Pathological significance of posttranslational modification of the viral proteins in the case of Cucumber mosaic virus infection

Final report

Generally the viruses has limited number of proteins, for example in the case of cucumber mosaic virus that means five proteins altogether. These proteins has to accomplish the complete life cycle of the virus coordinately with host proteins. That means, that all the viral proteins has several function during the infection cycle, and the post translational modification offer an excellent opportunity to switch between the different function of a protein.

Prior to our project, in the case of CMV the phosphorylation of the 2b protein was predicted according to the phosphorylation motifs for casein kinase II (S/TxxD/E) and cyclin-dependent kinase 2 (40SP41), but it was .not proved experimentally and its function was unnoted. The phosphorylation of the movement protein (MP) was identified only in MP transgenic plants, but not during the infection cycle of the virus and in the case of the coat protein (CP) the phosphorylation was not detected previously.

2b protein phosphorylation

The 2b protein of *Cucumber mosaic virus* has a role in nearly all steps of the viral life cycle including cell-to-cell movement, symptom induction and suppression of antiviral RNA silencing. Previous studies demonstrated the presence of 2b protein in the nucleus and in cytoplasm as well.

A putative phosphorylation site of CMV 2b protein (amino acids 39–43) located at the beginning of the second alpha helix is strictly conserved in all CMV isolates. KSPSE has been proposed to constitute phosphorylation motifs for casein kinase II (S/TxxD/E) and cyclin-dependent kinase 2 (40SP41). To examine the *in vivo* phosphorylation state of the 2b protein, Western blot analysis was carried out by using phosphoserine antibodies. Since antibodies for 2b protein detection were not available, the coding region of histidine tagged 2b protein was cloned into the infectious cDNA clone of Rs-CMVRNA 2. It was previously found that attachment of a hexahistidine sequence did not alter the subcellular localization and the ability to bind sRNAs. *Nicotiana benthamiana* plants were infected with the chimera Rs-2bHis *in vitro* transcripts in the presence of the Rs-CMV RNA 1 and 3 transcripts. Parallel to the infection, *N. benthamiana* plants were infiltrated with the binary vector expressing the 2bHis protein. Ten days after inoculation (symptoms appeared on the upper non-inoculated leaves) and 4 days post infiltration, the accumulation and the phosphorylation of 2b protein were analyzed by Western-blot using penta-his and phosphoserine antibodies. The Western blot analysis proved the 2b protein phosphorylation *in vivo* in infected plant as well as in infiltrated patches, but the mutant 2b/40-42/AAA was not phosphorylatedin in filtrated patches demonstrating that the 40/42 amino acids are the exclusive phosphorylated amino acids of the 2b protein.

In order to analyze the effect of the different phosphorylated state of 2b protein, seven different mutants were created. Substitutions were integrated into the Rs-CMV RNA 2

clone in the SPS motif for analysis of the effect of alanin (to mimic the non-phosphorylated state) and aspartic acid (to mimic the phosphorylated state) substitution in single or in both serine residues (40S, 42S). The substitution of serine amino acid with negatively charged aspartate residues, known to reproduce the electrostatic effect of phosphorylation. All of the mutations were integrated into the RNA 2 infectious clone of Rs-CMV resulting in the following constructs: 2b/40-42/SAS, 2b/40-42/APS, 2b/40-42/SPA, 2b/40-42/APA, 2b/40-42/DPS, 2b/40-42/SPD and 2b/40-42/DPD. To compare these mutants with wild-type virus (Rs-CMV) in terms of symptom induction and viral accumulation, plasmids containing the full-length clones of Rs-CMV RNAs were transcribed *in vitro* for inoculation of *N. benthamiana* plants. RNA 1 and 3 transcripts were combined with the wild-type RNA 2 and either with the mutated RNA 2 transcripts. The infection was followed by visual observation and northern blot analysis. Infections of *N. benthamiana* with Rs-SPS/40-42/APA and Rs-SPS/40-42/ DPD were asymptomatic similarly to the previously described Rs-SPS/40-42/AAA during the monitored period (six weeks). Infection with Rs-SPS/40-42/SPD and Rs-SPS/40-42/DPS induced mild symptoms: mild leaf distortion, mild systemic mosaic, and mild stunting. *N. benthamiana* plants infected with Rs-SPS/40-42/APS, Rs-SPS/40-42/SAS and Rs-SPS/40-42/SPA exhibited severe symptoms, including systemic mosaic, leaf distortion, and chlorosis. However, symptoms induced by these mutants were never as severe as those induced by Rs-CMV in *N. benthamiana*. The northern analysis was in concordance with the symptom appearance. The identity of each of the mutants was verified by RT/PCR followed by nucleic acid sequence determination.

