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Proteomic analysis of silkworm midgut cellular proteins interacting with the 5′ end of infectious flacherie virus genomic RNA

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Abstract

The flacherie disease in the silkworm is caused by the infectious flacherie virus (IFV). IFV relies on its 5′ region of genomic RNA to recruit host-related factors to implement viral translation and replication. To identify host proteins bound to the 5′-region of IFV RNA and identify proteins important for its function, mass spectrometry was used to identify proteins from silkworm midgut extracts that were obtained using RNA aptamer-labeled 5′ region of IFV RNA. We found 325 protein groups (unique peptide ≥2) bound to the 5′ region of IFV RNA including translation-related factors (16 ribosomal subunits, 3 eukaryotic initiation factor subunits, 1 elongation factor subunit and 6 potential internal ribosome entry site *trans*-acting factors), cytoskeleton-related proteins, membrane-related proteins, metabolism enzymes, and other proteins. These results can be used to study the translation and replication related factors of IFV interacting with host silkworm and to control flacherie disease in silkworm.

Key words: infectious flacherie virus, RNA-binding protein, mass spectrometry, silkworm, midgut cellular protein

Introduction

Bombyx mori infectious flacherie virus (IFV) is the causative agent of flacherie disease of the silkworm, which is a viral disease that causes serious sericulture losses in China, Japan, and other regions [1]. IFV is a positive-sense single-stranded RNA virus and a species of the family Iflaviridae, genus *Iflavirus* that encodes a single polyprotein of 3085 amino acids that is proteolytically processed into four structural proteins and a series of non-structural proteins by cellular and viral proteases [2]. The genomes of small positive-strand RNA viruses not only encode the viral proteins required for viral genome replication and infectious virus production, but also contain *cis*-acting RNA sequences and structures that play critical roles in the virus life cycle [3]. Highly conserved secondary RNA structures are known to be present at the 5′ region of the genomes of picornaviruses and hepatitis C virus (HCV) [4,5].

Host cell factors that interact with these RNA elements play important roles in all aspects of the virus life cycle [6,7]. Translation initiation of all picornavirus RNAs is dependent on the internal ribosome entry site (IRES) located in the long 5′ untranslated region (UTR) [8]. Various structural elements in the 5′ UTR, which differ among picornavirus genera, control the viral replication cycle in concerted action with the 3′ UTR [9,10]. RNA–protein complexes are formed when authentic viral RNA or *in vitro*-synthesized viral RNA transcripts are incubated with cell extracts. These complexes are involved in viral RNA replication and translation. RNA–protein interactions have been determined using different approaches, such as ultraviolet cross-linking, and electrophoretic mobility-shift assays. Although these techniques are useful for analyzing RNA-binding factors, mass spectrometry (MS) offers the advantage of identifying several proteins within a complex mixture [11–13]. RNA aptamers are short nucleic

acids (typically 12–80 nucleotides) capable of specific, tight binding to their target molecules. They may be used as RNA affinity tags for the rapid purification and investigation of RNAs and RNA–protein complexes [14]. At present, many viral RNA-interacting cellular proteins have been identified by riboproteomic affinity methods, such as several ribosomal subunits, eukaryotic initiation factors (eIFs), and IRES *trans*-acting factors (ITAFs) [10,15].

The target of IFV is the goblet cells of the midgut epithelium, and IFV is replicated in the cell cytoplasm. Without a susceptible cell line for IFV infection, it is difficult to study the biology of virus [16]. However, this problem prompted the development of alternative strategies to generate *in vitro* RNAs that are similar to the authentic viral genome. The RNA of IFV can also act as efficient mRNA in rabbit reticulocyte lysate and in wheat germ translation systems [17]. A previous study has revealed that the 5′ region of IFV has IRES activity, like *Ectropis obliqua* virus, *Perina nuda* virus, and *Varroa destructor* virus-1 in *Iflavirus* [18–21]. Interaction with these eIFs and ITAFs is necessary to promote IRES activity. The IRES region is a superior antivirus target. However, there were no reports regarding the interactions of host proteins with the 5′ region of *Iflavirus*.

In this study, we incubated the 5′ region of IFV genomic RNA (599 nt) that links the streptavidin aptamers [22] and the midgut extracts to perform affinity chromatography with streptavidin beads. The affinity of purified RNA–protein complexes was analyzed by MS. Our results demonstrated that the 5′ end of the IFV genome contains elements that bind specifically to different midgut cellular proteins, some of which include ribosomal protein, eIF, eukaryotic translation elongation factor (eEF), and others that are known or unknown factors involved in picornavirus and HCV IRES-associated translation and replication. These results would be useful for further study of the translation and replication of IFV and control of flacherie disease in silkworm.

