (21), and (iii) NP proteins are characterized by positively charged surfaces, yet, the majority of
TiLV proteins have relatively high isoelectric point (pI) values (10). Thus, TiLV is an example of an emerging pathogen for which only minimal information is available regarding the functionality of its proteins. Here we describe the application of a suite of bioinformatics, genetics, and biochemical tools that identified the protein encoded by Segment 4 of TiLV genome as the NP.

Results

A combined feature analysis predicts Protein 4 or Protein 6 as TiLV’s NP.

Although TiLV is defined as an orthomyxovirus-like agent (10), nine of its ten major ORFs show no homology to other known sequences. Even the homology to the ICV PB1 subunit, found in Protein 1 (TiLV proteins are numbered according to the segments from which they are expressed), is low (~17% amino acid identity (10)). Thus, TiLV is an example of a novel pathogen for which sequence homologies cannot be used to deduce the functions of many of its proteins.

Since all negative-stranded RNA viruses encode a single NP, of which multiple copies co-assemble with the viral genomic RNA to form nucleocapsids (reviewed in (21, 23)), we set out to deduce which of TiLV proteins serves as the NP. One common characteristic of orthomyxovirus NPs is a relatively high pI due to the high content of positively-charged residues; for example, the 498-residue influenza A virus (IAV) NP (strain A/WSN/33; (24)) has a calculated pI of 9.38 and bears 72 arginines and lysines. However, the calculated pI of most TiLV proteins is high (in fact, eight out of the ten proteins are basic (10)), making it challenging to identify TiLV NP based on this single criterion.
We reasoned that a given protein’s function is derived from a combination of multiple features and accordingly, proteins with similar characteristics are likely to share similar functions. We harnessed this notion to predict the function of TiLV proteins based on features extracted from their sequences, and compare these features to the characteristics of known proteins of other members of the orthomyxoviridae family. These features included the pI mentioned above, protein size (relative to the total coding size of the virus; Methods), and dN/dS, which is the ratio between the rate of non-synonymous (dN) and synonymous (dS) mutations (25). The latter reflects the type and level of selection operating on a protein. Our underlying assumption was that viral proteins with similar functions experience similar selection pressures, and thus, their dN/dS profiles will be similar. For example, positive diversifying selection operates on many viral proteins that directly interact with the adaptive immune system, such as glycoproteins (26), whereas non-structural proteins tend to be more conserved. Combining these features into a 3D scatter plot resulted in the clear segregation of the different proteins of the orthomyxoviruses into distinct functional groups (Fig. 1A, colored dots). By superimposing TiLV proteins onto this 3D plot (Fig. 1A, black dots), we assessed the similarity of TiLV proteins to the proteins of the orthomyxoviruses, based on our three features. Reassuringly, we noted that Protein 1, which is known to share homology to the ICV PB1 subunit, was indeed predicted as a polymerase subunit. When focusing on NPs of orthomyxoviruses, our analysis yielded either Protein 4 or Protein 6 as a candidate NP for TiLV (Fig. 1A, and see Fig. 1B for elaborated prediction probabilities); thus, we chose to probe both proteins’ RNA binding activities.

**Protein 4 binds RNA in the yeast three-hybrid system.**

To test the above predictions, we evaluated the interactions of Proteins 4 and 6 with RNA in the yeast three-hybrid (Y3H) system that detects protein-RNA interactions (27). To this analysis, we also added Proteins 7 and 8 (predicted as matrix proteins) since these are the two most basic proteins of TiLV.
(calculated pI values of 9.98 and 9.86, respectively). The Y3H system has successfully been used to analyze interactions of viral protein-RNA pairs (27–30), e.g., the RNA-binding activity of the human immunodeficiency virus (HIV) Gag and nucleocapsid (NC) proteins to HIV RNA encapsidation signal (HIV ψ RNA). In this genetic system, a hybrid RNA molecule bridges two fusion proteins (Fig. 2A). The first fusion protein, named LexA-MS2 coat, contains the LexA DNA-binding domain, fused to the coat protein of the MS2 bacteriophage that specifically interacts with MS2 sequences embedded within the RNA hybrid. The second fusion protein contains an N-terminal Gal4 transcriptional activation domain (Gal4AD) and a C-terminal protein; the latter is tested for binding to a specific sequence inserted into the RNA hybrid. The binding of the two fusion proteins to the same RNA hybrid results in the transcriptional activation of a lacZ reporter located downstream of the LexA DNA binding site. The S. cerevisiae strain L40 coat constitutively expresses the LexA-MS2 coat fusion protein and contains an integrated copy of the LexA-regulated lacZ reporter. To this end, we transformed the yeast strain L40-coat with plasmids expressing the Gal4AD fused to TiLV Protein 4, 6, 7, or 8, and plasmids expressing different RNA hybrids. These RNA hybrids consisted of a shared, primary RNA hybrid sequence linked to different TiLV genomic sequences: Segment 1 middle region ('Segment 1' hybrid) or the 5′ sequence of Segment 7 ('5′ Segment 7' hybrid). The primary RNA hybrid is composed of the 5′ stem-loop structure of the S. cerevisiae RNase P RNA gene (RPR1) leader, a linker (into which, TiLV sequences were inserted), two stem-loop structures that bind the MS2 coat protein, and the RPR1 3′ terminal sequence (27).

Qualitative colony-lift filter assay clearly demonstrated that co-expression of Gal4AD-Protein 4 fusion with each of the RNA hybrids resulted in robust activation of the lacZ reporter (depicted by yeast colonies that developed a dark cyan color, Fig. 2B). In contrast, co-expression of Gal4AD-Protein 6, 7, or 8 fusions with the same RNA hybrids did not activate the reporter gene (despite comparable expression levels of all tested TiLV proteins in the yeast, Fig. 2B). To test the RNA-binding specificity of Gal4AD-Protein 4, the assay was repeated with an RNA hybrid containing either the HIV ψ, or only the primary
RNA sequence (‘Primary RNA’) (27, 28, 30). Here too, co-expression of these RNA hybrids together with Gal4AD-Protein 4 fusion, but not with Gal4AD-Protein 6, 7, or 8 fusions, resulted in readily detected activation of the lacZ reporter, although this activation was weaker for the Primary RNA hybrid. No such activation occurred when we omitted the plasmid expressing the RNA hybrid from the transformation mix (‘No RNA’); excluding the possibility that reporter activation resulted from a direct, RNA-independent interaction between Gal4AD-Protein 4 and LexA-MS2 coat fusion proteins. A quantitative β-galactosidase liquid assay further established these results: we observed a significant activation of the lacZ reporter for Gal4AD-Protein 4 fusion (but not for Gal4AD-Protein 6, 7, or 8 fusions), only if an RNA hybrid was co-expressed with the fusion protein (Fig. 2C).