Since gene silencing suppressor activity is the primary function of 2b protein, this feature was analyzed in *Agrobacterium*-mediated co-infiltration assay. Binary vector expressing GFP reporter gene was agroinfiltrated into transgenic *N. benthamiana* (silenced for GFP expression) leaves together with the binary vector expressing the wild type 2b protein or the different mutant ones (2b/40-42/AAA, 2b/40-42/SAS, 2b/40-42/APS, 2b/40-42/SPA, 2b/40-42/APA2b/40-42/DPS, 2b/40-42/SPD, 2b/40-42/DPD) GFP silencing was visually observed at 4 days post agroinfiltration and the accumulation level of GFP RNA in the infiltrated leaves was quantitatively measured by qRT-PCR. We found that mutants 2b/40-42/SAS, 2b/40-42/APS and 2b/40-42/SPA retained partial suppressor activity. However, mutants 2b/40-42/DPS, 2b/40-42/SPD, 2b/40-42/DPD and 2b/40-42/APA failed to suppress GFP activity similarly to the previously described 2b/40-42/AAA.

We have analyzed the subcellular localization of 2b protein and its mutants by BiFC system. To determine the impact of phosphorylation state of 2b protein we monitored the subcellular localization of the wild-type and mutated 2b proteins with bimolecular fluorescent complementation assay (BiFC). Both the N-terminal and C-terminal fragments of YFP were fused to the C-terminus of 2b protein and also to the mutant 2b proteins with highly altered phenotype, generating fusion proteins (SPS/40-42/AAA and SPS/40-42/DPD). The 2b protein forms homodimers or higher oligomers therefore we used interaction of the wild-type 2b protein itself as a positive control applying BiFC in *N. benthamiana* plants. At 3 dpi, infiltrated leaves were subjected to confocal microscopy to monitor the reconstituted YFP signal. Strong YFP fluorescence in the cytoplasm and in the nucleus co-expressed Rs2b-N-YFPC and Rs2b-C-YFPC demonstrated that Rs-CMV was very apparent in cytoplasm as well as in nuclei. No fluorescence was observed by confocal microscopic imaging in cells within leaf patches co-infiltrated with *A. tumefaciens* cells expressing 2b/40-42/AAA. Interestingly, fluorescence was detectable only in cytoplasm in the case of

mutant 2b/40-42/DPD, but fluorescence was not detected in the nucleus, or in the nucleolus. This observation indicates that the phosphorylated 2b protein accumulated in cytoplasm, phosphorylation of 2b protein prevents its accumulation in the nucleus. In order to visualize the subcellular localization of the monomer form of the 2b protein EGFP was fused to the C-terminus of the Rs2b protein and to the 2b/40-42/APA and 2b/40-42/DPD mutants. In infiltration experiments the Rs2b-EGFP and the 2b/40-42/APA-EGFP were detected in the cytoplasm and the nuclei, while the 2b/40-42/DPD-EGFP was detected only in the cytoplasm, but not in the nuclei, confirming that the phosphorylated 2b protein is excluded from the nuclei.

To further analyze the subcellular localization of the mutants, nuclei were purified from agroinfiltrated leaf patches. The presence of the 2b proteins in the nuclei was verified by visual observation using microscopy as well as protein extraction and staining. Mutants SPS/40-42/ AAA, SPS/40-42/APA, and single mutants SPS/40-42/APS, SPS/40-42/SPA and SPS/40-42/SAS were detectable from purified nucleoli with penta-his antibody. The mutants 2b/40-42/DPS, 2b/40-42/SPD and 2b/40-42/DPD were not detectable in nuclei. These findings further demonstrate that the phosphorylated state of 2b protein precludes it from accumulating in the nucleus and restricts the location of 2b protein exclusively to the cytoplasm.