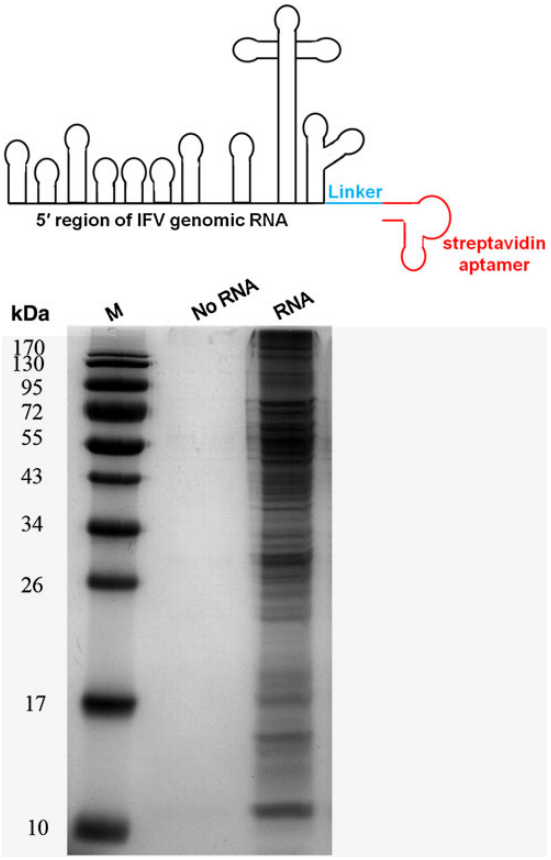

Figure 1. Identification of midgut cellular proteins that interact with the IFV 5′ region of genomic RNA (A) Schematic of the secondary RNA structure generated for chromatography. The RNA construct contains the SP6 promoter fused to sequences from IFV sequence (nts 1–599) with the streptavidin aptamer inserted. (B) SDS-PAGE of protein that interacted with IFV 5′ region of genomic RNA. Lane 'M' was prestained protein ladder, lane 'no RNA' was the supernatant that did not contain streptavidin aptamer-tagged RNA, lanes 'RNA' were RNA-binding proteins.

Materials and Methods

Construction of plasmids and *in vitro* RNA transcription

A cDNA clone of nt 1 to 599 of the 5′-region of IFV-CHN01 genome (GenBank: EU868609) was fused to the SP6 promoter and the RNA streptavidin aptamer and linker sequence using splice overlap extension polymerase chain reaction (PCR) (**Fig. 1A**) [22]. The PCR product was purified by a PCR purification kit (AXGEN, New York, USA) according to the manufacturer's instructions and inserted into plasmid vector pMD18 (TaKaRa, Dalian, China) linearized by the restriction enzyme *Xba*I. The linearized vectors were used for SP6 RNA polymerase (TaKaRa) *in vitro* transcription reactions to generate RNA according to the manufacturer's instructions.

Preparation of midgut cellular extracts

The midguts of 5th instar silkworm (strain P50) were collected, washed in physiologic saline and phosphate-buffered saline (PBS), and homogenized with insect cell protein extraction buffer by Precellys 24 (Bertin Technologies, Paris, France) [23]. The cell homogenates were centrifuged at 10,800 *g* for 15 min at 4°C. The supernatant was used to perform RNA affinity purification.

Enrichment of RNA-binding proteins using RNA affinity purification

The rapid purification of RNA-binding protein streptavidin affinity tags was performed as described previously [12] with some modifications. Briefly, the streptavidin aptamer tagged RNAs were incubated with midgut cellular extract (pre-cleared with streptavidin beads) in PBS containing 200 U of RNasin at 4°C for 2 h. RNA–protein complexes were affinity-purified with immobilized streptavidin beads. The RNA/protein/beads complexes were washed three times with cold PBS containing 200 U of RNasin. The elution was performed with saturated biotin solution and incubated at 4°C for 2 h. The beads without streptavidin aptamer-tagged RNA were used as the control. After centrifugation, the same volume of supernatant containing enriched proteins was resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Supernatant without streptavidin aptamer-tagged RNA was used as a control and stored at −70°C until Q Exactive MS.