Notably, while 30-32°C is the typical temperature range for the Y3H assay (two days of yeast growth, followed by β-galactosidase assay) (27–30), the optimal temperature for TiLV replication is 25°C (31). In line with these temperature differences, growing the yeast for two to three days at 30°C resulted in relatively strong activation of the lacZ reporter for the positive control (Gal4-HIV NC/HIV ψ pair), but no activation for Gal4-Protein 4, 7, or 8 fusions, co-expressed with HIV ψ RNA (Fig. 2D), presumably due to misfolding or reduced steady-state levels of TiLV protein(s), or both. Performing the assay for three days at 25°C resulted in no reporter activation for the TiLV pairs and only weak activation of the positive control, likely because of the suboptimal conditions for yeast growth. Therefore, we adapted the timelines of the filter and the liquid assays to include growth of the yeast at 30°C, followed by incubation at 25°C to allow protein-RNA pairing at the optimal temperature for TiLV replication. This design allowed evident reporter activation for either Gal4-Protein 4 or Gal4-HIV NC fusions, paired with the HIV ψ RNA (Fig. 2D). Accordingly, we applied these timelines (summarized in Fig. 2E) for the experiments described in Fig. 2 and Fig. 3.
Mutations in specific positively charged residues hinder Protein 4 binding to RNA in the Y3H system.

Next, we used the Y3H system to screen for mutations in Protein 4 that hamper interaction with RNA. We generated a library of yeast plasmids expressing the Gal4AD-Protein 4 fusion with randomly inserted linkers, encoding in-frame five amino acids additions. Proteins encoded by this library were tested for binding to TiLV RNA (5' Segment 7 hybrid RNA) in the Y3H system. Out of ~7000 yeast colonies of transformants, we isolated 45 colonies that did not stain blue by colony-lift filter assay. Sequence analyses of Protein 4 ORF expressing plasmids, extracted from these colonies, revealed 39 ORFs with in-frame linker insertions. Overall, the insertions were distributed along the entire ORF (Fig. 3A), suggesting that multiple sequences in Protein 4 contribute to its RNA-binding activity. For IAV, the structure of NP reveals that positively-charged residues, distributed along the NP polypeptide, fold to form the surface of the RNA-binding groove (24); and that mutations in specific basic residues (e.g., R267 and R416) greatly reduce NP RNA-binding activity (32, 33). Analogous organization and function of positively charged residues exist in the NP of a fish orthomyxovirus - the infectious salmon anemia virus (ISAV), and specific mutations in such residues disrupt RNA binding (34). Notably, the distribution of basic residues along Protein 4 polypeptide is also wide (Fig. 3A), and we observed a relatively high number of indel mutations in the region that spans residues K134 and K136 (four different linker insertions, of which one was accompanied by an in-frame deletion, Fig. 3A). Accordingly, we introduced, by site-directed mutagenesis, alanine substitutions in these residues (to generate K134A or K136A mutants). Since three linker insertions flanked residue R158, we also mutated this residue (to generate an R158A mutant). Finally, a cluster of four basic residues (K90, K91, R92, and R94) was mutagenized too to generate the K(90,91)A/R(92,94)A mutant. We introduced these mutations into the pACT2-ORF4 plasmid, and the binding of the parental (wt) or mutant Gal4AD-Protein 4 fusions to 5’ Segment 7 hybrid
RNA was tested in the Y3H system, using a quantitative β-galactosidase liquid assay (Fig. 3B). Of note, given that we carried out the site-directed mutagenesis on different clones of the pACT2-ORF4 plasmid (Materials and Methods), for each mutant, a corresponding control clone with wt sequence was generated and tested in parallel with the mutated cognate plasmid. These analyses demonstrated a significant reduction in β-galactosidase activity for the composite mutant K(90,91)A/R(92,94)A, relative to the wt Gal4AD-Protein 4 fusion (Fig. 3B). Of the single-point mutants, K134A showed a significant and robust reduction in β-galactosidase activity, while K136A or R158A did not (Fig. 3B). Immunoblotting confirmed the expression of all mutant and wt Gal4AD-Protein 4 fusions in the yeast (Fig. 3C).

Altogether, these results demonstrate that TiLV Protein 4 interacts with RNA in the Y3H system in a sequence-independent, temperature-dependent manner; and that specific positive residues contribute to Protein 4-RNA interactions.

**Multiple copies of Protein 4 are complexed with RNA in infected cells and virions.**

To test if Protein 4 is complexed with RNA in infected cells, we applied the R-DeeP method that screens for ‘RNA-dependent proteins’ - proteins that interact with RNA directly or indirectly (35). This unbiased and enrichment-free method is based on density gradient ultracentrifugation, where complexes of RNA-dependent proteins migrate to different locations in sucrose density gradients, depending on the presence or absence of intact RNA. Accordingly, we infected the tilapia OmB cell line with TiLV, extracted RNA-protein complexes from infected cultures, and separated in sucrose density gradients the extracts that were pretreated or not with RNase A. Then, we determined the migration of Protein 4 in the gradients by immunoblotting fractions of the gradients with αProtein4 antibodies. In the absence of RNase A treatment (and the presence of RNase inhibitor), most of Protein 4 migrated to fractions with
relatively high sucrose density (Fig. 4A, -RNase, Fractions 10-23). In contrast, RNase A treatment shifted the majority of this protein to fractions with lower sucrose densities (Fig. 4A, +RNase, Fractions 3-11). Such a ‘left shift’ is typical of many proteins enriched in domains linked to RNA binding (35) and suggests that in infected cells, Protein 4 is complexed with RNA.

The RNA segments of the IAV genome, multiple copies of the viral NP, and a small number of viral polymerase molecules form rod-like RNP complexes (see for example (15, 36, 37)). Imaging these rod-like structures by negative-staining immunoelectron microscopy (immuno-EM), using primary anti-NP antibodies and secondary gold particles-conjugated antibodies, detected multiple NP molecules along the entire length of the RNP rods (36). To test if Protein 4 is positioned in a similar pattern with respect to TiLV’s RNPs, obtained by the R-DeeP method, we pooled samples from high-sucrose-density fractions, enriched for Protein 4-RNA complexes (Fig. 4A, -RNase, Fractions 10-23). Then, RNPs were pelleted from these pooled samples by ultracentrifugation, stained with primary α-Protein4 antibodies and with secondary, gold particle-conjugated, anti-rabbit antibodies, and imaged. The imaging revealed rod-like structures with a clear alignment of the gold particles and these elongated structures (Fig. 4B; Cellular RNPs), highly resembling the organization of NP along IAV RNPs (36). The length of the stained rods varied, likely reflecting the different lengths of TiLV genomic RNA segments. RNP filaments of varying lengths were reported before for IAV (37). We also observed the same organization of Protein 4 along with rod-like structures by immuno-EM when we isolated RNPs from concentrated TiLV virions, using a method described before for IAV (36) (Fig. 4B; Viron RNPs). Altogether, the pattern of Protein 4 staining resembles the one observed for IAV NP and suggests that multiple copies of Protein 4 are part of TiLV RNPs and cover TiLV RNA segments.

