# Final report of the "Exploring the protein functions of Cucumoviruses with bioinformatics and proteomics" project

## Introduction

Two original research papers with high impact factors were published in the field of our cucumovirus research [1][2] and a third will be submitted soon enough. My research interests is expanded towards animal virology, so I performed successful cooperation with Veterinary medical research groups therefore the grant number of this project was indicated in the common papers because the licence fee of the used molecular modelling software package (Schrödinger Suite) was paid from this grant. First, I summarize the main results on our cucumovirus research then I give a short report about my on other research activities.

## Scientific results on Cucumoviruses

### *Alanine-scanning of CMV 2b protein*

I took part as a collaborator in Katalin Nemes and Katalin Salánki's (Plant Protection Institute, MTA ATK) very comprehensive research project on the alanine-scanning of CMV 2b protein. CMV 2b-siRNA ribonucleoprotein molecular models were created to illustrate the experimental results on structural biological basis. We identified two triplets necessary for the suppressor function of the 2b protein and two other positions were required for cell-to-cell movement of the virus, which are not essential for suppressor activity. The results of this work was published in Plos One [1].

### *Role of phosphorylation in CMV 2b protein*

We had new results in connection with the CMV 2b protein function. Three years ago a paper was published with this title: "Nuclear-cytoplasmic partitioning of CMV protein 2b determines the balance between its roles as a virulence determinant and an RNA-silencing suppressor" [3]. We identified a conserved dual phosphorylation switch in CMV 2b protein, which equilibrates the shuttling of the 2b protein between the nucleus and the cytoplasm, and regulates the suppressor activity of the 2b protein. Molecular dynamics (MD) simulations were computed the native and modified 2b protein tetramer–siRNA ribonucleoprotein complexes in order to see the main conformational changes. These great results were published in Scientific Reports [2].

### *Unpublished results, fails (detailed description)*

*CMV capsid and host factor interaction*

Unfortunately, a promising and exciting part of the research plan is still unpublished, but at last I have new good results which will raise the chance of acceptance in a scientific journal.
In a short summary, I paste here the paper abstract recently submitted:
"A previous study showed that a single amino acid difference in the cucumber mosaic virus (CMV) coat protein (CP) elicits unusual symptoms. The wild type strain (CMV-R) causes green mosaic symptoms and malformation, whereas the mutant strain (CMV-R3E79R) causes chlorotic lesions on inoculated leaves and strong stunting with necrosis on systemic leaves. Virion preparations of CMV-R and CMV-R3E79R were partially purified from Nicotiana clevelandii A. Gray and analysed by two-dimensional gel electrophoresis. Their separated

protein patterns showed remarkable differences at the 50-75 kDa range, both in numbers and intensity of spots, with more protein spots for CMV-R3E79R. Mass spectrometry analysis demonstrated that the virion preparations contained host proteins identified as ATP synthase alpha and beta subunits as well as small and large RUBISCO subunits, respectively. The presence of ATP synthase was confirmed by western blot analysis, while virus overlay protein binding assay (VOPBA) and immunogold electron microscopy were used to prove the interactions between the virus particle and the host proteins. Molecular modelling study revealed that the electrostatic change in the CP of the CMV-R3E79R can result in stronger interactions with ATP synthase subunits. Based on our findings we suggest that the mutation present in the CP can have a direct effect on the long–distance movement and systemic symptoms. In molecular view the mutant CMV virion can lethally block the rotation of the ATP synthase F1 motor complex which may lead to cell apoptosis, and finally to plant death."

This paper was submitted to the Archives of Virology but unfortunately it was rejected. There were three major critiques about the manuscript:

1. We used only 6 nm IgG-gold conjugated secondary antibodies, so we could only present the localizations of the virus and the ATP synthase F1 subunits in different electron micrographs. The 6 nm in diameter gold particles formed "hedgehog-like" patterns by surrounding the virus particles, but this indirect evidence was not satisfactory for the reviewers. They suggest double-labelling to see the direct interactions. We have done the double-labelled EM experiments in the Department of Plant Anatomy (ELTE). (Károly Bóka carried out the EM experiments.) The 6 nm gold in diameter particles indicated the virus localization while the 10 nm in diameter gold particles indicated the ATP synthase F1 complex localization. We found double labelled regions in the chloroplast stroma, but the frequency of occurrence was not significant between the wild-type and the mutant virus. We assume that there is a steric hindrance which blocks the anti-ATPase antibody binding to the F1 complex. Namely, the anchored CMV virion may not let enough space for the anti-ATPase antibody. So, we can only use EM experiments to detect the proper localization of the virus particles. These EM micrographs serve clear evidence for that the CMV not only localises in the cytoplasm (viroplasm) but it can also be found in the chloroplast stroma.