Mass spectrometry

The supernatant proteins (with and without aptamer-tagged RNA) were enriched three times, and pooled. The 150 μg protein of elution samples (RNA and no RNA system) was then digested using filter-aided proteome preparation, lyophilized, and dissolved in 0.1% formic acid (FA). All of the peptide fractions were injected into the liquid chromatography (LC) system. The time of the LC gradient separation is 60 min. Experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Thermo Fisher Scientific, Waltham, USA). The precursor-ion mass tolerance was 20 ppm, and

fragment-ion mass tolerance was 0.1 D. Each fraction (10 µl) was injected for nano-LC-MS/MS analysis. The peptide mixture (5 µg) was loaded onto a the C18-reversed phase column (Thermo Scientific Easy Column; 10 cm long, 75 µm inner diameter, and 3 µm resin) in buffer A (0.1% FA) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% FA) at a flow rate of 250 nl/min controlled by IntelliFlow technology over 60 min. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for higher-energy collisional dissociation (HCD) fragmentation. Determination of the target value is based on predictive automatic gain control. Dynamic exclusion duration was 40 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled.

Western blot analysis

The supernatant proteins (with and without aptamer-tagged RNA) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (PALL, New York, USA) The membranes were blocked with 5% non-fat dried milk for 1 h at 37°C, and incubated with rabbit anti-coatomer subunit delta polyclonal antibodies (Abcam, Cambridge, UK), and rabbit anti-La protein homolog polyclonal antibodies which were prepared in our laboratory (data not shown). After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dingguo, Shanghai, China) and visualized by ECL Plus chemiluminescence reagent kit (Perkin-Elmer, Waltham, USA).

Data analysis

The raw MS/MS results were searched against the UniProt KB_ *Bombyx mori* database using Proteomics Tools (version 3.1.6) and Mascot (version 2.2). Database search parameters were as follows: enzyme was trypsin, missed cleavage was 2, static modifier was carbamidomethyl (C), dynamic modification was oxidation (M), and the filter parameters were protein FDR ≤0.01 and peptide FDR ≤0.01.

The chromosome *loci* of the mapped genes were shown using SilkMap tool (http://www.silkdb.org/silksoft/silkmap.html). The protein sequences were subject to BLAST query against the AmiGO database (http://amigo.geneontology.org/cgi-bin/amigo/blast.cgi) and clusters of orthologous groups (COGs) (http://www.ncbi.nlm.nih.gov/COG/). The corresponding gene ontology (GO) terms were extracted from the most homologous proteins, and the GO annotation results were plotted using the Web Gene Ontology Annotation Plotting (WEGO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) by uploading compiled WEGO native format files containing the obtained GO terms [24].

The FASTA protein sequences of identified proteins were searched against KEGG (http://www.genome.jp/kegg/), and the corresponding KEGG pathways were extracted. Meanwhile, the interactional sites of the proteins of interest and IFV RNA were analyzed with catRAPID [25].

Results

RNA affinity capture of 5′ region of IFV RNA-binding proteins

Silkworm midgut cellular proteins were identified that associate with an RNA containing sequences from the 5′-region of IFV and a streptavidin RNA aptamer (**Fig. 1A**). Sequences from the 5′-region (nts 1–599) were amplified from IFV-CHN01 (GenBank: EU868609) [26]. In addition, the oligonucleotide used to amplify the 5′ region incorporated a SP6 promoter for *in vitro* transcription. The obtained RNA-protein complexes were tested by SDS-PAGE. The results revealed that there were several weak stripes in systems with no RNA, while there were more bands in system with the 5′ region of IFV RNA at the same volume supernatant (**Fig. 1B**).

Protein identification

In order to facilitate identification of proteins that associate with the IFV 5′ region, the proteins were identified by PAGE-free MS. Thisc--286.82(h)-14.p-