**Protein 4 directly interacts with RNA in infected cells.**
To examine if Protein 4 directly binds RNA in infected cells, we applied the XRNAX method that purifies cellular cross-linked protein-RNA complexes (38). In this method, proteins and RNAs are extracted from UV-irradiated cells with the classic acid guanidinium thiocyanate-phenol-chloroform extraction mix (39), and a subsequent organic phase separation step separates free RNAs (aqueous phase) and free proteins (organic phase) from cross-linked protein-RNA complexes (enriched in the interphase). To this end, we extracted TiLV-infected OmB cells that were UV-irradiated or not. Only the extract of the UV-irradiated sample formed sponge-like, insoluble interphase, while the sample that was not irradiated formed only fluid-like interphase, as described before (38). We purified the proteins from these interphase fractions and treated them, or not, with DNase or RNase. Next, we analyzed these preparations by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, using αProtein4 antibodies (Fig. 5A). Protein 4 was present in the interphase fraction only when the cells were UV-irradiated, suggesting that it interacts with RNA. In line with this notion, a high molecular weight (MW), Protein 4-reactive band (>250 kDa, marked by a black rectangle in Fig. 5A) was observed only in the UV-crosslinked sample and was sensitive to RNase, but not to DNase. Thus, this slow-migrating band is likely composed of high MW complexes of Protein 4 cross-linked to RNA. Of note, in the UV-irradiated samples, we observed an additional Protein 4-reactive band, migrating at Protein 4 calculated MW (~38 kDa, (10), marked by an asterisk in Fig. 5A). This band likely consists of free proteins trapped in the sponge-like interphase, as no additional, silica-based purification steps were included to eliminate such free proteins (38). No reacting bands were observed when we re-probed the membrane with control, rabbit polyclonal antibodies, raised against a TiLV Protein 5-derived peptide (αProtein5 antibodies; see Fig. 5B for immunoblotting specificity of αProtein4 and αProtein5 antibodies and Fig. 5C for re-probing results). Altogether, these results further demonstrate that Protein 4 binds RNA in infected cells. Moreover, the cross-linking by UV suggests a direct binding (40) of Protein 4 to the RNA.

**Protein 4 binds TiLV RNA in infected cells and virions.**
Next, we tested if Protein 4 binds TiLV RNA in infected cells. To this end, we infected susceptible E-11 cells (1) with TiLV, and four days postinfection (dpi), when first signs of cytopathic effect (CPE) were visible, we lysed the cells with a non-denaturing buffer. Then, we immunoprecipitated Protein 4 from the cleared lysates using α-Protein4 antibodies and tested the pellets for the presence of Protein 4 (by immunoblotting) and all ten segments of TiLV genomic RNA (by qRT-PCR). Controls included immunoprecipitation (IP) of lysates of infected cells, using either α-Protein5 antibodies or pre-immune rabbit serum (collected before the vaccination with Protein 4), and IP of lysates of uninfected E-11 cells with α-Protein4 antibodies. These analyses revealed that the pellet obtained with α-Protein4 antibodies contained high amounts of each of the ten viral RNAs, compared to pellets generated with control antibodies (Fig. 6A). This differential co-IP was in contrast to equal, low levels of actin RNA (Ct equals ~26), detected in all pellets (likely reflecting nonspecific binding of this abundant RNA). Accordingly, actin RNA was used as a reference for each pellet, and ΔΔCt values and relative RNA levels (‘Relative Co-IP’) were calculated (Fig. 6A). Averaging the normalized levels of the ten RNA segments in each of the pellets revealed a significant co-IP of TiLV RNA with Protein 4, with fold differences of two to three orders of magnitude, compared to the controls (Fig. 6C). No viral RNAs were detected in the pellet obtained with α-Protein4 antibodies from uninfected cells, ensuring the absence of cross-contamination. Immunoblotting revealed an efficient IP of Protein 4 or Protein 5 when the cognate antibodies were used (Fig. 6D). To analyze Protein 4-genomic RNA interactions in TiLV virions, we concentrated virus particles from culture supernatants of TiLV-infected E-11 cells and repeated the above co-IP in non-denaturing conditions with α-Protein4, α-Protein5, or pre-immune antibodies. qRT-PCR (here, virion RNA levels were not normalized to actin RNA) and immunoblotting demonstrated efficient and significant co-IP of Protein 4 with all ten segments of TiLV RNA genome, compared to controls (Fig. 6B, E and F). Together, these results suggest that Protein 4 binds the ten RNA segments of TiLV, both in infected cells and in free virions.
Cytoplasmic and nuclear distribution of Protein 4.

Orthomyxoviruses replicate in the nucleus. The NP escorts the RNA genome to the nucleus and out of the nucleus (41, 42). To monitor the intracellular distribution of Protein 4, we stained TiLV-infected OmB cells at one dpi, with primary α-Protein4 antibodies and a secondary Alexa Fluor 488-conjugated anti-rabbit antibody; and imaged the cells by fluorescence microscopy. This analysis revealed Protein 4 in both the cytoplasm and the nuclei of infected cells (Fig. 7A, B), while no fluorescent signals were detected in the negative control (Fig. 7A; noninfected), stained cells. Notably, a portion of Protein 4 formed discrete cytoplasmic puncta with variable sizes (Fig. 7A, B). Measuring the average Protein 4 signal in the nucleus and the cytoplasm among infected, untreated cells revealed that 52% of cellular Protein 4 was nuclear (Fig. 7C, UT). This nuclear localization increased to 82% when we treated TiLV-infected cells with leptomycin B (LMB) - an inhibitor of nuclear protein export, mediated by CRM1 (43–45) (Fig. 7B, C; LMB). Overall, these results suggest that Protein 4 shuttles between the cytoplasm and the nucleus at one dpi and that it uses CRM1 for nuclear export.