2. The VOBPA experiments presented in the MS, technically and methodically was not clearly carried out. One of the reviewers gave clear instructions to do it properly.

3. The reviewers claimed direct evidence to verify the hypothesised CMV-ATPase F1 interaction: "Finally, the authors could also use purified chloroplasts or mitochondria as starting material to confirm binding." "Finally, much more strong evidences are required to confirm this interaction…." In order to prove the direct interaction between CMV capsid and CMV-ATPase F1, I designed a modified ELISA experiment. In the last year, I managed to isolate chloroplasts from Nicotiana clevelandii A. Gray and I successfully purified ATP synthase F1 complex from isolated chloroplasts. In the modified ELISA experiment the purified ATPase F1 complex was immobilized to the ELISA plate. The second layers were highly purified wild-type CMV and highly purified mutant CMV dilution series and a negative control. The third layer was a para-nitrophenyl-phosphatase (pnp) conjugated anti-CMV antibody (Bioreba). We've got the expected result: the mutant CMV binds almost four times stronger the ATP synthase F1 complex than the wild virus.

*Possible role of the CMV 2b C-terminal domain*

Previous modelling results [4] showed that the 40 aa long C-terminal domain of the CMV 2b protein is stabilized by a divalent metal ion. This computational result was verified by experimental molecular biology. A quadruple mutation (Rs2DDTD/95–98/AAAA) was introduced into the position of the putative divalent metal ion binding site to analyse the biological relevance of molecular modelling derived hypothesis. The plant inoculation experiments proved that the movement of the mutant virus was slower and the symptoms were milder comparing to the wild type virus. These results demonstrated that the quadruple mutation weakens the stability of the 2b protein tetramer–siRNA ribonucleoprotein complex. Currently, molecular dynamics (MD) simulations were carried out to check the role of the conserved Trp99 and Trp105 which are probable involved in the RNA binding. These tryptophan residues form π-π stacking effect with the nucleotide bases on each end of the siRNA duplex. Two double mutations Rs2WW/99,105/AA and Rs2WW/99,105/FF were designed to investigate the kinetics of virus movement inside the plant. These plant experiments were in progress, but unfortunately, we still haven't got significant differences between the two mutant types and the wild type virus. On the contrary, I expect that the movement of the double alanine mutant will slower than the double phenylalanine mutant because the alanine residue is unable to form stabilizing pi-pi interaction with the last base of the bound siRNA.

*CMV movement protein, expression and characterization*

In the past four years I've made huge effort to purify CMV movement protein (3a) for protein crystallography and for biochemical characterization experiments. I've developed a new native protein purification procedure on the basis of numerous recently published Nature and Science papers. The highly hydrophobic CMV movement protein is extracted under native conditions using decyl-maltoside, a non-ionic detergent which has large solubilizing effect. I carried out this purification process in small scale, but I didn't have enough time to do the purification in larger scale. Actually, the protein expression was initiated in five litres of e. coli, but the further purification steps (extraction, ion exchange and size-exclusion chromatography) still haven't done. I intend to continue these experiments after closing this project.

**Other research activities**

***Virology***

I was a co-author in a paper where I created a homology model of a new pig Picobirnavirus (PBV) coat protein. The novelty was that we identified four additional epitope sequences in the antigenic region of the PBV coat protein [5]. An important new result was published on rotaviruses in the Emerging Infectious Diseases [6], where we identified unusual rotavirus strains in fecal specimens from sheltered dogs in Hungary by viral metagenomics. The novel rotavirus species displayed limited genome sequence homology to representatives of the eight rotavirus species, A–H, and qualifies as a candidate new rotavirus species that we tentatively named Rotavirus I. Finally, I am pleased to report a published paper about new bat caliciviruses [7]. Phylogenetic analyses and molecular modelling identified firmly the two viruses as candidate members within the Caliciviridae family, with one calicivirus strain resembling members of the Sapovirus genus and the other bat calicivirus being more related to porcine caliciviruses of the proposed genus Valovirus. This data serves the effort for

detecting reservoir hosts for potential emerging viruses and recognize important evolutionary relationships.

***Biotechnology***

I gave permission to Gergely Bánóczi (BME) to use the Schrödinger Suite molecular modelling software package in his research projects, therefore the grant number of this project were indicated in those papers. I specified these papers in the Selected publication list [8][9][10][11].

**References**


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