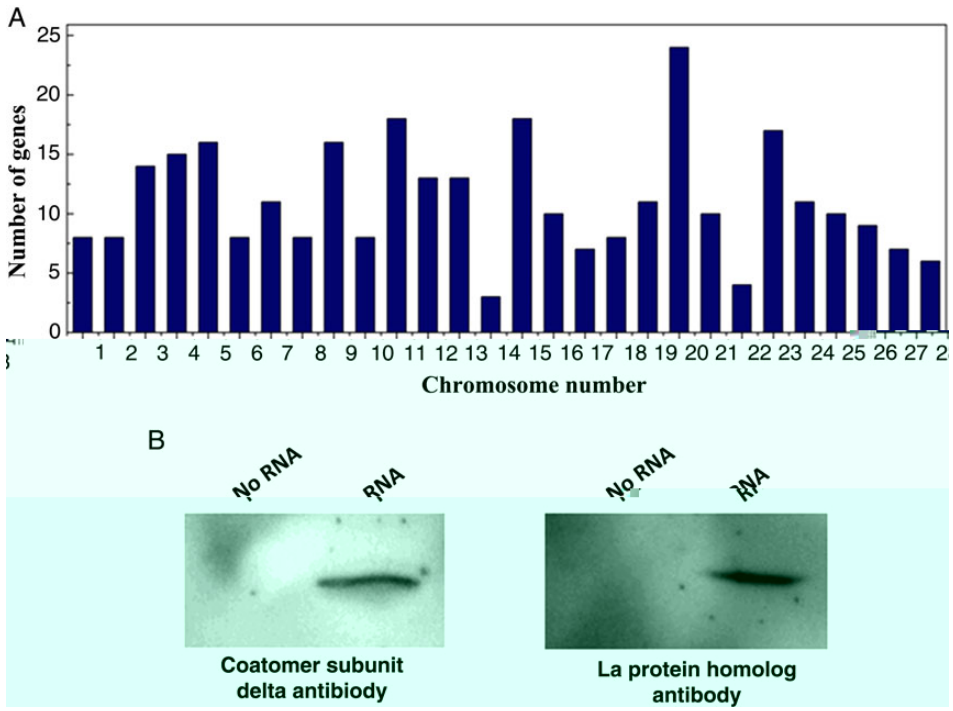

Figure 2. The confirmation of the identified proteins interacted with IFV 5′ region of genomic RNA (A) Chromosome distribution of the identified proteins interacted with IFV 5′ region of genomic RNA. (B) The western blot results of coatomer subunit delta and La protein homolog.

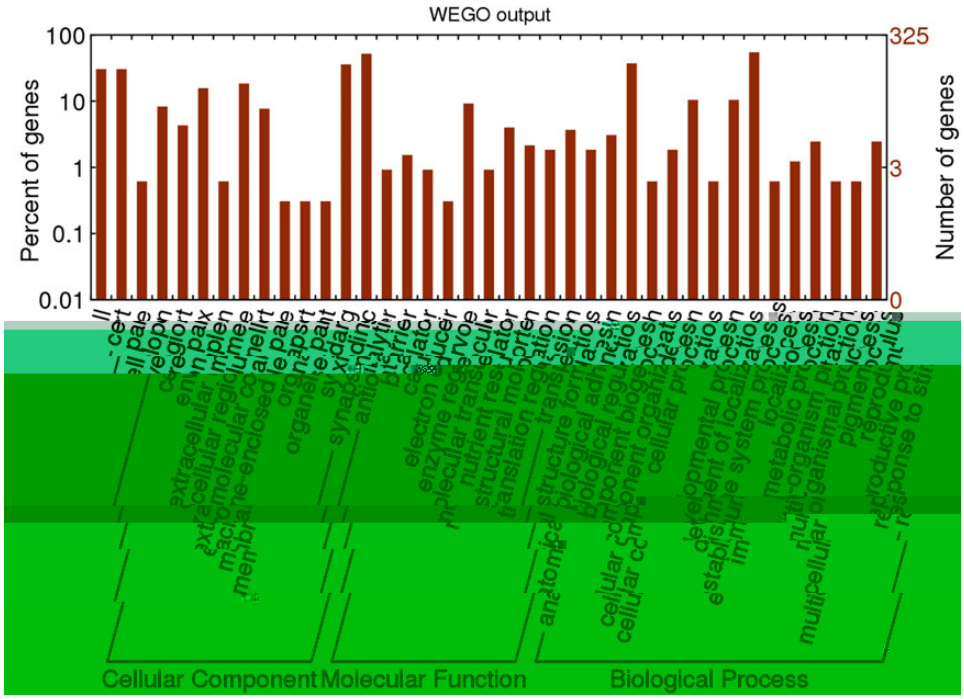

Figure 3. Gene ontology categories for the 325 proteins that interacted with IFV 5′ region of genomic RNA The number of genes indicates those with available GO terms. The percentages indicate the proportion of the total genes.

the majority for the BP category. Picornavirus IRES-driven translation initiation depends on the recognition of the IRES by specific cellular proteins such as ATP, eIFs, and ribosome [8].

The COGs of proteins were generated by comparing the protein sequences of complete genomes [30]. Posttranslational modification, protein turnover, chaperones/translation, ribosomal structure, and

biogenesis/energy production and conversion were the top 3 identified functional classifications (**Supplementary Fig. S2** and **Table S3**).