We have also analyzed the structural consequence of the 2b protein phosphorylation. Serine 40 and 42 are located in the forepart of the second α-helix. The hydroxyl groups of serine 40 and 42 form H-bond(s) with the phosphate oxygen atoms of the cytosine-14 (C14_PO) and/or guanosine-13 (G13_PO). These H-bonds give high stability to the whole 2b tetramer – siRNA complex. But, in the course of the MD simulation for only very short time periods (5–50 ps) these serine side chains swing toward the solvent where a host kinase can phosphorylatethese serine residues. After the phosphorylation event the phospho-serine side chains cannot turn back to the negatively charged sugar-phosphate backbone of the siRNA, since their negative charges repel the 2b protein chain from the siRNA. Beside phosphorylation domain, 2b protein also contains nuclear localization signals (NLS1 and NLS2). The molecular models of 2b dimmers suggest that the NLS regions located at the first alpha-helix are involved not only in siRNA binding but act as interaction interface for importin protein, probably interaction with karyopherin α as suggested previously. The presented results regarding to the 2b protein phosphorylation were published. (**Nemes et al, PLOS ONE 9:11, Nemes et al, SCIENTIFIC REPORTS 7:(1)p. 13444**).

CP phosphorylation

Previously phosphorylation of the CMV CP was not reported. In order to determine whether CMV CP is phosphorylated *in vivo*, total protein was extracted from infected *Nicotiana tabacum* L. cv Xanthi leaf tissue and subjected to western blot analyzes using CP and phosphoserine antibody. The corresponding band at the CP size confirmed that CP is phosphorylated *in vivo*. Phospho.ELM database and NetPhosK server were used to analyze CMV CP for phosphorylation sites, and the 148 Ser aa was suggested as a feasible site of phosphorylation. Several CMV CP amino acid sequences were compared and Ser proved to be highly conserved in this position. To investigate the phosphorylation of this aa position, we created mutant CP/S148A to introduce alanin to position 148. Rs CP and CP/S148A mutant were expressed in bacteria and subjected to western blot analyses using

phosphoserine and CP antibodies. We performed phosphoprotein staining with the bacterially expressed CP as well, and this experiment also proved the phosphorylation ofthisposition.

To investigate the role of CMV CP phosphorylation *in vivo*, we introduced mutations to the position 148 creating mutant CP/S148A mimicking the non-phosphorylated state, and CP/S148D to mimics the phosphorylated state of the serine at position 148 in the infectious clones of Rs-CMV. All the transcripts were infectious on *N. tabacum* L. cv Xanthi plants. Two weeks after inoculation the identity of the mutants was analyzed in the systematically infected leaves with RT/PCR followed by nucleotide sequence determination. While the Rs-CMV and CP/S148A were stable in five independent experiments, the CP/S148D mutant was unstable as it was mutated to phosphorylatable amino acids, namely Y (four times) or S (once), respectively.

Next we compared the symptom induction and virus accumulation of the stable CP/S148A mutant and the wild-type virus (Rs-CMV) in *N. tabacum* L. cv Xanthi plants. Rs-CMV induces mosaic, mottled leaf and distortion symptoms in *N. tabacum* L. cv Xanthi plants followed by symptom recovery (asymptomatic leaf stages during infection) (Fig 1A). Symptomless leaves stages are followed by a newly, mosaic leaf development. CP/S148A mutant caused severe mosaic in all leaf stages. We found that substituting CP amino acid S148 to A altered the symptoms as the phenomenon of recovery did not occur so the formation of cyclic symptoms ceased (Fig 1A,). The visual observation was compared to the virus quantity two weeks after inoculation (recovered state of the Rs-CMV). The accumulation of CP in the systemic leaves was assessed with anti-CP monoclonal antibody and the viral RNA accumulation was detected with northern analyses. The virus accumulation was severely different in the upper non inoculated leaves. Both the viral RNA and the CP amounts were greatly higher in the CP/S148A infected plants than those of the parental strainRs-CMV (Fig 1B).