Discussion

The largest of the ten major proteins of TiLV has weak homology to the polymerase subunit of ICV and is proposed to subserve that function for TiLV. The functions of the other nine proteins are unknown. The findings presented here are consistent with the identity of Protein 4 as the TiLV NP. These findings include bioinformatic combined feature analysis and experimental characterization of Protein 4 as an RNA-binding protein that binds RNA independently of a specific RNA sequence, interacts with all ten segments of the TiLV genome, and localizes with RNPs. The migration of Protein 4, extracted from infected cells, in density gradients was typical of RNA-dependent proteins - proteins that are complexed
with RNA (Caudron-Herger et al., 2019). Indeed, RNase pretreatment of these cellular extracts changed
the pattern of Protein 4 migration and shifted it to lesser dense fractions. In addition, Protein 4 from
both infected cells and purified virions co-immunoprecipitated in non-denaturing conditions with all ten
segments of the TiLV RNA genome. While these results imply that Protein 4 is complexed with TiLV RNA,
they cannot distinguish between direct and indirect interactions. The binding of Protein 4 to RNA in the
Y3H system strongly suggests a direct Protein 4-RNA interaction but cannot exclude the presence of an
additional cellular factor that mediates this interaction. Yet, UV-irradiation cross-linked Protein 4 to RNA
in infected cells strongly implies a direct interaction (40) between these molecules. Several results
suggest that Protein 4 molecules cover TiLV genome in a manner that is independent of the RNA
sequence: the localization of multiple copies of Protein 4 along with rod-like assemblies that highly
resemble IAV RNPs (36); the interaction of Protein 4 with all ten genomic RNA segments; and the
nonspecific RNA-binding activity of Protein 4 in the Y3H system. Binding single-stranded RNA with no
sequence specificity (46) or some specificity to G-rich and U-poor sequences (47) has been
demonstrated for IAV NP. The multiple copies of Protein 4 presented in infected cells and virions should
assist the development of diagnostics means for TiLV based on antibodies against this protein.
Like IAV NP (48), Protein 4 does not contain canonical consensus sequences of either RGG, KH, arginine-
rich, or RRM RNA binding motifs (49). Instead, many positively charged residues likely mediate the
interaction with RNA, as is the case for NPs of members of the orthomyxoviridae family, including IAV
(24), influenza B virus (IBV) (50), and ISAV (34). These basic residues, scattered throughout the NP
polypeptides, fold to make an electropositive, RNA-binding groove. Thus, a similar fold may be essential
for Protein 4 RNA binding activity. The linker insertions that disrupted Protein 4 RNA binding activity
were scattered throughout ORF4, supporting the notion that multiple regions in Protein 4 contribute to
this function. Since many positively charged residues contribute to NP-RNA interaction in the case of
IAV, IBV, and ISAV, point mutations in only portion of these basic residues significantly disrupt this
This is similar to the observation we made for Protein 4 in the Y3H system: specific charged-to-alanine mutations, such as K(90,91)A/R(92,94)A or K134, significantly disrupted the interaction of Protein 4 with RNA, while other point mutations, such as K136A or R158A, did not. Of note, the adaptation, described here, of the Y3H system to study protein-RNA interactions at 25°C should assist the investigation of additional RNA-binding proteins, derived from poikilothermic animals and their pathogens.

During the course of our analyses, we noted an absence of structural homology between IAV/ISAV NPs and Protein 4. The predicted structures of Protein 4 obtained by both AlphaFold (51) or RoseTTAFold (Robetta server; (52)) differed substantially one from another and from the solved crystal structures of IAV or ISAV NPs; specifically, we noted very high root-mean-square deviation (RMSD) values between IAV/ISAV NPs and Protein 4, ranging between 14-42 Å (calculated by PyMOL software). The apparent lack of structural homology adds to the lack of sequence homology among TiLV sequences and other known sequences and further demonstrates the uniqueness of TiLV. Moreover, the lack of sequence and structural similarities highlights the importance of the procedures described herein that revealed the function for Protein 4.

The fact that an abundant cellular RNA like actin mRNA did not efficiently co-immunoprecipitate with Protein 4 suggests that Protein 4 can preferentially interact with TiLV RNA in infected cells, despite its apparent RNA-binding activity, which is independent of a specific RNA sequence. Such preferential binding may result from coordinated co-expression of NP and viral RNA in a specific cellular compartment(s). Indeed, viruses with negative RNA genome encapsidate their genomes in nucleocapsids concomitantly with viral genome replication (17). In addition, efficient replication of the IAV genome may require nucleolus targeting by NP early in infection (42, 53, 54). The nuclear localization of Protein 4, described in this manuscript, may provide the cellular compartment needed for...
TiLV RNP assembly. Currently, we do not know if specific sub-nuclear compartments are required for such assembly.

The nuclear and cytoplasmic localization of Protein 4 matches the presence of TiLV RNA in both compartments (10) and supports the notion that Protein 4 functions as NP. Accordingly, we suggest that Protein 4 shuttles TiLV RNA between these two compartments by providing the required nuclear localization signal (NLS) and nuclear export signal (NES). This scenario resembles the cytoplasmic/nuclear distribution of IAV NP (41, 55), mediated by its NLS and NES (reviewed in (23)).

While the balance between NLS and NES activities of IAV NP may be dynamic (23), inhibition of the cellular NES receptor, CRM1/exportin-1, by LMB (43–45), increases the nuclear distribution of IAV NP (56). The nuclear distribution of Protein 4 increased too upon LMB-treatment of TiLV infected cells, further emphasizing the nucleus-cytoplasm shuttling of this protein, its dependency on CRM1 and the overall similarity to influenza virus NP. Currently, we do not know if Protein 4, like IAV NP, directly binds CRM1 (56), or the identity of specific residues in Protein 4 that function as the NLS or NES. Of note, the matrix (M1) protein of influenza viruses shares some characteristics with the nucleoprotein, including nucleocytoplasmic shuttling as part of the viral RNPs (vRNPs) (57, 58), and thus, Protein 4 could have been assigned as TiLV M1 protein. However, we consider this possibility unlikely since M1 does not bind the RNA directly; in contrast, Protein 4 does (demonstrated by the Y3H and XRNAX assays).

Another similarity between TiLV and IAV is that the NPs of the two viruses form discrete puncta. In the case of IAV, after the nuclear-to-cytoplasm export of vRNPs, these complexes form discrete puncta (also named ‘hot spots’) that enlarge upon the progresses of infection, while accumulating different vRNP segments (59–64). Similarly, our immunofluorescence microscopy analyses demonstrate that TiLV protein 4 forms discrete cytoplasmic puncta with varying sizes. It will be interesting to see if these viral inclusions are also located in close proximity to endoplasmic reticulum (ER) exit sites (63) and if their biogenesis affects (or affected by) the cellular GTPase Rab11 pathway, as was demonstrated for IAV (59, 65).
61–63, 65–68). Overall, the many similarities, described here, that exist between Protein 4 and NPs of influenza viruses further strengthen the idea that TiLV is a member of the orthomyxoviridae family.

In addition to elucidating the probable identity of the NP of a novel virus that is a threat to global food security, our work illustrates a strategy for functional analyses in viral discovery where sequence analysis does not reveal homologies to known viral or cellular proteins. This strategy is important in light of the expected discovery of many unknown viruses and the zoonotic potential of a portion of such pathogens.

Materials and Methods

Feature analysis for TiLV and influenza viruses.

Coding sequences of TiLV, influenza D virus (IDV), ISAV and Thogotovirus were downloaded from NCBI (72); coding sequences of IAV, IBV, and ICV were downloaded from the NIAID Influenza Research Database (IRD) (73). Only sequences that maintained a reading frame were further analyzed. The sequences were classified in datasets based on the type of the virus (IAV, IBV, ICV, IDV, ISAV, Thogotovirus or TiLV) and the type of the ORF (e.g., PB1, M2, or NP). IAV datasets were further classified by the host (e.g., avian or human) and strain (e.g., H1N1 or H5N1). This classification resulted in overall 431 datasets, which were aligned using Mafft alignment (74) with default parameters and, due to computational intensity, the most distant 35 sequences were sampled. Next, we codon-aligned the sequences using PRANK (75) and constructed a maximum-likelihood phylogeny using PhyML (76). Overall, the total number of sequences in our datasets was 14025. Dataset alignments and tree files were uploaded to Zenodo and can be accessed using the following link:
For each dataset, we calculated three parameters: pI values, ‘relative ORF length’, and dN/dS ratio. Specifically, we determined the average pI for each dataset using an isoelectric point calculator (77). The relative ORF length was calculated by dividing the average length of an ORF (a dataset) by the total coding length of the specific cognate virus (e.g., the average length of PB1 of ICV divided by the total coding length of ICV). For dN/dS calculation, we ran the Selecton software (78) with both the M8 model, which allows for positive selection detection, and the null M8a model. For each dataset, a likelihood ratio test was performed following multiple testing corrections with FDR (79) to determine which model fits the data better. For each dataset, the dN/dS values were averaged across all positions so that each dataset was represented by one dN/dS value. A 3D scatter plot was generated using ggplot2 (80) and gg3D (https://github.com/AckerDWM/gg3D).