KEGG pathway analysis

To provide a global view of functions of proteins that were identified, all proteins with different KO ids were submitted and mapped to 184 pathways (**Supplementary Table S4**). The top 24 pathways for identified proteins numbers are shown in **Fig. 4**. Metabolic pathway, biosynthesis, and microbial metabolism in diverse environments were the top three comprehensive pathways that were mapped. Ribosome and protein processing in endoplasmic reticulum were among the fourth and fifth pathways that were mapped (**Figs. 4** and **5**). These results further confirmed that 5′ RNA region of IFV genome plays very important role in protein translation of IFV [18]. In addition, mRNA surveillance pathway (H9IYK6, H9JCI5, H9JG54, Q1HPL5, and H9JD63) and RNA transport (H9J5Q7, H9J7V0, H9JQ89, and Q684K3) were also found (**Supplementary Table S2** and **S3**). These proteins may play important roles in RNA process of IFV.

Discussion

The 5′ region of IFV possesses an IRES [18], so the region might bind to translation-related factors and ribosomal proteins. In our results, 16 ribosomal proteins, 3 eIFs (eIF2, eIF3, and eIF4 subunit), elongation factor 1 (EF1), and six potential ITAFs were found to have RNA-binding motif (unique peptide ≥2) (**Table 1**).

Initiation factors are necessary for processing mRNA prior to 40S subunit binding and the 60S subunit to form the 80S initiation complex [31]. However, different picornavirus IRESs need different eIFs [32]. For example, eIF4G, eIF4A, and eIF4B were shown to interact with type II IRES; however, eIF4E, eIF1, or eIF1A was not needed [33,34]. Poliovirus (PV) RNA utilizes eIF2 for the initiation of translation in cell-free system, and translation directed by PV RNA is blocked when eIF2 is inactivated at earlier time [35]. The two highly conserved RNA-binding motifs in eIF3 that direct translation initiation from the HCV IRES RNA [36]. The eIF4A subunits contain DEAD box domains, found within RNA helicases [37].

Elongation factors are a set of proteins in cellular protein synthesis. In the ribosome, they facilitate translational elongation, from the formation of the first peptide bond to the last one. Interactions between viral genomic RNA and eEF1 have been reported for several plant viruses, including tomato bushy stunt virus [38], tobacco mosaic virus [39], brome mosaic virus [40], turnip mosaic virus [41], turnip yellow mosaic virus [42], and animal viruses including bovine viral diarrhea virus [43], PV [44], West Nile virus [45,46], and Dengue 4 virus [47]. Elongation factors play important roles in viral translation and replication.

Several potential picornavirus ITAFs were found in this study, such as La (**Table 1**). The ITAFs are important *trans*-acting factors required for viral translation and replication [10]. La could enhance IRES-mediated translation in PV, EMCV, and HCV [48–50]. The importance of nucleoplasmin family in cellular processes such as chromatin remodeling, genome stability, ribosome biogenesis, DNA duplication, and transcriptional regulation has led to the rapid growth of information available on their structure and function [51]. A conserved RNA recognition motif protein encoded by *LARK* gene is eliminated to determine the consequences for circadian timing *in vivo* [52] that might affect the IFV replication and host life cycle.

The cytoskeleton and motor proteins have been found to play important roles in RNA intracellular localization and may also regulate translation and viral replication [53–55]. Twelve cytoskeleton and proteins were identified as tubulin and myosin etc. (**Table 2**). Riboproteomic analysis of polypeptides interacting with the IRES of foot-and-mouth disease virus and HCV RNA revealed cytoskeleton-related proteins [14,15]. Myosin plays a key role in the transport of cargo to the plasma membrane. Its cargo includes cell-surface receptors, pigment, and organelles such as the endoplasmic reticulum. It is also emerging that RNA and RNA–BPs (RNA-binding proteins) make up another class of myosin cargo [56].

Membrane lipid composition is important for RNA replication of positive-strand RNA viruses [57–60]. Several kinds of membrane-related proteins were found as shown in **Table 3**. The membrane-shaping host reticulon proteins play crucial roles in viral RNA replication compartment formation and function [61,62]. With IFV infected silkworm, goblet cell cytoplasm was filled with specific membranous vesicles and