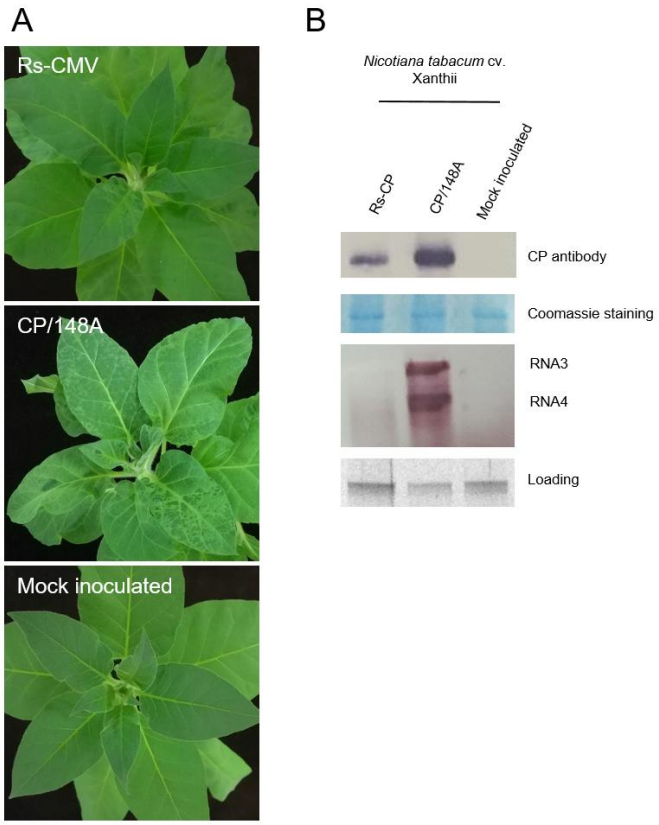

Figure 1. (A) Symptoms induced by Rs-CMV and CP/148A mutant on *Nicotiana tabacum* L. cv Xanthi plants. (B) Coat protein and viral RNA accumulation in systemically infected leaves

The accumulation of CP was assessed in 14 vertical leaf positions during one month period using anti-CP monoclonal antibody (Fig 2B). The level of CP in different leaves were in accordance with the symptoms as two recovery stages were detectable in the case of Rs-CMV during the experiment where the level of CP was drastically reduced while infection with CP/S148A caused equable CP level in all leaves during infection (Fig 2B). These results indicated that the substitution of amino acid S148 with Ala prevent the recovery phenotype of CMV in tobacco plants, parallel to increased amount of CP. Next determining whether the RNA or the amino acid sequence is crucial in symptom formation (since previously gene silencing was connected with symptom recovery) a silent mutant was created, namely CP/S148sil. In this mutant the coding region of position 148 was mutated but the aa sequence of the Rs CMV CP was unaffected. The silent mutant caused similar symptoms as the wild-type virus, with cyclic recovered leaf stages (Fig 2A, B lower part). As mentioned before, CP/S148D mutant was unstable, so we could not involve this mutant to this experiment.