Predicting the protein label for each TiLV protein was performed with Linear Discriminant Analysis of the Scikit-learn python module (81).

Y3H plasmids.

TiLV ORFs (of Protein 4, 6, 7, or 8), or portions of TiLV genome (Segment 1 middle region or the S’ sequence of Segment 7), were amplified from TiLV genomic RNA by RT-PCR (see Table 1 for a list of oligonucleotides used in this work). Amplified ORFs were cloned into BamHI and EcoRI-digested pACT2 (a yeast expression vector that carries the LEU2 marker; Clontech), using the Gibson Assembly method (82). The resulting plasmids (pACT2-ORF4, 6, 7, or 8) encode fusion proteins with an N-terminal Gal4AD and a C-terminal TiLV protein (Gal4AD-Protein 4, 6 7 or 8, respectively). Plasmids expressing RNA hybrids were generated by homologous recombination in the S. cerevisiae, Y3H strain, L40-coat (27). To this end, plIIA/MS2-2 (a yeast RNA expression vector that carries the URA3 marker; (27)) was linearized with SmaI and SphI and co-transformed into yeast with the above Segment 1 or 7-derived fragments (tailed, by PCR, with sequences homologous to the termini of the linearized plIIA/MS2-2). The resulting RNA hybrid
expressing plasmids were extracted from the yeast with Zymoprep Yeast Plasmid Miniprep I (ZYMO RESEARCH; #D2001). All the above plasmids were propagated in E. coli, purified with NucleoBond Xtra Midi Plus (MACHEREY-NAGEL; #740412.50), and their inserts’ sequence was verified. RNA hybrids containing either the HIV-1 encapsidation or only the primary sequence of the RNA bridge were described before (27, 28, 30).

**Y3H assay.**

Colonies of yeast transformants, selected for uracil (Ura) and leucine (Leu) prototrophy, were analyzed for reporter activation using filter lift assays (27, 28, 30), with the indicated modified incubation times and temperatures. For liquid β-galactosidase assays, yeast transformants (3 colonies per condition) were randomly picked from selection plates and grown overnight in a selective medium (5ml, 25°C). To synchronize the growth of the yeast, the overnight cultures were diluted in a fresh selective medium (OD₆₀₀=0.004) and grown overnight to OD₆₀₀ of 0.8-1 at 25°C. The β-galactosidase activity of the liquid culture was determined with ortho-Nitrophenyl-β-galactoside (ONPG) substrate, according to Clontech Yeast Protocols Handbook (PT3024-1).

**Linker insertion mutagenesis.**

Using pACT2-ORF4 plasmid as a template, ORF4 and flanking sequences (100 bp long each) were PCR-amplified. Using this reaction, we added 40 bp homologous to sequences in pBluescript SK+ plasmid (located upstream and downstream of its EcoRI site) and EcoRI sites to the termini of the amplified fragment. This addition enabled the cloning of the amplified fragment into EcoRI-digested pBluescript SK+ by Gibson assembly reaction. The resulting pBluescript-ORF4 plasmid was subjected to in vitro transposon-insertion-mutagenesis, using MuA transposase (Mutation Generation System Kit, Thermo Scientific; #F-701). Plasmids with random transposon insertions were transformed into E. coli (JM109...
strain) and selected with kanamycin and ampicillin, as the transposon contained the kanamycin resistance gene. ~21000 kanamycin-resistant colonies were pooled, and their plasmids were extracted. To enrich for ORF4 sequences bearing the transposon insertions, the plasmids (2 μg) were digested with EcoRI, separated in 1% agarose gel, and transposon-containing ORF4 fragments (2.4 kb long) were extracted and cloned into new EcoRI-digested pBluescript SK+ plasmid. The resulting plasmids were transformed and selected as above. ~3400 colonies were pooled, their plasmids were extracted and digested with NotI to remove the majority of the transposon body. The digested plasmids were self-ligated to generate a library of plasmids, encoding ORF4 sequences with randomly inserted, in-frame, 15 bp linker insertions. This library was amplified (electroporated into E. coli, and plasmids were extracted from ~55000 ampicillin-resistant colonies), digested with EcoRI, and ORF4 fragments with random linker insertions were extracted after separation in 1% agarose gel. The sequences that flank ORF4 in these fragments, derived from pACT2 plasmid, allowed the cloning of the fragments into this plasmid by homologous recombination. Accordingly, the fragments were transformed, together with NcoI and SacI-digested pACT2, into yeast (L40-coat strain) (83), expressing TiLV RNA (5’ Segment 7 hybrid RNA) from plasmid pIIIA/MS2-2. Transformants were grown on selective plates (SD-Leu-Ura), and ~7000 colonies were screened for lacZ reporter activation, or lack of activation, using Y3H, X-gal colony-lift filter assay. Colonies with no lacZ reporter activation (‘white’ colonies) were expanded, retested for the lack of reporter activation, and subjected to colony PCR with ORF4-specific primers. Amplified products were sequenced to determine the position of the inserted linkers in ORF4.

**Site-directed mutagenesis.**

To generate ORF4 sequences with point mutations and to test them in the Y3H assay, we used pACT2-ORF4 plasmids with linker insertions (isolated from the above mutagenesis screen), since these linkers contained a unique NotI site, allowing cloning in the yeast by homologous recombination (see below).
Specifically, plasmids pACT2-ORF4 CC38W, CC25W, or CC6W (harboring linker insertions downstream of nucleotide 920, 813 or 731 of Segment 4 genomic RNA, respectively; GenBank accession no. KU751817), were used to generate ORF4 mutants K(90,91)A/R(92,94)A, K134A, and K136A, or R158A, respectively. Mutations-containing ORF4 fragments were generated by overlapping PCR with primers harboring the indicated mutations (Table 1). The resulting PCR fragments termini contained sequences homologous to ORF4 sequences, found upstream and downstream of the NotI sites in the cognate pACT2-ORF4 CC38W, CC25W, or CC6W plasmids. These plasmids were digested with NotI and co-transformed with their related PCR fragments into yeast L40-coat strain to allow homologous recombination. In the resulting plasmids, the PCR fragment harboring the point mutation(s) replaced the linker-containing sequence. These plasmids were extracted from the yeast, amplified in *E. coli* and the sequences of ORF4 mutants were confirmed. Next, the plasmids were tested for loss-of-function (lack of lacZ reporter activation) in the Y3H assay. To verify that this loss-of-function was the result of the mutations in ORF4 and not of unidentified mutations in the plasmid backbone, ORF4 wt sequences (with no point mutations) were PCR amplified and introduced into NotI-digested, pACT2-ORF4 CC38W, CC25W or CC6W plasmids, to confirm gain-of-function (activation of the lacZ reporter) of the resulting plasmids in the Y3H assay.