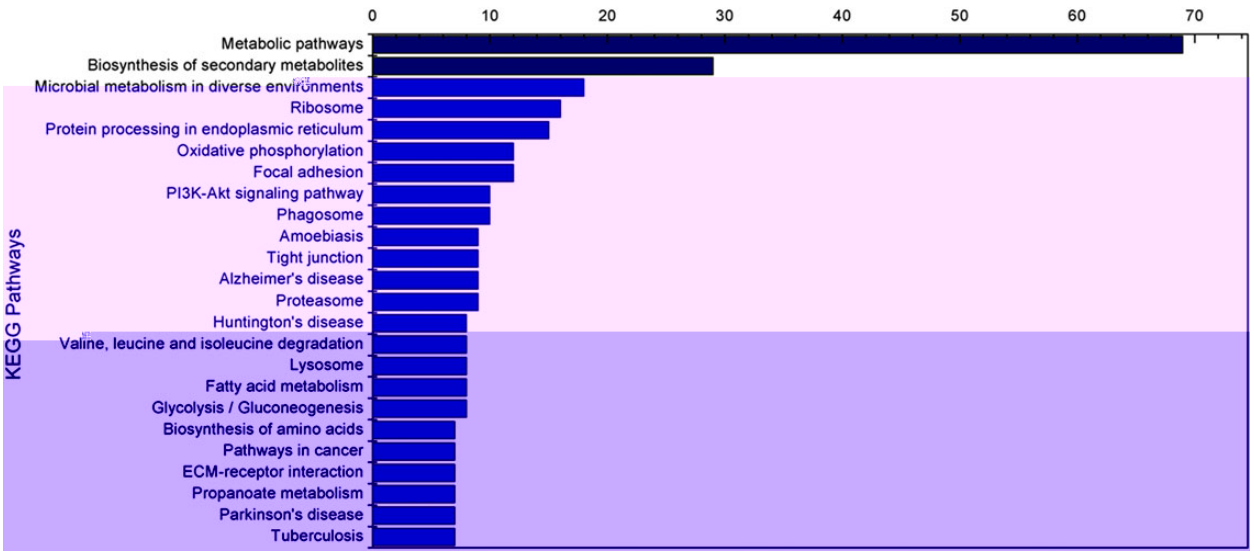

Figure 4. Top 24 KEGG pathways for identified protein genes

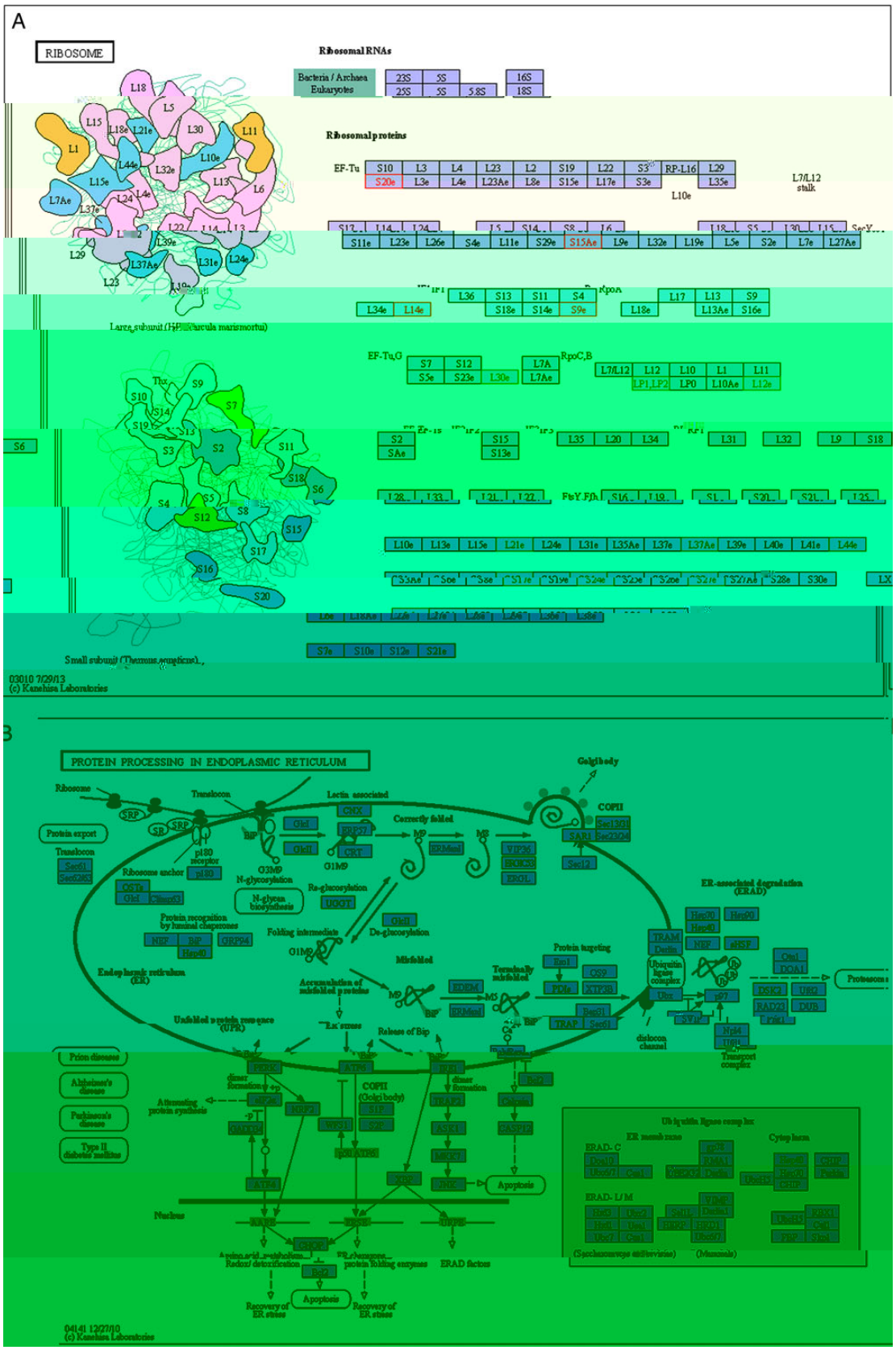

Figure 5. Distribution of the 16 identified ribosome subunits and 15 proteins in protein processing in endoplasmic reticulum pathway interacted with IFV 5′ region of genomic RNA Red labeled proteins were identified by this study.

Table 1. Identification of translation initiation, elongation, and potential IRES *trans*-acting factors that interact with IFV 5′-region RNA

Accession number	Protein description	PepCount	Unique PepCount	Cover percent	MW (Da)	PI	Length
Q5UAL6	40S ribosomal protein S27	3	2	27.38	9433.04	9.91	84
Q5UAN5	Ribosomal protein S9	4	4	19.07	22,576.11	10.76	194
Q6PUF7	40S ribosomal protein S24	4	2	23.48	15,171.86	10.91	132
Q5UAM7	Ribosomal protein S15A	2	2	13.18	14,692.17	10.01	129
Q5UAS7	Ribosomal protein L12	3	3	19.51	17,565.2	9.58	164
Q5UAM2	Ribosomal protein S20	4	2	19.51	13,731.99	10.14	123
Q5UAR8	Ribosomal protein L21	2	2	12.58	17,984.96	10.96	159
Q5UAQ1	Ribosomal protein L36A	2	2	20.19	12,294.53	10.53	104