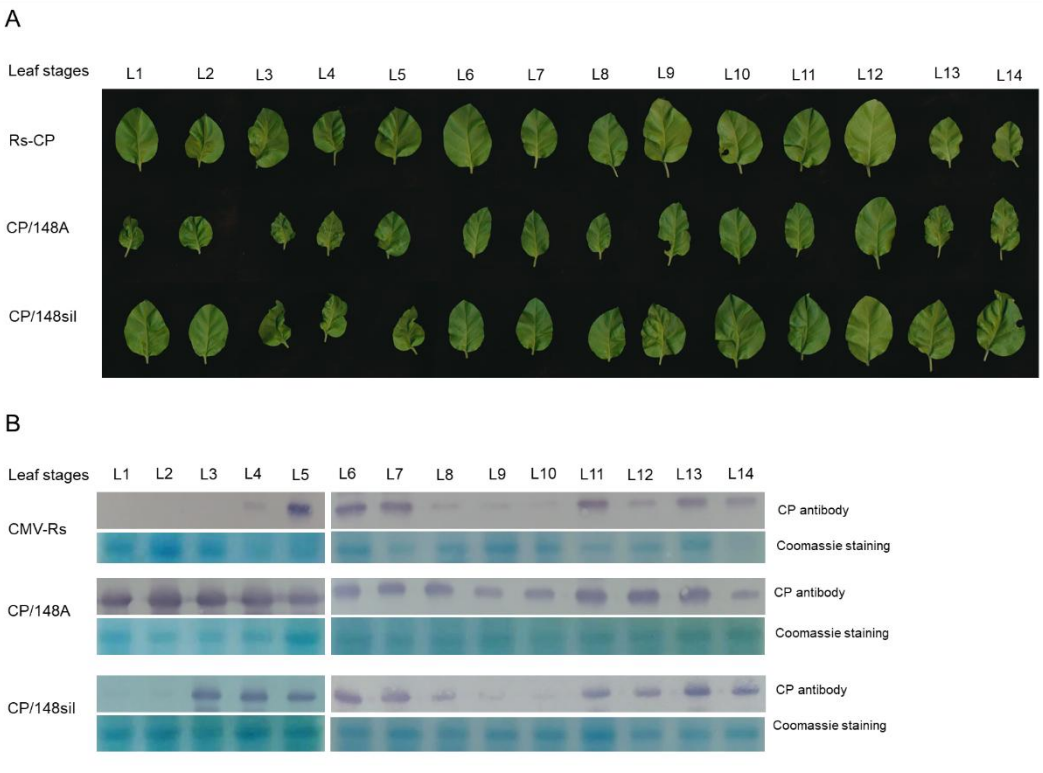

Fig.2. (A) Symptoms induced by Rs-CMV, CP/148A and CP/148sil on *Nicotiana tabacum* L. cv Xanthi plants in different leaf stages. The different leaf stages were numbered from the top to the bottom (L1-14, L1 is the youngest leaf while L14 signify the oldest leaves). (B) CP accumulation in different leaf stages.

After determining that aa position 148 is involved in formation of cyclic phenomenon of CMV infection, and stable virions are essential for systemic infection, we analyzed the virion stability of the wild-type and the CP/S148A mutant virus. Transmission electron microscopy observation of virions purified from infected *N. tabacum* L. cv Xanthi leaves showed no difference, Rs/148A could form similar viral particles as the wilt-type virus (Rs-CMV). This suggests that the formation of the virions were not affected by the S/148A alanin mutation. RNase sensitivity assay was performed by incubating the purified virions for a time period of 0-120 minutes at room temperature (RT). Rs-CMV gRNAs were partially degraded after 30 minutes, and in this case after 2 hours gRNAs were completely degraded, while gRNAs from Rs/S148A mutant were only partially degraded even after 120 minutes. The results showed that Rs/S148A virion is more stable and effective to bind gRNA than the wild-type virion. Alanine substitution at position 148 increase the RNA-binding capability of CP.

Previous studies demonstrated that symptom recovery is associated with RNA silencing and a recent study demonstrated that CP has negative effects on VSR activities of 2b protein (Zhang et al 2017). We examined the effects of wild-type CP (Rs-CMV) and the mutants (CP/148A and CP/148D) on silencing activity of 2b in infiltrated patches using Agrobacterium-mediated transient assay. Binary vector expressing GFP reporter gene was

agroinfiltrated into transgenic *Nicotiana benthamiana* (silenced for GFP expression) leaves together with the binary vector expressing the wild type 2b protein co-expressed with vector expressing wild-type CP, CP/S148A and CP/S148D, respectively. The suppressor activities were monitored by visual observation of the GFP fluorescence and quantitatively by measuring the accumulation level of GFP RNA in the infiltrated leaves by qRT-PCR (Fig 3A, C).