**Generation of rabbit polyclonal antibodies.**

Polyclonal antibodies were raised in rabbits, according to Tel Aviv University Animal Care guidelines. To generate antibodies against Protein 4 (αProtein4), ORF4 was PCR-amplified from pACT2-ORF4 plasmid and cloned into pET28b(+) bacterial expression vector between NdeI and XhoI restriction sites, thus attaching a 6xHis-tag to the N-terminus of Protein 4. The resulting pET28-ORF4 plasmid was transformed into *E. coli* BL21(DE3) and grown in LB under kanamycin selection. An overnight starter was diluted 1:50, grown in 200 ml LB+ kanamycin at 37°C t and at OD<sub>600</sub> of 0.5, protein expression was induced with 0.1 mM IPTG for 3 h. Next, cells were pelleted and resuspended in 4 ml of native Suspension Buffer (20 mM...
Tris, 500 mM NaCl, pH 8.0, complete EDTA-free protease inhibitor cocktail (Roche; #11836170001) and 0.1% Triton. Lysozyme was added (1 mg/ml final concentration), and the suspension was kept on ice for 20 min with an occasional vortex. Cells were disrupted by sonication on ice, using a microtip-equipped sonicator (Fisher Scientific Series 60 Sonic Dismembrator Model F60, 5x 10 sec pulses with 10 sec intervals). The lysate was then treated with 10 μg/ml RNase A and 5 μg/ml DNase I (Sigma Aldrich; #R4642 and #D4263, respectively) (10 min on ice) with occasional vortex and centrifuged (Sorvall RC 6 plus, SS-34 rotor, 20,200 x g) for 20 min at 4°C. The pellet containing Protein 4 in inclusion bodies was resuspended in 10 ml of Buffer-II (50 mM Na₂HPO₄, 300 mM NaCl, 8 M urea, pH 8.0) and stirred at room temperature to solubilize the lysate (~30 min). The lysate was then centrifuged (20 min at 10,000 x g at room temperature), and the soluble Protein 4 was purified from the clear supernatant by Nickel-agarose beads batch purification. Specifically, Nickel-agarose slurry (2 ml, Adar-Biotech; #1018-25), equilibrated with Equilibration Buffer (10 mM Tris, 50 mM Na₂HPO₄, 500 mM NaCl, 8 M urea, pH 8.0), were mixed with Protein 4 lysate and stirred on a rotary shaker (60 min at 4°C). The beads were washed (on a column) with Washing Buffer (10 mM Tris, 50 mM Na₂HPO₄, 500 mM NaCl, 8 M urea, 10 mM imidazole, pH 8.0) until no protein was detected in the flow-through, as tested by Bradford reagent. Protein 4 was eluted with 5 ml Elution Buffer (10 mM Tris, 50 mM Na₂HPO₄, 250 mM NaCl, 8 M urea, 300 mM imidazole, pH 8.0), collecting 1 ml fractions. Peak fractions were pooled and dialyzed twice at 4°C against Dialysis Buffer (20 mM Tris, 300 mM NaCl, 0.01% NP40, 10% glycerol, 1 mM MgCl₂, pH 7.5; the first dialysis buffer also contained 1 mM DTT, while the second did not). Dialyzed protein was stored in aliquots at -20°C.

The Bradford assay determined protein concentration. Two albinoe New Zealand female rabbits (two-month-old) were injected with Protein 4 (100 μg/rabbit), mixed with 1.5 volume of complete Freund’s adjuvant (Sigma; #F5881). Immunization was followed by two booster immunizations with the same dose of Protein 4, mixed with incomplete Freund’s adjuvant (Sigma; #F5506). The rabbits were continuously immunized every three to four weeks (up to five injections). Rabbit sera samples were...
collected one week following the second boost (and subsequently every three weeks). Pre-immunized sera were collected before immunization. To generate antibodies against Protein 5 (αProtein5), a peptide derived from the ORF5 sequence was synthesized using the Liberty Blue Automated Microwave Peptide Synthesizer (Medicinal Chemistry Laboratory, the Blavatnik Center for Drug Discovery, Tel Aviv University). One rabbit was immunized with KLH-conjugated peptide (100 µg/injection) as above.

**Immunoblotting and Antibodies.**

Proteins in extracts of yeast, fish cells and pellets of co-IP or XRNAX experiments were mixed with loading buffer (final concentration of 0.05 M Tris-HCl pH 6.8, 0.1 M DTT, 2 % SDS, 10 % Glycerol, 0.1 % bromophenol blue), heated (100°C, 5min), separated by SDS-PAGE and transferred to nitrocellulose membrane (iBlot 2 Transfer Stacks, Invitrogen; #IB23001). Western blot analyses were performed according to (84), with the following modifications: membranes were incubated with primary antibodies overnight at 4°C, and detection of the horseradish peroxidase (HRP), conjugated to the secondary antibodies was performed with Immobilon Forte Western HRP substrate (Millipore; #WBLUF0500).

Primary antibodies included mouse anti-Actin (MP Biomedicals; #69100; 1:10,000 dilution); mouse anti-HA epitope tag (Biolegend; #MMS-101R; 1:1000 dilution); Rabbit anti-Protein 4 (1:10,000 dilution); Rabbit anti-Protein 5 (1:1,000 dilution). Secondary antibodies included HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch; #115-035-003; 1:10,000 dilution); HRP-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch; #111-035-003; 1:15,000 dilution).

**Cell lines.**

The spontaneously immortalized Omb cell line, derived from Mozambique tilapia (Oreochromis mossambicus) brain (85) and the E-11 cell line, derived from the striped snakehead (Ophicephalus striatus) (86), were grown as described before (31).
Migration of Protein 4 in sucrose density gradients.

TiLV-infected OmB cells (confluent culture in two 10 cm dishes) were trypsinized when the first signs of CPE appeared (4 dpi). Cells were combined, pelleted, extracted, and the extract was treated, or not, with RNase, according to the R-DeeP method (35), with the following modifications: in the absence of RNase treatment, RNase inhibitor (RNasin, Promega; #N251B) was added to the extract (100U/ml); for RNA digestion, only RNase A (Sigma; #R4642) was used. Treated extracts were loaded on 5% to 50% sucrose density gradients, which were fractionated into 23 fractions (500 µl each) after ultracentrifugation (Beckman Optima XPN-80 ultracentrifugation, SW 41 Ti Swinging-Bucket Rotor, 160,000 x g, 18 h, at 4°C). 20 µl of each fraction were analyzed by immunoblotting. For immuno-EM, 100 µl of the indicated fractions were analyzed.