Q5UAP9	Ribosomal protein L37A	2	2	16.67	10,031.87	10.6	90
Q5UAQ8	Ribosomal protein L30	6	5	35.40	12,346.36	9.65	113
H9J1M5	Ribosomal protein LP1	6	4	56.25	11,478.77	4.18	112
H9IVT2	40S ribosomal protein S21	2	2	23.46	8811.06	7.67	81
Q5UAN7	Ribosomal protein S7	6	2	18.95	21,858.28	9.82	190
H9JX29	Ribosomal protein L14	7	3	20.61	18,623.75	11.27	165
Q5UAM5	Ribosomal protein S17	5	4	42.11	15,421.65	9.71	133
H9JU64	Ribosomal protein L6	8	6	20.38	28,999.88	10.89	260
Q684K3	Translation initiation factor 2 gamma	5	4	10.90	50,600.37	9.03	468
H9JTH1	Eukaryotic translation initiation factor	2	2	11.03	31,411.22	9.76	290
H9J7V0	Eukaryotic translation initiation factor 3	2	2	2.91	62,410.69	7.58	549
H9JCI5	EF1 alpha-like factor isoform 1	2	2	6.97	65,964.74	5.16	603
Q2F5N9	Nucleoplasmin isoform 2	3	2	22.99	20,843.06	4.86	187
H9JJU3	Aspartyl-tRNA synthetase	2	2	4.36	46,355.27	9.03	413
H9J930	La protein homolog	2	2	8.53	45,133.49	6.01	387
Q5I7M6	RNA-binding protein lark	5	5	15.74	38,515.19	9.31	343
Q17201	Bmsqd-2	4	2	9.24	32,552.06	8.66	303

Table 2. Identification of cytoskeleton and motor-related that interact with IFV 5′-region RNA