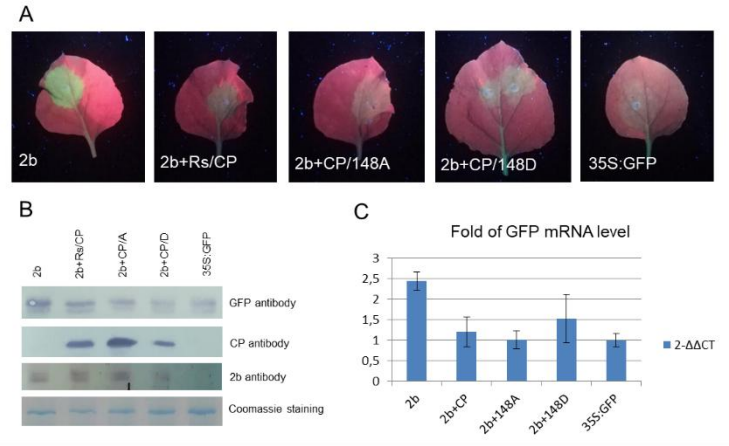

Fig. 3.(A) GFP fluorescence in *N. benthamiana* patches agroinfiltrated with binary vector expressing GFP rep
agroinfiltrated patches measured by qRT-PCR.

Coincident with a previous study, we found that co-infiltrating with CP caused reduction in the silencing suppressor activity of the 2b protein so in the presence of CP, 2b protein is less capable to suppress local silencing. Replacement of 148 S to A modulates the effect of CP to RNA silencing, co-infiltrating with CP/S148A was more significantly attenuate the 2b-mediated suppression of GFP silencing (Fig 3). The visual observation revealed that in the case of CP/S148D mutant the GFP fluorescence is less reduced than in the case of the wild-type CP. The presence of the CPs, GFP and 2b protein were verified by western blot analysis using monoclonal CP, GFP and 2b protein antibodies, respectively (Fig 3B). GFP mRNA levels in the presence of the suppressor and CPs were determined by qRT-PCR, which confirmed the visual observation.

To examine the effect of the CP phosphorylation in economically important hosts, *Capsicum annuum* cv. Brendon and *Solanum lycopersicum* cv. Money maker plants were infected with the wild-type and the CP/S148A mutant virus. Rs-CMV induce mild mosaic symptoms on pepper and on tomato as well. CP/S148A mutant caused more severe symptoms with severe stunting on pepper and on tomato as well (Fig 4A,B). Interestingly, CP accumulation did not increased parallel to the severity of the symptoms (Fig. 4C).

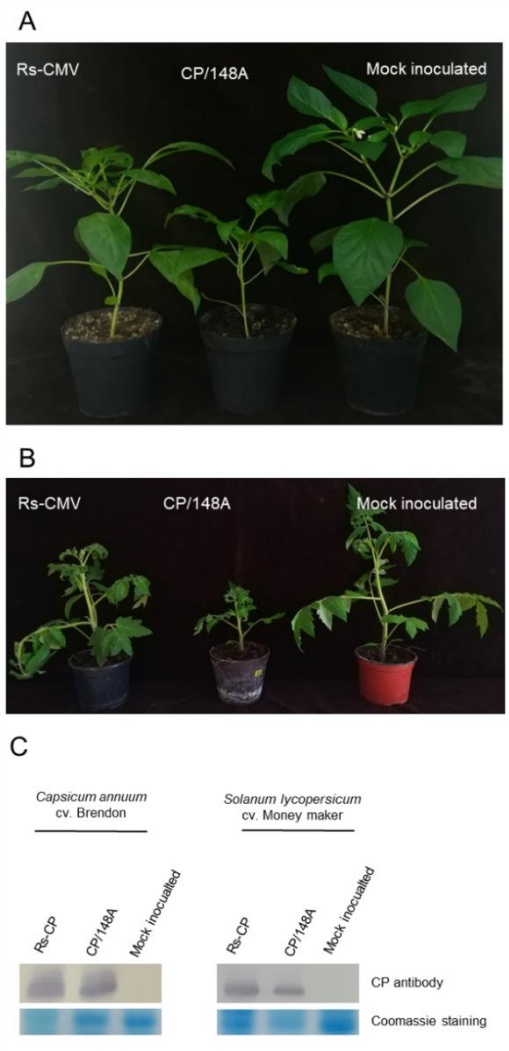

Fig.4. Symptoms induced by Rs-CMV and CP/S148A mutant on Capsicum annuumm cv. Brendon (A) and on Solanum lycopersicum c. Money maker plants (B) and CP accumulation in systemically infected leaves (C)

Manuscript regarding to the CP phosphorylation results is submitted to Virology.