Negative-staining immuno-EM.

Samples (100 µl each) from fractions of the sucrose density gradient (not treated with RNase and enriched with RNP/Protein 4 complexes), were pooled and mixed with R-DeeP lysis buffer (35) to a final volume of 13 ml. RNPs were pelleted by ultracentrifugation (Beckman Optima XPN-80 ultracentrifugation, SW 41 Ti Swinging-Bucket Rotor, 160,000 x g, 3 h, at 4°C), resuspended in 90 µl of DNase/RNase-free water and adsorbed to nickel formvar/carbon coated grids (30 µl/grid). RNPs from PEG-concentrated virions (see above) were isolated as described before (36) and adsorbed to the grids. The grids were washed with PBS, blocked with bovine serum albumin (1%)-containing PBS (30 min), and incubated (40 min at room temperature) with α-Protein4 antibodies (diluted 1:500 in blocking solution). Grids were further washed with blocking solution, incubated (40 min at room temperature) with a secondary, 12nm gold particles-conjugated, goat anti-rabbit antibody (Jackson ImmunoResearch; #111-205-144), washed with PBS, fixed with 2.5% glutaraldehyde, and washed with PBS and ddH2O. The grids
were contrasted with aqueous 2% uranyl acetate and examined with JEM 1400plus transmission electron microscope (Jeol, Japan). Images were captured using SIS Megaview III and iTEM the Tem imaging platform (Olympus).

**XRNAX.**

Confluent OmB cells, in a 10 cm plate, were infected with TiLV, and when first signs of CPE appeared (4 dpi), the culture was processed according to the XRNAX method (38) and the online protocol (https://www.xrnax.com/). Briefly, the supernatant was discarded, and cold phosphate-buffered saline (PBS, 6 ml) was added. The cells were irradiated with UV (150 mJ/cm²), trypsinized, and pelleted. The pellet was extracted with acid guanidinium thiocyanate-phenol-chloroform (EZ-RNA kit, Biological Industries; #20-400-100), and phases were separated by centrifugation. Aqueous and organic phases (containing free RNA and proteins, respectively) were discarded. The sponge-like, insoluble interphase (enriched for protein-RNA crosslinked complexes) was washed with TE+SDS 0.1% buffer (10 mM Tris-Cl, 1 mM EDTA, 0.1% SDS), disintegrated in TE+SDS 0.1% and in TE+SDS 0.5% buffer (10 mM Tris-Cl, 1 mM EDTA, 0.5% SDS), and isopropanol-precipitated according to the XRNAX protocol. The pellet was resuspended in 21 µl ultra-pure water. Equal portions of the cross-linked RNA-Protein suspension were digested with DNase or RNase or left untreated. Specifically, 7 µl of the suspension were mixed with 10 µl ultra-pure water, 2 µl 10X DNase buffer, 1 µl DNase (Baseline-ZERO DNase, Epicentre; #DB0711K) and incubated for 1 h at 37°C. Alternatively, 7 µl of the cross-linked RNA-Protein suspension were mixed with 13 µl ultra-pure water, 0.1 µl RNase A, and incubated for 1 h at room temperature. The digested and undigested suspensions were analyzed by immunoblotting with rabbit anti-Protein 4 polyclonal (αProtein4) antibodies.

**Co-IP of Protein 4 and TiLV RNA.**
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**Immunofluorescence.**

OmB cells were seeded on collagen-coated, 13 mm glass coverslips (4 mg/ml collagen type I from rat tail, Sigma; #C3867) in a 24-well plate, treated, or not, with 45 nM LMB (Merck; #L2913) for 2 h before TiLV addition. 1 dpi, media with LMB/virus were removed, cells were washed twice with cold PBS, fixed with 4% paraformaldehyde for 30 min, and washed three times with PBS. The cells were blocked and permeabilized in blocking solution (1% bovine serum albumin/ 0.1% Triton X-100 in PBS; 45 minute), incubated with αProtein4 antibodies (diluted 1:1000 in blocking solution; 45 min), washed three times
with PBS, incubated with fluorescently labeled, secondary goat anti-rabbit antibodies (Alexa fluor 488, Thermo Fisher Scientific; #A-11034; diluted 1:250 in blocking solution; 45 min). DAPI stain (4′,6-
diamidino-2-phenylindole; 2.5 μg/ml final concentration) was added to the slides with the secondary antibodies. Cells were washed three times with PBS, and the coverslips were glued to glass slides with Fluorescent Mounting Medium (GBI Labs; #E18-18). All the above steps were carried out at room temperature. To calculate the nuclear-cytoplasmic distribution of Protein 4, TiLV-infected OmB cells, treated or not with LMB, were stained as above and imaged with spinning disk confocal (Yokogawa CSU-22 Confocal Head) microscope (Axiovert 200 M, Carl Zeiss MicroImaging) as described before (89, 90).

Calculations were performed on single confocal midplanes of cells, using the DAPI signal to define the nuclei, while the entire cell area was manually defined. Protein 4 signal of selected areas (whole cell, nucleus, and background areas) was measured using the SlideBook program and the Mask function. For each area, the mean pixel intensity of Protein 4 signal and the number of pixels of the area were determined. Total Protein 4 signal of each area was calculated by subtracting the mean pixel intensity of the background from the mean pixel intensity of the selected area and multiplying by the number of pixels per area. The nuclear Protein 4 signal percentage was determined by dividing the Protein 4 signal in the nucleus by the whole-cell Protein 4 signal.

Acknowledgments

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Aviv University) for negative-staining immuno-EM procedures; Prof. Jeroen Krijgsfeld (DKFZ) for advice regarding the XRNAX method.

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References


Figure legends

FIG 1 Combined feature analyses. (A) A 3D scatter plot of IAV, IBV, ICV, IDV, ISAV, Thogotovirus and TiLV proteins, based on relative ORF length, pl values, and evolutionary rates (dN/dS). Each point corresponds to a single dataset, and the colors represent different protein functions. TiLV ORFs are in black, and the numbers correspond to the protein (segment) number. A total of 431 alignments encompassing 14,025 sequences were used to generate the plot (see Methods). (B) Quantitative prediction of the linear discriminant analysis. Shown is a heatmap displaying the probability of each
TiLVs’ proteins assigned to one of orthomyxoviruses’ six known protein functions. Colors correspond to the prediction probability.