Accession number	Protein description	PepCount	Unique PepCount	Cover percent	MW (Da)	PI	Length
H9J4V2	Tubulin beta-4b chain	9	4	13.86	37,243.67	4.91	332
H9JHX3	Isoform F	3	3	1.85	209,582.44	6.49	1943
H9J4H4	Short isoform H	2	2	1.37	203,746.09	5.72	1829
H9J1Q5	Tubulin beta-4b chain-like isoform x1	15	7	20.96	49,056.33	5.79	439
H9J3F1	Microtubule-associated protein	2	2	8.57	39,211.21	5.74	350
H9JGR0	Actin-interacting protein 1-like	3	2	18.63	17,422.34	7.93	161
B3VTP0	Long form-like	18	16	21.41	102,749.53	5.43	878
H9JXG0	Myosin heavy muscle	26	23	18.98	134,836.77	5.97	1175
H9JXG1	Muscle myosin heavy chain	78	53	53.33	123,511.13	5.36	1082
B5M9A1	Myosin heavy chain	37	28	36.54	91,159.53	6.54	791
C4P7H2	Myosin heavy chain variant A	17	15	25.43	65,828.31	6.24	582

electron-dense bodies [16]. PV replicates its genome in association with membranous vesicles in the cytoplasm of infected cells, and cellular COPII coat proteins are involved in production of the vesicles that form the PV replication complex [63–65]. Although we did not found COPII coat proteins, three subunits of coatomer protein that own COPI vesicle coat (GO:0030126) and retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum (GO:0006890) were found (**Table 3**). In addition, other found membrane-related proteins (**Table 3**) could better explore the mechanism of membranous vesicles forming in the cytoplasm. The forming mechanism of specific vesicles and electron-dense bodies may be related to IFV 5′-region RNA.

Interestingly, we identified a number of enzymes involved in ATP, mitochondrial-related and metabolic enzymes (**Supplementary Tables S5** and **S6**). Mitochondrial ATP synthase is a factor that mediates HIV-1 transfer between APCs and CD4(+) target cells [66]. Purified mitochondria from HIV infected cells are infectious, and mitochondrial inhibitors block HIV transmission [67]. After silkworms are infected with IFV, a number of mitochondria surround the virions [16]. Based on this, we hypothesize that ATP synthase and mitochondrial-related proteins play important roles in viral transmission and life cycle. Picornavirus IRES-driven translation initiation depends on the recognition of the IRES by ATP [8].

Metabolic enzymes that bind RNA form another level of the cellular regulatory network [68]. However, the function of nucleotide-binding activity by carbohydrate metabolic enzyme is unclear. RNA-binding activity has been reported, such as for alpha-glucosidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [69]. The NAD^+-binding Rossman domain found within GAPDH and other dehydrogenase- and nucleotide-binding family proteins should be designated as a general RNA-binding motif [69,70].

Table 3. Identification of membrane-related that interact with [illegible]-region RNA

Accession number	Protein description	bCount	Unique	Count	Co[illegible]percen[illegible]	(Da)	PI	Lengt[illegible]
Q1HQA9	Transmembrane trafficking protein		2		12.20	681.2	6.0	
Q14UC7	Aquaporin AQP-Bom2		2		10.42	693.2	6.1	25
Q6SA04	Innexin 2		3		12	810	6.	59
H9IT08	Leucine-rich repeat-containing protein 15-like							

Cellular chaperones and folding enzymes are vital contributors to membrane-bound replication and movement complexes during RNA virus infection [71–73]. The folding enzymes (**Supplementary Table S7**) and GTP-binding proteins (**Supplementary Table S8**) were also found. The small GTP-binding protein ARL8 plays crucial roles in tobamovirus RNA replication [74]. Histone deacetylase activity may be required for the virus replication [75]. Specifically, the histones H2B and H4 were identified. The phosphorylation of viral and cellular proteins can have major impacts on viral infection, replication, and cytotoxicity in the host cell [76]. Three serine/threonine–protein phosphatases (Q1HPL5, H9JG54, and H9JD63) were found. The replication of positive-strand RNA viruses, the largest class of viruses, including significant human, animal, and plant pathogens, may also be regulated by phosphorylation events [77,78]. In addition, 30 KD proteins (30KPs) (**Supplementary Table S9**) may play an important role in translation and replication of IFV. The 30KPs were the major plasma proteins and classified into the lepidopteran-specific lipoprotein 11 family [79]. The 30KPs in silkworm may serve as the amino acid source during embryo development and play a role in the inhibition of apoptosis [79,80].

This study aimed to identify proteins that interacted with host factors and the 5′ end of IFV genomic RNA. The identification of many proteins (ribosome, endoplasmic reticulum, membrane, mitochondrial, and IRES-related proteins) with potential roles in viral translation and replication indicates that much work is needed to better understand these complex functions and pathways (**Fig. 6**). These results also revealed many enzymes that may serve multiple roles during IFV infection of the silkworm midgut. Functional investigations of identified RNA-binding proteins of interest are needed to determine their role in viral translation and replication, and to provide potential therapeutic targets.

Supplementary Data

Supplementary Data is available at *ABBS* online.

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