MP phosphorylation

Since the MP concentration in CMV infected plant is low, and predominantly localized in plazmodezmata, the phosphorylation of the MP protein was investigated in E. coli expressed MP. The MP was successfully expressed, and Western analysis with phosphoserine antibody proved the phosphorylation of the MP. Since penta-Hist region was included at the carboxi terminus of the MP, the identity of the protein was detected with penta-His antibody (Fig. 5).

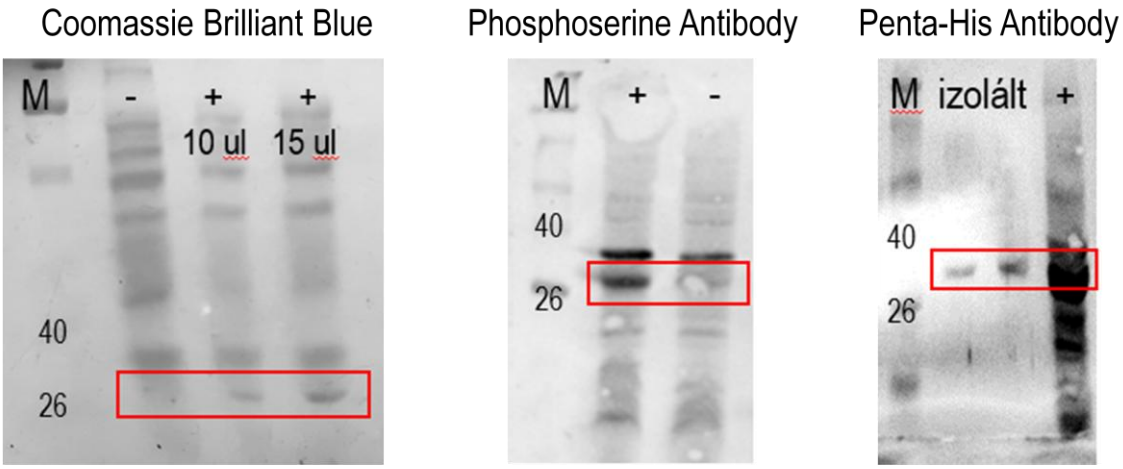

Fig 5. MP expression and phosphorylation

Phospho.ELM database and NetPhosK server were used to analyze CMV MP for phosphorylation sites, and the 28, 120 and 255 Ser aa was suggested as a feasible site of phosphorylation. Numerous CMV MP amino acid sequences were compared and these Ser positions proved to be highly conserved. To investigate the possible role of phosphorylation of these aa positions, the 28, 120 and 255 S separately was replaced to A (mimicking non-phosphorylated state; 28A, 120A, 255A) or D (mimicking the phosphorylated state; 28D, 120D, 255D) in the infectious clone of Rs CMV. All the mutant clones were infectious on *Nicotiana tabacum* L. cv Xanthi plants, even if the symptoms were clearly different. In the case of the MP28 D, 120A and 255A the symptoms were similar to the original Rs-CMV with mosaic symptoms and leaf deformation, while in the case of MP 28A, 120D and 255D the symptoms were milder, with weaker mosaic and no leaf deformation (Fig 6). The Norther analysis supported these observations.