FIG 2 Protein 4 binds RNA in the Y3H system. (A) Schematic presentation of the Y3H system. A hybrid RNA molecule bridges the LexA-MS2 coat and the Gal4AD-TiLV chimeric proteins, resulting in transcriptional activation (arrow) of a lacZ reporter (modified from (27)). (B) Filter assay for β-galactosidase activity. Yeast colonies expressing the indicated RNA-protein pairs were tested for β-galactosidase activity using colony lift colorimetric assay. Upper panels show images of sections of the filters with stained yeast colonies. Lower panels show Western blot analyses of the expression of the HA-tagged, TiLV protein-Gal4AD fusions in yeast, probed with anti-HA and anti-Actin (loading control) antibodies. All fusion proteins showed their expected MW (approximately 56, 55, 39, and 37 kDa for Gal4AD fused to Protein 4, 6, 7, and 8, respectively). For Protein 8-Gal4AD, the lower band (asterisk) matches the calculated MW. (C) Quantitative β-galactosidase liquid assay. For each indicated RNA-protein pair, three yeast colonies were randomly picked from a plate of yeast transformants, expanded, and assayed for β-galactosidase activity using the colorimetric liquid assay. The dashed red line indicates the average β-galactosidase activity of No RNA control for all tested proteins. **p≤0.05; ***p≤0.005 (Student’s t-test, accounting for multiple testing using the Bonferroni correction). (D) Filter assay for β-galactosidase activity in different time and temperature settings. Panels show images of yeast colonies, expressing the HIVΨ RNA and the indicated TiLV proteins, grown at the indicated periods and temperatures and stained as in (B). HIV NC served as a positive control. (E) Timeline and conditions for testing Protein 4 RNA-binding activity in the Y3H filter and liquid assays.

FIG 3 Protein 4 mutants with reduced RNA-binding activity in the Y3H system. (A) Random linker insertion mutagenesis. Schematic presentation of Protein 4 ORF (black horizontal line) with randomly inserted linkers (arrows) positions, each disrupted lacZ activation in the Y3H system. A stack of two arrows represents insertions of two different linkers at the same position. An arrow with a pentagon
represents two identical insertions and thus, may not represent two independent insertion events. Grey bars denote positions of arginine or lysine residues. Black bars mark the position of positive residues subjected to site-directed mutagenesis. ‘W’ marks a region with multiple in-frame insertions and a deletion. (B) Effects of point mutations in Protein 4 on its RNA-binding activity. Indicated Protein 4 mutants were tested in the Y3H system for lacZ activation by quantitative β-galactosidase liquid assay. Each mutant and its cognate wt clone were tested in parallel for binding to 5’ Segment 7 hybrid RNA. The boxplot presents the average β-galactosidase activity (with error bars; n = 3 yeast colonies per protein-RNA pair). Significance between pairs of wt and mutant was calculated using Student’s t-test (**p≤0.001; NS, not significant). (C) Expression of wt and Protein 4 mutants in the yeast. Protein extracts of yeast expressing wt and the indicated Protein 4 mutants were analyzed by Western blotting using antibodies against Protein 4 and actin.

FIG 4 Protein 4 is complexed with RNA. (A) Protein 4 migration in sucrose density gradients. RNA-protein complexes were extracted from TiLV-infected OmB cells, treated or not with RNase A, and separated in sucrose density gradients, by ultracentrifugation, according to the R-DeeP method. Fractions (1-23) of the sucrose gradients were collected from top to bottom (low to high density, respectively). The fractions were analyzed by Western blotting using α-Protein4 antibodies. The black arrow at the bottom represents the shift of Protein 4 from dense to light fractions, following RNase A treatment. (B) Negative-staining immuno-EM of RNPs. Fractions of the gradient in (A) (-RNase, Fractions 10-23), containing cellular RNPs that were extracted by the R-DeeP method and enriched for TiLV RNPs, were pooled, pelleted, and stained by immune-EM with primary α-Protein4 antibodies and secondary gold particle-conjugated anti-rabbit antibodies (upper panel). Virion RNPs, extracted as described before (36), were stained as the cellular RNPs (lower panel).
FIG 5 Protein 4 directly interacts with RNA in TiLV-infected cells. (A) TiLV-infected OmB cells were UV-irradiated or not and extracted according to the XRNAX method. Cross-linked protein-RNA complexes were further extracted from a fraction (interphase) enriched with these complexes and were treated, or not, with DNase or RNase. Complexes were analyzed by immunoblotting with αProtein4 antibodies. Reactive bands include free Protein 4 (~38 kDa; asterisk) and a high MW, RNase-sensitive complex (>250 kDa; rectangle). (B) TmB cells were infected (MOI=5) with TiLV (Infected), or not (Naïve), proteins were extracted from the cultured cells at 1 dpi and analyzed by immunoblotting with the indicated antibodies.

A pellet of TiLV virions was extracted and analyzed too (Virions). The majority of Protein 5 (calculated MW of ~38 kDa) appeared as a slower migrating band (~50 kDa), for both cellular and virions extracts.

(C) The blot of Fig. 5A was re-probed with αProtein5 antibodies.

FIG 6 Co-IP of TiLV RNA with Protein 4. TiLV-infected E-11 cells (A) or virions (B) were lysed in a non-denaturing buffer, and the extracts were immunoprecipitated with αProtein4 or αProtein5 antibodies, or with a pre-immune serum. Co-IP of the ten segments (Seg1-Seg10) of TiLV RNA genome (A, B) and actin mRNA (A) was quantified by qRT-PCR. (A) For each pellet, the ∆∆Ct value of each of the viral RNA segments was determined, using actin mRNA as a reference gene (actin CT values were 25.99, 25.84 and 25.97 for pre-immune, αProtein5 or αProtein4 pellets). For each pellet in (B), the ∆Ct value of each of the viral RNA segments was determined. The fold change of RNA levels in pellets obtained with αProtein4 or αProtein5 antibodies (‘Relative Co-IP’) in (A, B) were calculated relative to the RNA levels in the pre-immune pellet (which was set to 1). Boxplots (C, E) show the average (with error bars) of the co-IP of all ten viral RNA segments (dots) in the αProtein4 or αProtein5 pellets, relative to the pre-immune pellet. ***p<0.001 (Student’s t-test). Immunoblots (D, F) with αProtein4 or αProtein5 antibodies. Pellets correspond to 4% of the cell or virion extracts, while the input corresponds to 2.5% or 0.8 % of the extract analyzed in (D) or (F), respectively.
FIG 7 Cytoplasmic and nuclear distribution of Protein 4. (A) OmB cell cultures were infected (MOI=0.04) with TiLV (TiLV) or not (noninfected), or were treated with 45 nM LMB for 2 h and then infected with TiLV in the presence of LMB (LMB+TiLV). 24 h postinfection, the cells were stained with αProtein4 antibodies and DAPI and imaged with a confocal microscope. Single optical sections of the cultures are shown, with DAPI and Protein 4 signals in greyscale and merged signals in color. (B) Serial optical sections of the TiLV-infected cell in (A) were reconstituted into a 3D image. Arrows point to Protein 4 puncta. Grid and bars represent 10 μm. (C) Boxplot of the average percentage of the nuclear signal of Protein 4 of 16 TiLV-infected and untreated cells (UT) and 19 infected, LMB-treated cells (LMB), described in (A). For each cell, the percentage of the nuclear signal was determined by dividing the nuclear signal of Protein 4 by the total signal of this protein in the whole cell. ***p≤0.001 (Student’s t-test).
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<td>pACT2 downstream to ORF4 REV</td>
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<td>Site directed mutagenesis- K134A</td>
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<tr>
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*TiLV Sequences are in italics.

* Mutated nucleotides are in bold.