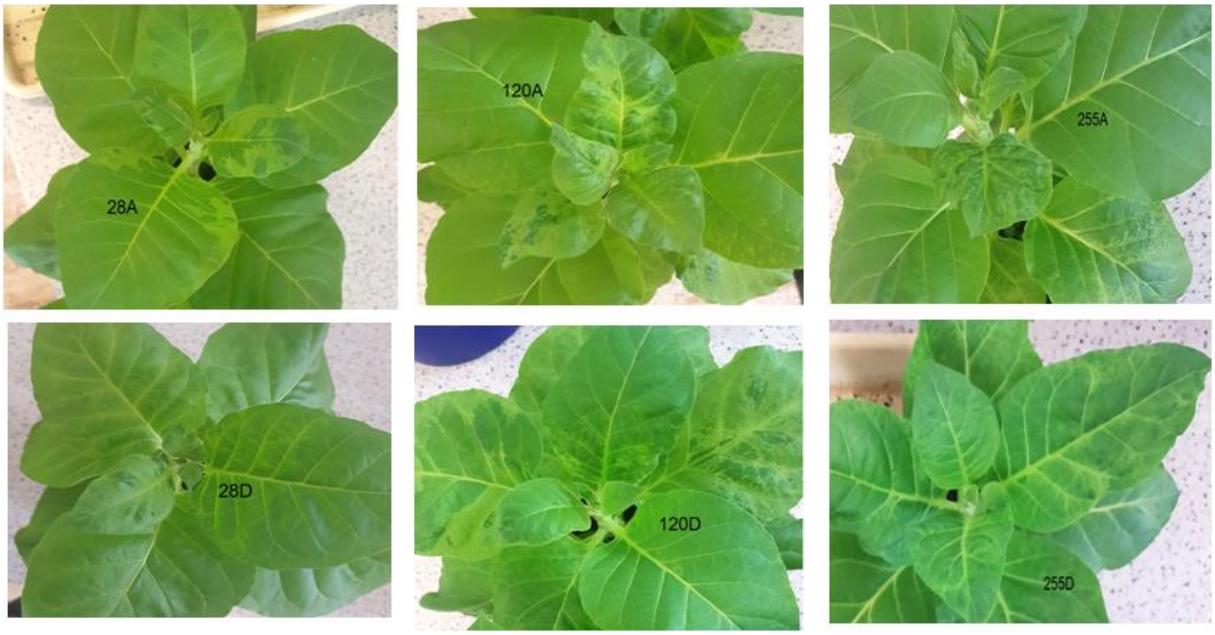

Fig 6 The infection phenotype of the different CMV MP mutants

The experiment system for the *in vivo* localozation of the MP was developed. The MP-GFP fusion protein was infiltrated in *N. benthamiana* plant leaves. The fusion protein was

detected at the plasmodesmata in plants and also in protoplasts. The MP localization was detected near to the cell wall, likely in the plasmodesmata. In the case of the mutants with stronger symptoms, the cell wall location of the mutant MP was more pronounced (Fig7 in the case of MP 255 mutants.

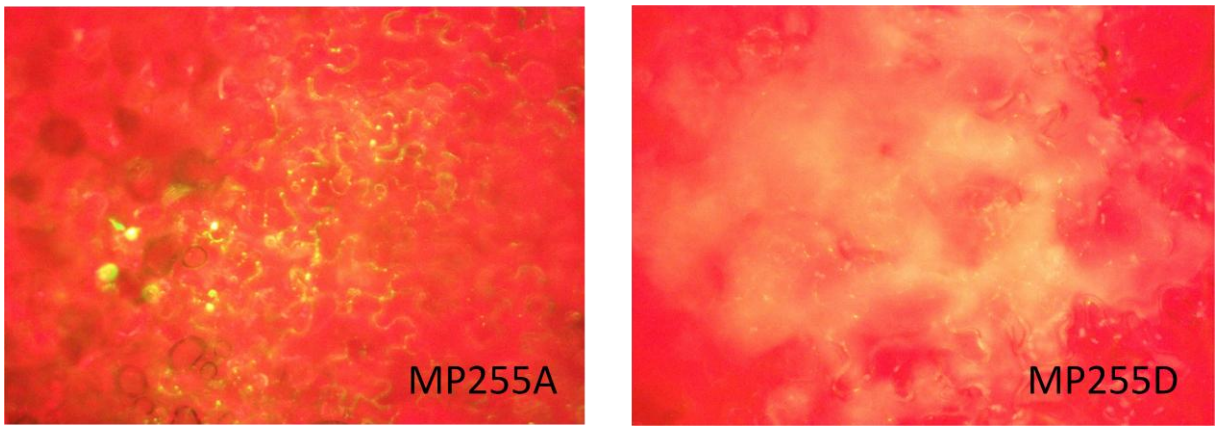

Fig. 7 Localization of MP-GFP fusion protein in infiltrated *N. benthamiana* leaves.

Publication is in preparation from this part of the project.